Effects of Calcium Oxalate on Expression of Clusterin and Lower Urinary Tract Symptoms in Prostatitis and Benign Prostatic Hyperplasia Patients with Calculi

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Background: Prostatic calculi are common in urological treatments. Our major purpose in the present study was to explore the occurrence and composition of prostatic calculi, and investigate the effect of calcium oxalate (CaOx) on clusterin expression and lower urinary tract symptom (LUTS) in prostatitis and benign prostatic hyperplasia (BPH) patients with calculi.

Material/Methods: From December 2016 to January 2017, a total of 79 prostatitis patients aged more than 50 years were enrolled. The patients were divided into 3 groups: group A had small calculi (discrete, small echoes); group B had large calculi (large masses of multiple echoes, much coarser), and group C had no calculi. Immunohistochemical analysis was performed to evaluate the tissue scores. The clusterin expression was detected by quantitative real-time CR (qRT-PCR), Western blot, and immunofluorescence.

Results: According to multifactor analysis, age was significantly associated with prostatic calculus. The composition of prostatic calculus was an independent factor of LUTS. The clusterin expression was elevated in group B. The mRNA and protein levels of clusterin in prostatitis and BPH patients with stones were obviously higher than those in prostatitis and BPH patients without stones. CaOx enhanced clusterin expression in a dose-dependent manner.

Conclusions: Large prostatic calculi were associated with LUST. Furthermore, CaOx enhanced clusterin expression, leading to large prostatic calculi. These results may provide a therapeutic strategy for prostatitis and BPH.

MeSH Keywords: Calcium Oxalate • Calculi • Clusterin • Lower Urinary Tract Symptoms • Prostatic Hyperplasia

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Prostatitis syndromes include acute and chronic infections, asymptomatic prostatitis, and chronic pelvic pain syndrome (CPPS) [1]. Despite various treatments, some patients have treatment failure, which is relevant to prostatic calculi [2]. Most prostatic calculi are found by radiological examination for prostate cancer or benign prostatic hyperplasia (BPH) [3]. Moreover, prostatic calculi are relevant to the mild chronic infections of the acini and secretory ducts. However, the clinic significance of prostatic calculi is unclear and the relationship between prostatoliths and lower urinary tract symptoms (LUTS) remains unknown [4]. Studies on the role of prostatic calculi in LUTS or CPPS-related symptoms were contradictory [2,5,6]. It was demonstrated that prostatic calculi are produced by calcification of the corpora amylacea and sediment of prostatic secretions under inflammatory conditions [7], so it is crucial to explore the chemical composition of calculi to understand the pathogenesis of prostatic calculi.

Previous studies revealed that calcium oxalate (CaOx) or calcium phosphate (CaP) with carbonate-apatite and hydroxyapatite is the major component of prostate calculi [3], which are usually discovered in men with acute or chronic prostatitis and can cause chronic prostatitis [8]. It is reported that calcium oxalate monohydrate (COM) is involved in hyperoxaluria, while calcium oxalate dihydrate (COD) is related to hypercalciuria [9]. The CaOx formation process comprises nucleation, development, clustering, and cell adhesion [10,11]. CaOx stones frequently possess a core-shell structure. The composition of stones on the surface and interior layer are determined using multiple methods. It is reported that CaOx and CaP are the major components of the interior layer, while CaOx is the main component on the surface, suggesting that CaP triggers COM growth by heterogeneous nucleations [12].

Calcium oxalate crystals were demonstrated to induce the production of renal cell reactive oxygen species, which can regulate the expression of inflammation-related molecules [13]. Clusterin, an inflammation-related protein, is reported to exert a protective effect on epithelial cell injury [14]. As a heterodimeric disulfide-linked glycoprotein, clusterin can be expressed in nearly all human tissues [15]. Moreover, clusterin is involved in multiple physiologic processes, such as morphologic transformation, cell adhesion, and cell interactions [16]. However, the effect of clusterin on prostatic calculi is unknown. Therefore, in the present study we assessed clusterin expression in tissues of prostatitis and BPH patients and in human prostate epithelial cells after CaOx treatment.

### Material and Methods

#### Clinical data and specimens

From December 2016 to January 2017 in Wuhan Asia General Hospital, we enrolled 47 prostatitis and BPH patients with stones and 32 prostatitis and BPH patients without stones (controls). These patients had various degrees of clinical symptoms, including symptoms of bladder irritation and/or LUTS, and were treated by transurethral plasmakinetic bipolar vaporization (TUPK). Exclusion criteria were: medication or stoma therapy without TUPK and pathological diagnosis, merged prostate cancer, neurogenic bladder, urethral malformation or stricture, severe neurosis, no available standard medical history, and undiscovered prostatic stones specimens after TUPK. Body mass index (BMI) and international prostate symptom score (IPSS) were obtained. IPSS score ≥8 represented severe LUTS [17]. Prostate-specific antigen (PSA) and prostate volume (PV) were also been determined. All experiments were approved by Wuhan Asia General Hospital Institutional Ethics Committee and informed consent was provided by all patients. Six patients were randomly selected from the severe LUTS and non-severe LUTS groups (n=3 per group) by use of a random number generator.

#### Immunohistochemistry

Sections (4-µm) were deparaffinized and rehydrated in alcohol. After blocking in 3% H2O2, and antigen recovery in boiling 10% citrate buffer, the sections were incubated with anti-clusterin (1: 200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C followed by horseradish peroxidase-labeled secondary antibody (Zhongshan Golden Bridge, Beijing, China) for 1 h. The sections were washed in tris-buffered saline-Tween 20 (Dako, Glostrup, Denmark). Finally, sections were stained with 3, 3'-diaminobenzidine (DAB, Zhongshan Golden Bridge) for 10 min and then stained with hematoxylin after washing. The images were observed by an E-400 microscope (Nikon, Tokyo, Japan) and integral optical density (IOD) was calculated using Image Pro plus 6.0 software (Microsoft Media Cybernetics, Bethesda, MD, USA). The percentage of cells was calculated and expressed as follows: 0=no cell staining, 1=less than 1/3 of cell staining, 2=1/3–2/3 of cell staining, and 3=more than 2/3 of cell staining. Tintorial strength was evaluated as follows: 0=no color, 1=canary yellow, 2=pale brown, and 3=dark brown. The clusterin expression was determined using a scoring system based on the tintorial strength and the positive ratio of tissue.

#### Cell culture and treatment

The RWPE-1 (human prostate epithelial cell line) cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The RWPE-1 cells were maintained in RPMI 1640 medium (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO2. The RWPE-1 cells were treated with CaOx (purchased from the American Type Culture Collection, ATCC, Rockville, MD, USA). The percentage of cells was assessed using Image Pro plus 6.0 software (Microsoft Media Cybernetics, Bethesda, MD, USA). The percentage of cells was calculated and expressed as follows: 0=no cell staining, 1=less than 1/3 of cell staining, 2=1/3–2/3 of cell staining, and 3=more than 2/3 of cell staining. Tintorial strength was evaluated as follows: 0=no color, 1=canary yellow, 2=pale brown, and 3=dark brown. The clusterin expression was determined using a scoring system based on the tintorial strength and the positive ratio of tissue.
Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum at 37°C with 5% CO₂. Then, the cells were incubated with different concentrations of CaOx (0, 100, 300, and 600 μg/mL) for 24 h.

**Real-time PCR**

Total RNAs of prostate tissues and cells were isolated using Trizol (Invitrogen, San Diego, CA, USA). Briefly, samples were homogenized in Trizol reagent followed by chloroform and mixed for 5 min. After centrifugation (12 000 g for 15 min at 4°C), the supernatant was carefully drawn into a new tube. An equal volume of isopropyl alcohol was added and incubated at room temperature for 20 min. Following centrifugation (12 000 g for 4°C for 10 min), the supernatants were removed completely and the precipitate was washed twice by 75% ethanol. Finally, the RNA was eluted by nuclease-free water. The concentration and purity were detected using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The cDNA was obtained by 1 μg RNA using the High-Capacity CDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). In brief, the RNA was incubated with 2X RT master mix containing 10×RT Buffer, 25×dNTP Mix, 10×RT Random Primers and MultiScribe™ Reverse Transcriptase at 25°C for 10 min followed by 37°C for 2 h and 85°C for 5 min. The expressions of clusterin (forward: 5’-GAA GTC TCC AGG GAC ATG G-3’ and reverse: 5’-GCG TAC AGG GAT AGC ACA GC-3’) were assessed in the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following reaction conditions: 94°C for 5 min, 40 cycles of 96°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Then, the PCR products were separated on a 1% agarose gel with ethidium bromide staining. Densitometry was analyzed with 200TM-Image software (Bio-Rad, USA) and β-actin served as an internal reference gene.

**Western blot**

Prostate tissues and cells were washed by PBS and lysed in lysis buffer (Beyotime, Beijing, China). Then, the lysates were incubated on ice for 30 min and oscillated for 30 s. After centrifugation at 10 000 g for 30 min at 4°C, the supernatant was collected to measure the protein concentrations by BCA kit (Solarbio, Beijing, China). The proteins separated by the SDS-PAGE were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Then, the membranes were incubated overnight at 4°C for 1 h after blocking with primary anti-clusterin (1: 1000, Santa Cruz Biotechnology) and anti-β-actin (1: 1000, Santa Cruz Biotechnology). The membranes were then probed with anti-rabbit IgG (1: 50 000, Cell Signaling Technology, Beverly, MA, USA). The bands were determined by a Molecular Imager VersaDoc MP 5000 System (Bio-Rad, Hercules, CA) and densitometry was determined with Quantity One (Bio-Rad).

**Measurement of clusterin by immunofluorescence**

Cells were seeded onto slides and were fixed with 2% paraformaldehyde. Subsequently, the fixed cells were permeabilized by 0.1% Triton X-100 followed by incubation with anti-clusterin primary antibody (1: 100, Santa Cruz Biotechnology) and goat anti-mouse secondary antibody (Alexa Fluor 488). Thereafter, cells were counterstained in 4’, 6-Diamidino-2-phenylindole dihydrochloride (DAPI, 1: 1000, Life Technologies, Grand Island, NY, USA) at room temperature for 1 h and then observed under a confocal microscope (Zeiss LSM, Carl Zeiss, Germany).

**Statistical analysis**

Statistical analysis was conducted by SPSS (SPSS, Inc., Chicago, IL) and data are expressed as mean ±SD. The independent-samples t test and analysis of variance (ANOVA) were used for feature analysis. Categorical variables were compared using the Pearson chi-square test. Multivariate logistic regression was used to determine the associated factors for severe LUTS. P <0.05 was set as statistically significant.

**Results**

**Clinical pathological parameters**

A total of 79 patients were studied, including 47 prostatitis and BPH patients and 32 prostatitis and BPH patients. Among them, no significant differences were observed between several parameters (BMI, PV, PSA, and IPSS) and prostatic calculi except for age, while mean IPSS in the patients with prostatic calculi (9.87) was significantly higher than in those without prostatic calculi (7.5). The mean PV (26.18) of the patients with prostatic calculi had no obvious difference from those without stones (24.06) (Table 1). Moreover, the components of prostatic calculi were clearly related to the severe LUTS (IPSS ≥8) (Table 2). In all the prostatitis and BPH patients, clusterin expression was significant related to the severe LUTS (Table 3). Furthermore, in prostatitis and BPH patients with stones, the clusterin expression was significantly associated with the CaOx in calculi (Table 4).

**The expression of clusterin in the patient**

The expression of clusterin was weakly positive in the prostatitis and BPH patients with severe LUTS, whereas clusterin was overexpressed in the prostatitis patients with severe LUTS (Figure 1A, 1B). Moreover, the mRNA and protein expression...
of clusterin in the tissues of prostatitis and BPH patients with stones was clearly higher than that in the tissues of prostatitis and BPH patients without calculi (P<0.0001; Figure 2A, 2B).

**CaOx enhanced the clusterin expression**

After the RWPE-1 cells were treated by different concentrations of CaOx for 24 h, the mRNA expression of clusterin level was enhanced in a dose-dependent manner and the mRNA level of clusterin with 1000 μg/mL CaOx was approximately 8-fold higher than that without CaOx treatment (P<0.01; Figure 3A). Furthermore, the protein level of clusterin was also markedly increased by the high concentration of CaOx (P<0.01; Figure 3B), and a remarkable increase was observed in the clusterin expression induced by the high concentration of CaOx (Figure 4).

**Discussion**

Prostatic calculi, which are considered to be asymptomatic and are associated with LUTS, are common in urology practice. However, the morbidity rate of the prostatic calculi in the general population is uncertain [18]. BPH is also common in older men, and the morbidity rate of patients older than age 50 years was reported to be 48.91% [19]. In our study, we found that age was associated with prostatic calculi, and the composition of prostatic calculi might affect the severity of LUTS. Moreover, the clusterin expression level was relatively higher in prostatitis and BPH patients with stones than in prostatitis and BPH patients without stones. The positive clusterin expression was more common in patients with severe LUTS than in patients with non-severe LUTS. Furthermore, CaOx enhanced the clusterin expression in a dose-dependent manner in human prostate epithelial cells.

Most prostatic calculi are found in middle-aged and older men. The morbidity rate of prostatic calculi with hyperplasia was reported to be 68.52% [20], suggesting that prostatic calculi are associated with aging. Older patients were reported be more likely to have prostatic calculi, but the association between age and prostatic calculi was not statistically significant [21]. In our study, prostatitis patients with BPH were enrolled, including 47 men with prostatic calculi and 32 patients without prostatic calculi. All were over age 50 years, indicating that age is a critical factor in the formation of prostatic calculi. Additionally, men with large prostate glands are reported to be older and exhibit higher levels of prostate-specific antigen (PSA) [22], but we found that large PV was not strongly associated with prostatic calculi, in contrast to a previous report [4].

The mechanism of prostatic calculi formation is still unclear. The incidence of prostatic calculi is associated with prostatitis, prostatic hyperplasia, and prostatic cancer [23]. Prostatic calculi are reported to form by the sedimentation of prostatic secretions and sloughed epithelium, and calcium phosphate and calcium carbonate appear to be the main constituents [24].

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**Table 1. Clinical parameters.**

|                        | Case       | Control    | P     |
|------------------------|------------|------------|-------|
| Age (year)             | 51.3±4.69  | 48.84±3.89 | 0.015 |
| BMI (Kg/m²)            | 24.99±2.37 | 24.61±1.85 | 0.448 |
| PV (ml)                | 26.18±5.57 | 24.06±5.23 | 0.09  |
| PSA (ng/ml)            | 1.20±0.59  | 1.19±0.67  | 0.944 |
| IPSS                    | 9.87±5.97  | 7.50±4.1   | 0.08  |
| Severe LUTS            |            |            | 0.142 |
| Yes                    | 27         | 13         |       |
| No                     | 20         | 19         |       |

**Table 2. The effect of the composition of the prostate calculi on LUTS.**

|                       | Severe LUTS | Non-severe LUTS | P     |
|-----------------------|-------------|-----------------|-------|
| CaOx                  | 23          | 12              | 0.05  |
| Non-CaOx              | 4           | 8               |       |

**Table 3. Relationship between clinical parameters and clusterin expression in prostatitis and BPH patients.**

|                       | Clusterin expression |       |       |
|-----------------------|----------------------|-------|-------|
|                        | Positive | Negative | P     |
| Case                  | 28       | 19       | 0.006 |
| Control               | 9        | 23       |       |
| Severe LUTS           | 29       | 11       | <0.001|
| Non-severe LUTS       | 8        | 31       |       |

**Table 4. Relationship between prostatic calculi component and clusterin expression in prostatitis and BPH patients with calculi.**

|                      | Clusterin expression |       |       |
|----------------------|----------------------|-------|-------|
|                       | Positive | Negative | P     |
| CaOx                 | 25       | 10       | 0.04  |
| Non-CaOx             | 3        | 9        |       |
| Severe LUTS          | 22       | 5        | <0.001|
| Non-severe LUTS      | 6        | 14       |       |
Figure 1. Clusterin expression in prostate tissues. (A) Clusterin expression in patients with non-severe LUTS or severe LUTS. (a–c) Patients with non-severe LUTS; (d–f) Patients with severe LUTS. (B) The mean absorbance of clusterin in prostate tissues.
CaOx calculi are commonly encountered in clinical practice in all age groups. Additionally, symptomatic prostatic calculi can lead to urinary stasis and infection of the prostatic urethra [25], and prostatic calculi have been demonstrated to be involved in non-specific LUTS [26]. In our study, we found that CaOx had a significant correlation with severe LUTS, and older men tend to have more severe LUTS.

Clusterin is involved multiple biological processes, such as cell-cell interaction, apoptosis, and tissue remodeling, responding to cytotoxic injuries or degenerative diseases [27,28]. Generally, clusterin includes the secreted clusterin (sCLU) and nucleus-targeted clusterin (nCLU). The former may exert a cytoprotective effect while the latter is related to proapoptotic processes [29,30]. Moreover, the balance between sCLU and nCLU may determine

Figure 2. Clusterin expression in the prostatitis and BPH patients. The mRNA (A) and protein levels (B) of clusterin in the prostatitis and BPH patients with and without calculi, respectively. *P<0.0001.

Figure 3. Clusterin expression in the RWPE-1 cells with CaOx treatment. The mRNA (A) and protein levels (B) of clusterin were enhanced by CaOx in a dose-dependent manner. ** P<0.01 versus the no-CaOx group.
Figure 4. Clusterin expression was enhanced by CaOx in RWPE-1 cells. DAPI was used to visualize nuclei. Scale bar=20 μm.
the cell fate [31], and sCLU in plasma is a marker of prostate cancer [32]. Clusterin is reported to suppress apoptosis through the NF-κB pathway [33], and clusterin level was enhanced in kidney cells following CaOx treatment [16]. However, the role of clusterin in prostate cells is not clear. In the present study, strong expression of clusterin was found in patients with severe LUTS, and clusterin was overexpressed in the prostatitis and BPH patients with stones. Furthermore, the clusterin expression in RWPE-1 was increased by CaOx, suggesting that CaOx can trigger human prostate epithelial cell injury and up-regulate clusterin expression. During the calculus formation, clusterin can be initiated in CaOx-treated prostate cells and exert protective and/or reparative effects by improving cell interactions and eliminating necrotic cells and toxic denatured macromolecules.

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Conclusions

We demonstrated that age is significantly associated with prostatic calculi, and the composition of prostatic calculi is an independent factor of LUTS. Clusterin expression was elevated in the prostatitis and BPH patients with prostatic calculi. Furthermore, CaOx enhanced clusterin expression in prostate cells in a dose-dependent manner.

Conflict of Interest

None.