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Short Communication

Electron imaging of calcium oxalate crystals in beagle dogs' urine

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KEYWORDS

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Abstract Calcium oxalate crystalluria appears to be a common problem in most of small animal clinics. This current study aimed at inducing a condition of oxalate crystalluria in beagles and record the primary changes in canine blood and urine on response to oxalates injection. 15 dogs were divided into two groups; those in the treatment group were injected intravenously with 0.5 M potassium oxalate and the dogs of control group were injected with physiological saline for five successive days. Urine test revealed a significant decrease in urinary creatinine and urinary urea nitrogen levels. The ultrastructural examination of urine sediment showed typical and atypical phases of calcium oxalate crystals and the X-ray defractionation of these crystals showed high content of calcium in addition to other minerals. Therefore potassium oxalate injection may provide an example of calcium oxalate crystalluria which may answer some question around the pathogenesis of this problem in dogs.

1. Introduction

Urolithiasis is a recurrent clinical problem in dogs throughout the world. Although struvite uroliths have been reported as the most common uroliths worldwide [1,2], recent studies evaluated the trends in stone submissions in the period of last ten to twenty years and have revealed equalization of calcium oxalate and struvite-containing calculi, and subsequent increases in calcium oxalate-containing uroliths thereafter [3]. The main reasons of the long-term changes in mineral composition of uroliths are yet unknown [4]. However, scientists found out some predisposing factors which might incorporate in stone formation. Some of these factors might include demographic and nutritional changes, preference to certain types of breed, and complex interaction of other multiple factors [5].

The process of stone formation starts with precipitation of crystals in when the urine is oversaturated with calcium oxalate, colloquially the urine where the ion product in excess of the level at which spontaneous precipitation occurs. However, urine contains inhibitor(s) that can withhold the process of supersaturation. Most of this activity can be ascribed to macromolecules and a considerable participation by citric acid and some ions [6]. Magnesium can be considered to act as an

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inhibitor of Calcium oxalate (CaOx) crystallization because of its capacity as a chelator of oxalate. Magnesium oxalate is more soluble than CaOx which enables oxalate excretion outside the urinary system. Although it has been reported that magnesium could inhibit crystal nucleation [7], growth [8] and aggregation [9], However, there is little evidence to recommend magnesium therapy in patients with urolithiasis. Oxalates are highly toxic agents to the renal tissue. They are believed to play an infamous role in calcium oxalate urinary stone formation [10] through induction of severe tubular injury, cell sloughing [11], and renal insufficiency or renal failure in some case. Moreover, oxalates may lead to changes of the whole blood picture where Robinson [12] reported a significantly decreased hemoglobin, hematocrit, erythrocytes, and neutrophilia in female rats after receiving 4% EG for 10 days. In addition, severe anemia and acute renal failure were recorded in a patient with chronic exposure to both very large doses of oxalate precursor [13].

After urinary supersaturation, the next step in stone formation process is nucleation which means formation of a solid crystal phase in a solution followed by retention of these crystals within the renal tissue to act as nucleus to simply permit further crystals deposition, growth and aggregation [14]. Urinary macromolecules prevent the nucleation step by binding within pre-formed crystals or adsorption on a crystal surface to induce degradation and dissolution of crystals faces and edges [15]. Only scanning electron microscopy (SEM) can provide these information and record the completeness of urinary inhibitors against crystallization. Furthermore SEM is supplied by additional equipment for analysis of the crystals and measure mineral and other elemental content.

In the current study we had exposed beagle dogs to potassium oxalate injection in order to induce crystalluria. The aim of this study is to assess the hematological and urinary changes accompany the process of crystalluria in canines. We also aimed at examining the ultrastructure and the chemical compositions of the biologically produced CaOx crystals after frequent injection of oxalates.

2. Materials and methods

2.1. Animals

Fifteen healthy intact adult dogs (beagles and mongrel breeds) aged between 2.5 and 4 years old were selected in this study. Seven dogs (two males and five females) were assigned to the control group, and the other eight dogs (four males and four females) were considered the treatment group. Dogs were housed in stainless net cages (1 dog per cage) with ten-day quarantine and acclimatization periods in the department of clinical veterinary medicine, Yangzhou University, Yangzhou city, China. Dry mixed food was provided for dogs at twice daily and tap-water was provided ad libitum.

2.2. Study design

0.5 M potassium oxalate solution ($K_2C_2O_4 \cdot H_2O$) was prepared and sterilized by passing through a 0.22-μm filter. Animals in the treatment group were given 0.5 M KOx at a dose of 0.13 ml/kg, while the same volumes of 0.9% physiological saline were given to the control ones. The Dose was chosen according to [16]. Butterfly catheters were inserted into the cephalic veins and fixed. Each group was injected with the assigned solutions three times a day for 5 consecutive days. All experiments and procedures performed on the animals were approved by the Animal Care and Use Committee of Yangzhou University.

2.3. Hematological picture

Blood samples were collected just prior to injection, and on days one, three and five. Five mL-blood samples were collected each time from the cephalic veins of injected dogs. Two-hundred microlitre of blood samples were added into tubes along with EDTA. The whole blood was anti-coagulated by adding EDTA and analyzed within 20 min by automatic blood analyzers (BC-2800, Mindray biomedical electronics, China). White blood cell (WBC) count, differential leukocyte, red blood cell (RBC) count, hemoglobin (HGB) hematocrit (HCT), and platelet count were measured.

2.4. Urine analyses

Urine samples were collected daily from day 0 till day 5 by indwelling catheters. Every urine sample was divided into two parts. One part was examined for urine pH immediately. The other part was placed in a tube and stored at −20° C for further biochemical analyses to determine the urinary levels of calcium (Ca), magnesium (Mg), urea nitrogen (UN), and creatinine (CR). The previous elements were analyzed by automatic biochemical analyser (AU 480, Backman, USA).

2.5. Urine sediment examination

Five millilitre of urine were centrifuged at 6000*g* for 10 min; the sediments were spread on a microscopical slide and examined by light microscopy. Sediment suspensions were dried overnight at 37° C, mounted on aluminium stubs and coated with gold 3-nm thickness using a gold sputter. The stubs were examined using an S-4800; Hitachi SEM equipped with an X-ray defractometer.

2.6. Statistical analyses

Mixed ANOVA using SPSS (Version 16.00) software was used for statistical analysis. Time (Day 0, Day1, Day3 and Day5) was used as a within-subject factor meanwhile the treatment was used as a between-subject factor (Saline Vs Potassium oxalate). Bonferroni's post hoc test was used to identify specific differences. A Pearson correlation test was used to determine the relationship between different urine parameters. The level of significance at which the null hypothesis was rejected was $\alpha = 0.05$. Values in the figures are means ± SE.

3. Results

3.1. Hematological findings

The hematological parameters showed non-significant differences ($P > 0.05$) between treatment and control groups.

3.2. Urine measurements

Statistical analysis indicated that both time ($F_{3,57} = 0.603$, $P = 0.623$) and treatment ($F_{1,58} = 0.448$, $P = 0.533$) variables when work independently had non-significant effects on the urinary urea nitrogen. However, when they concurrently work, we observed that the (time X treatment) interaction had a significant effect ($F_{3,57} = 3.268$, $P = 0.03$) where the post hoc test showed that the treatment group showed reduced levels of urinary urea nitrogen on D3 and D5 post injection (pi) (Fig. 1A). Similarly time ($F_{3,57} = 2.81$, $P = 0.071$) and treatment ($F_{1,58} = 0.951$, $P = 0.374$) effects did not significantly influence the urinary creatinine level but the (time X treatment) interaction did ($F_{3,57} = 4.12$, $P = 0.02$) where the post hoc test showed that the dogs showed reduced levels of creatinine on D5 (Fig. 1B). Finally, urinary Mg and urinary Ca did not show any significant changes between saline and potassium-oxalate injected dogs.

Pearson correlation coefficient showed a significant correlation between urea nitrogen and creatinine levels of urine ($r = 0.724$, $P = 0.01$). There was a significant correlation between urinary Mg and urinary urea nitrogen ($r = 0.64$, $P = 0.01$), with creatinine ($r = 0.51$, $P = 0.04$) in control group. There were not any significant correlations between urine parameters in potassium oxalate treated dogs. The mean urine pH of the treatment group was about < 6.7 and did not express any significant change compared to control group.

3.3. Urine sediment examination

Under light microscopy (LM): appearance of both types of calcium oxalate dihydrate (COD), and calcium oxalate monohydrate (COM) crystals in different sizes in solitary or in aggregation.

Under scanning electron microscopy (SEM): Isolated crystals or clusters of CaOx crystals aggregation fused with some tissue debris. Solitary tetrahedral shape of COD crystals and adhered COD were observed under light microscope and SEM. Atypical shapes of COM crystals were detected by SEM as some COM crystals exhibited rounding at the edges, bow–tie appearance. Some crystals appeared extensively eroded and sub crystalline particles were detected. X-ray defractionation of crystals revealed large content of Ca, Na, Cl, K in addition to carbon and oxygen indicating for organic matter (probably oxalate) and some cells of urinary sediment contained calcium (Figs. 2 and 3).

4. Discussion

Oxalates are very toxic agents that have hazardous effects on animal's body. We designed this experiment to explore the changes occurred in the hematology and urine parameters after oxalate precursor injection for 5 consecutive days in beagle dogs. We didn't find any significant changes in the hematological picture after oxalate injection. Our findings are similar to

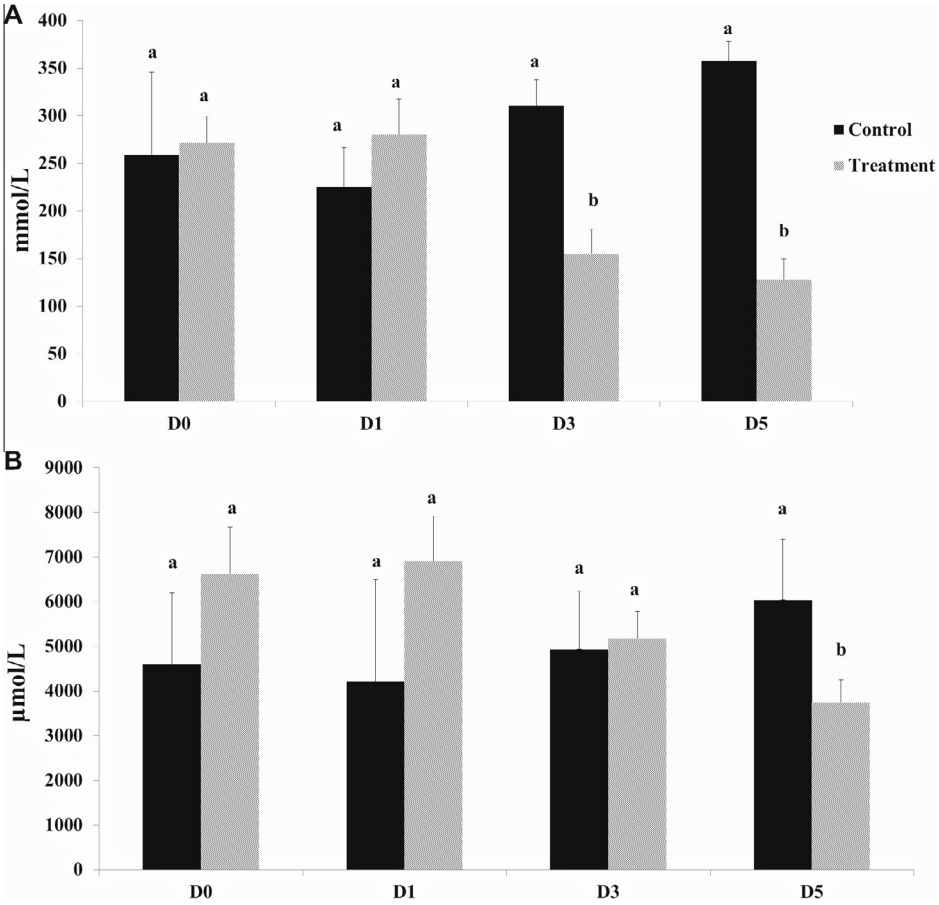

Figure. 1 Urinary concentrations of urea nitrogen (A) and creatinine concentration (B) in control and treatment groups.

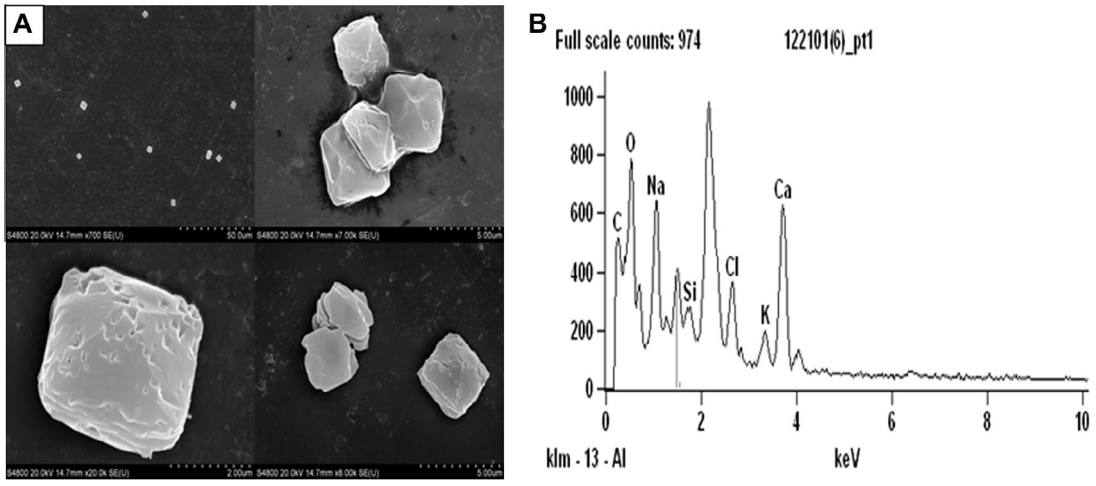

Figure. 2 Urinary sediment under scanning electron microscopy. (A) Tetrahedral calcium oxalate dihydrate crystals aggregation and eroded surfaces. (B) X-ray defractionation results of crystals showing high content of Ca, Na, Cl and little content of K, Si.

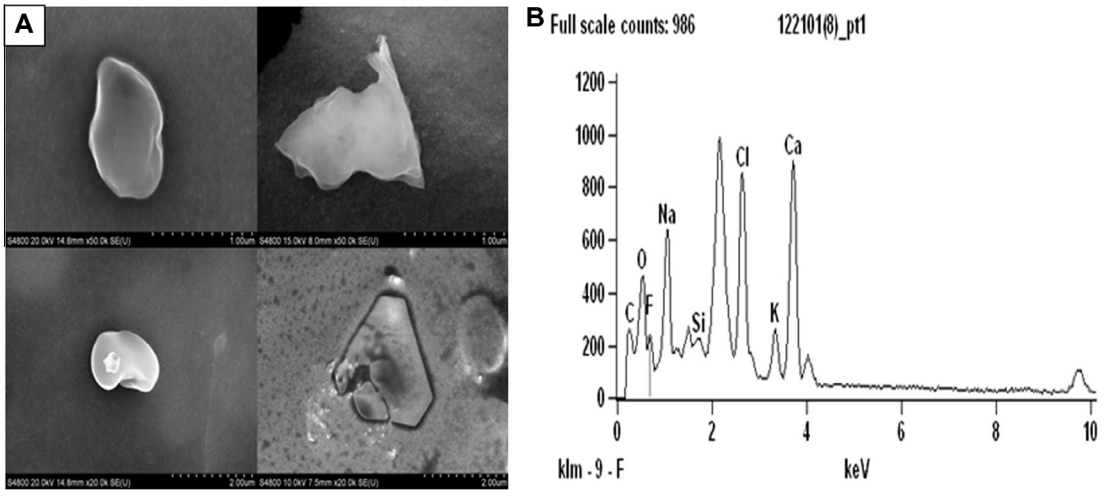

Figure. 3 Calcium oxalate monohydrate under scanning electron microscopy. (A) Atypical calcium oxalate monohydrate crystals with round edges and boa tie appearance. (B) X-ray defractionation results of crystals showing high content of Ca, Cl, Na, and little content of K and Fe.

DePass [17] where no significant changes in the hematological picture were recorded.

Our results also showed significant decreases in urinary levels of creatinine and BUN as if they were retained inside the body which suggests a disruption in the glomerular filtration due to the secondary effect oxalates changes in the renal physiology. Although magnesium has been consistently reported to inhibit CaOx crystal deposition in both inorganic solutions and urine, but in the current experiment the measurements of magnesium and calcium levels in urine did not show any significantly change throughout the experiment. These findings are similar to the results in Stevenson in dogs [18] and Mochizuki [19] in urolithiatic cats. Practically there is no convincing evidence that stone disease can be unequivocally ascribed to a reduction in magnesium urinary output. Several studies failed to detect any difference between stone forming patients and healthy controls [20]. Although magnesium was significantly correlated to creatinine, BUN in urine of control group, but we did not find any significant correlations in the treatment group between the urine parameters which might be because of the decrease in the concentration levels of urinary UN and creatinine but magnesium and calcium remained unchanged.

Kienyes secrete several substances including undifferentiated proteins, and glycosaminoglycans that bound to the surfaces of COM crystals [21], incarcerated within them [22]. These substances are supposed to inhibit crystals retention and nucleation by reducing the ability of attachment of COM-more adherent phase-to renal epithelial cell membranes. Therefore converting COM phase to a COD- less adherent phase- is a mean of inhibiting crystals attachment and nucleation [23]. The imperfect shapes of COM and COD crystals observed under electron microscope were excreted by natural urine which means that beside the mineral content, urine also contains different proteins, lytic enzymes and other organic matrix. These substances attack crystal faces and edges to induce dissolution and degradation of the urinary crystals and

decrease crystals chance for deposition on the renal tissue; for instance osteopontin is incriminated in crystals erosion and the appearance of cracked surfaces [24].

In addition, CaOx crystals themselves contain intracrystalline proteins, which create discontinuities within the mineral phase, increase the nonuniform crystals and reduce the average size of their crystallites component [25], in other meaning the intracrystalline proteins that induce deformity and atypical shape of the crystals. Grover [26] recorded massive erosions and different degrees of crystals degradation of CaOx crystals after incubation in MDCK containing crystals matrix extract CME, while crystals incubated with cultured cells alone were not eroded. In another way some crystals aggregated in amorphous shape together with tissue debris that might be due to the THP urinary protein that may act as a glue which increases the viscous binding between crystals and allow the crystals to stick together in order to be expelled in urine [27]. High content of K salts in the urine sediments might be due to the overloading the circulation with potassium source through the frequent injection of potassium oxalate.

5. Conclusion

CaOx urolithiasis in dogs is a growing problem of concern in the veterinary field. From this study we found that overloading of oxalates into the canines body may change the urine parameters. We concluded that kidneys can defend against stone formation by hindering the retention of oxalate crystals within the renal tissue through excretion of natural urine which contains potent inhibitors capable of degradation and dissolution of the crystals. These findings assist in understanding the pathogenesis of the early events of urolithiasis in canines. Future research may be directed toward analysis of the crystals matrix proteins and measuring their concentration in canines' urine by use of high techniques to assess their role in inhibiting stone formation.

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