Effect of Potassium Oxalate injection on Serum and Kidney Tissue of Beagle Dogs

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Abstract: Calcium oxalate (CaOx) crystalluria is a problem of growing concern in dogs. Reports describing pathologic findings of this condition are uncommon and ultrastructural descriptions are rare. We evaluated the consequences of calcium oxalate crystal deposition in renal tissue. Six dogs were intravenously injected with 0.5 M potassium oxalate (Kox) for 7 consecutive days. Ultrasonography revealed mild hyperechogenicity in both cortical and medullary renal tissue after treatment with Kox and the biopolar length and width also increased compared to the pre-injection value. Serum creatinine and blood urea nitrogen levels gradually increased. Tissue examination by light and electron microscopy demonstrated that CaOx crystal deposition was accompanied by morphologic changes. Canine renal oxalosis resembles the histological and ultrastructural findings reported from rats, mice and tissue cultures.


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Key words: CaOx, dog, kidney, serum, SEM, TEM

1. Introduction

Oxalates are highly potent nephrotoxins. They are poorly soluble in the presence of calcium which results in precipitation as calcium oxalate crystals in renal tubules (Cruz et al., 2004). Increasing oxalate concentrations and crystal deposition produce proximal tubular cell (PCT) necrosis (Thamilselvan and Khan, 1998; Miller et al., 2000; Guo and mMartn, 2005). Tissue culture studies confirm that calcium oxalate monohydrate (COM) is cytotoxic and triggers the release of renal injury markers(Guo et al., 2007). Even without crystal deposition, hyperoxaluria induces apoptosis in distal convoluted tubules (DCT) and collecting ducts (CD) in rabbits (Sarica et al., 2001). The dead renal epithelial cells serve as a nidus for the accumulation and aggregation of crystals within tubules with the possible formation of CaOx microliths (Khan, 2004). Deposition of oxalate crystals results in release of inflammatory markers and mediators (Thamilselvan and Khan, 1998) and activates the expression of some interleukins and cytokines during the formation of nephroliths (Okada et al., 2010 and Yuen et al., 2010).

In recent years, the incidence of CaOx urolithiasis has become a problem in dogs worldwide (Houston and Moor, 2009; Del Angel-Caraza et al., 2010; Vrabelova et al., 2011) due to changes in the lifestyle of dogs such as increased feeding of acidified commercial diets, changes in dietary calcium and magnesium content, and the increased popularity of small breeds of dogs which are more prone to CaOx urolithiasis (Stevenson, 2001; Ling et al., 2003; Houston and Moor, 2009).

We set out to investigate the clinicopathological effects and histopathologic changes associated with canine CaOx urolithiasis. Others (Gauer et al., 1984; Thrall et al., 1984; Thrall et al., 1985; Smith et al., 1990) have employed varying methodologies to induce CaOx crystalluria in dogs, principally through ethylene glycol (EG) administration. Few studies have documented the biochemical, histologic and ultrastructural changes accompanying CaOx nephrolithiasis. Most studies of CaOx nephrolithiasis were conducted in mice (Mo et al., 2004; Khan and Gelenton, 2010), rats (Hossain et al., 2005; Marengo et al., 2006; Khan et al., 2007) and tissue culture (Khaskhali et al., 2009; Chutipongtanate and Thongboonkerd, 2010; Ouyang et al., 2011). The results of those studies may not completely explain the pathological mechanism during the procedure of CaOx formation. So, artificial model of CaOx crystals formation was established and studied from biochemical detection, imaging examination and histological and ultrastructural detection to explore the pathological mechanism of CaOx crystals.

2. Materials and methods

2.1. Chemicals

0.9% physiological saline was prepared for control dogs. 0.5 M potassium oxalate solution(K2C2O4·H2O) was prepared and sterilized by passing through a 0.22-μm filter. Then the dogs of treatment group were given 0.5 M KOx at dose 0.13ml/kg and the same volumes were given to control dogs.

2.2. Experimental design
Ten healthy intact adult beagles over the age of range 2.5-3 years, were used in this study and divided into treatment group(n=6, four males and two females) and control group(n=4, one male and three females). All dogs were raised for 10 days and fed full diet twice daily in order to accommodate the environment. Tap water was provided ad libitum. Before study, all dogs received clinical examination and no diseases were detected. Butterfly catheters were inserted into the canine cephalic veins and fixed. Each group was injected with the corresponding solution at post 8 hours after the last injection. Kidney And biochemical analyser (AST), nitrogen (BUN), creatinine (CR), calcium (Ca) and magnesium (Mg) were analyzed by automatic biochemical analyser (AU 480, Backman, USA).

2.4. Biochemical investigations

Blood samples were collected from the cephalic vein of all dogs on days 0 (just prior to injection), 1, 3 and 5. Serum samples were preserved in a refrigerator(-20°C) till analysis. Blood urea nitrogen (BUN), creatinine (CR), alanine aminotransferase (ALT), aspartate aminotransferase (AST), calcium (Ca) and magnesium (Mg) were analyzed by automatic biochemical analyser (AU 480, Backman, USA).

2.5. Pathological examinations

Both kidneys were collected by surgical method at post 8 hours after the last injection. Kidney samples were detected as follows:

2.5.1. Light microscopic examination

Sections of kidney tissue were fixed in 10% neutral-buffered formalin, routinely processed, embedded in paraffin, cut into approximately 4μm thick section, and then stained with H&E, PAS and Pizzolato’s stains. Sections were examined and photos were taken by a light microscope (Leica, Germany).

2.5.2. Electron microscopic examination

Kidney tissues were cut into small pieces (1 mm³) and fixed in 2.5% glutaraldehyde. After rinsed with distilled water, sections were dehydrated in a graded ethanol series and critical-point dried. After mounting on stubs, specimens were coated with a conductive layer and examined by scanning electron microscopy(S-4800; Hitachi, Tokyo, Japan). In the meanwhile, crystal ingredients were analyzed by energy disperse spectroscopy (Noran; Thermo, American). For transmission electron microscopy (TEM), after fixation in 2.5% glutaraldehyde the tissues were washed several times in 0.1 M PBS buffer (pH, 7.4), post-fixed in 2% osmium tetroxide in the same buffer, dehydrated in a graded ethanol series, passed through several changes of 100% acetone, and embedded in epoxy resin. Ultrathin sections of the embedded tissue were examined unstained and observed by transmission electron microscopy (CM100; Philips, Holland).

2.6. Statistical analyses

SPSS software (version 16.0. SPSS Inc., IL, USA) was used for all analyses. Paired t-test was used to compare renal bipolar length and width in each animal before and after injection. One way repeated measures (ANOVA) were used with the data set meeting the assumptions of normality of distribution (Shapiro-Wilk’s test) and sphericity (Mauchly’s test). Figures and tables were constructed using means ± SEM. The time of injection (0, 1, 3 and 5 d) was used as a within subject factor. Bonferroni’s post hoc test helped identify specific differences. Differences were considered significant at P<0.05.

| Table1. Serum concentrations of AST, ALT, CR, Mg and Ca in control dogs on days 0, 1, 3, and 5 (mean ± SEM) |
|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
|                  | Day 0                          | Day 1                          | Day 3                          | Day 5                          |
| AST (U/L)        | 43.33±6.01                     | 40.67±5.46                     | 30.33±2.91                     | 34.67±2.33                    |
| ALT (U/L)        | 58.00±5.86                     | 56.33±2.963                    | 54.67±6.17                     | 56.67±6.89                    |
| BUN (mmol/L)     | 6.33±0.97                      | 5.25±0.38                      | 5.28±1.20                      | 6.50±1.57                     |
| CR (μmol/L)      | 72.93±10.08                    | 76.00±9.07                     | 76.97±15.51                    | 65.77±10.83                   |
| Mg (mmol/L)      | 0.97±0.08                      | 0.89±0.03                      | 0.86±0.07                      | 1.33±0.49                     |
| Ca (mmol/L)      | 2.37±0.04                      | 2.38±0.05                      | 2.25±0.09                      | 2.35±0.04                     |
Table 2. Serum concentrations of AST, ALT, CR, Mg and Ca in treatment dogs injected with 0.5M KOx on 0, 1, 3, and 5 days. (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>44.5±2.21*</td>
<td>36.82±3.60*</td>
<td>44.12±3.56*</td>
<td>41.56±4.66*</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>69.72±19.98*</td>
<td>71.12±25.12*</td>
<td>66.32±23.42*</td>
<td>54.52±17.45*</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>8.04±1.32*</td>
<td>9.38±1.54*</td>
<td>15.44±2.01*</td>
<td>19.96±4.30*</td>
</tr>
<tr>
<td>CR (µmol/L)</td>
<td>100.48±15.29*</td>
<td>118.46±15.61*</td>
<td>163.76±20.59*</td>
<td>206.78±37.57*</td>
</tr>
<tr>
<td>Mg (mmol/L)</td>
<td>0.85±0.06*</td>
<td>0.69±0.19*</td>
<td>0.96±0.08*</td>
<td>0.99±0.08*</td>
</tr>
<tr>
<td>Ca (mmol/L)</td>
<td>2.57±0.15*</td>
<td>1.81±0.58*</td>
<td>2.61±0.10*</td>
<td>2.67±0.07*</td>
</tr>
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Note: (a, b) Within a row, values with different superscripts are significantly different (P < 0.05).

3. Results
3.1. Ultrasonographic findings
There was a mild hyperechogenicity in both cortical and medullary renal tissue after treatment with KOx. There was a significant increase in the biopolar length to 7.14±0.09 cm of the left kidney compared to the pre-injection value (6.052±0.09 cm). The width of left kidney changed from 2.58 ± 0.11 cm before treatment to 3.70 ± 0.07 cm after treatment. Similarly, the biopolar length of the right kidney increased significantly from 5.60 ± 0.13 cm to 6.55 ± 0.18 cm, and the width increased from 2.85 ± 0.11 cm to 3.63 ± 0.29 cm. In contrast, no significant differences were found in the biopolar length, width and echogenicity in control group.

3.2. Serum analysis.
There was no change in serum CR and BUN in the control group (Table 1), but, significant gradual increase in the treatment group on 0, 1, 3, and 5 d post injection (Table 2). There was no significant change in AST, ALT, Ca and Mg levels in any group at any time point.

3.3. Morphological changes
3.3.1. Histopathological findings
Tissue from the control group had no significant lesions. In the treated group, crystals reacted positively with Pizzolato’s stain and localized in the lumens of PCTs, DCTs and CDs (Figure 1). Tissues with crystals had the most severe tubular dilation and hydropic change. Tubular cell necrosis was also present in areas free of crystals. Fan shape intraluminal CaOx crystals rested on the epithelial surface and were associated with microvillus detachment. The medulla had multiple microliths surrounded by congestion, focal fibrin and lymphocyte infiltrates. Some glomeruli had CaOx crystal aggregation within Bowman’s space. H&E and PAS staining showed a few atrophic glomeruli containing eosinophilic proteinaceous material within Bowman’s capsules and tubular lumens (Figure 2).

3.3.2. Scanning electron microscopic examination and energy dispersive spectroscopy analysis
SEM illustrated that crystals were bound to injured epithelial cells. CaOx crystals aggregated and blocked tubular lumens (Figure 3). Microliths were present in renal medullary tubules.

Energy dispersive spectroscopy analysis results revealed that the crystals were high calcium concentration along with carbon and oxygen, which indicated CaOx crystals. CaOx crystals were not detected in control group.
3.3.3. Transmission electron microscopic examination

Ultrastructural abnormalities in treated group included the presence of excessive cytoplasmic vacuoles in PCTs and DCTs. Some PCT cells had clubbing of microvilli, basal infoldings, and trapped mitochondria. Mitochondria swelled and mitochondrial ridge was broken. Rupture and lysis of tubular cells were present. Necrotic cells contained shrunken, abnormal nuclei. Bush border were partly rupture. Crystals were arranged in alternating layers with a thick black matrix composed of protein and cell debris alternating with white layers of ghost crystals (Figure 4).

4. Discussion

Urine concentrations of oxalate and calcium play an important role in CaOx urolith formation in dogs. This process is associated with a complex and incompletely understood sequence of events.

Ultrasonography is used to aid in early disease diagnosis. In the present study, the dogs’ kidneys had an increased cortical and medullary echogenicity. Two previous studies had similar results when ethylene glycol was administered to dogs (Adams et al. 1989; Adams et al. 1991). The authors of that study attributed this change to calcium oxalate crystal deposition within renal tissue. In the study described herein, the renal mass of animals in the experimental group was significantly increased after 7 days of KOx injection. This was manifested by a significant increase in renal BPL and MW of both kidneys. Such an effect may occur as a result of early inflammation accompanying CaOx crystal deposition as evidenced by histopathologic examination.

The significant gradual increase in both serum creatinine and BUN levels after KOx injection in this study indicates renal dysfunction (Grauer et al., 1984 and Emeigh Hart, 2005). Unlike other studies in rats (Marengo et al., 2004; Marengo et al., 2006), KOx did not induce significant changes in either serum or urinary creatinine. The disturbance in normal renal function may be due to the injurious effects of KOx with resultant renal crystal deposition, in addition to hindering the normal filtration process leading to retention of toxic elements.

In the present study, KOx injection in dogs led to crystal deposition in tubular lumens. These crystals migrated to inter- and intracellular locations and eventually into the interstitium. Their movement into the interstitium was associated with inflammatory cell accumulation including lymphocytes and macrophages (de Water et al., 1999; Schepers et al., 2005). Interstitial inflammatory cell infiltrates around crystals may play an important role in renal tissue damage through the production of proteolytic enzymes, cytokines, and chemokines (Lieske and Deganello, 1999 and Khan et al., 2002). Pizzolato’s stain and TEM imaging revealed internalized crystals in tubular epithelial cells which have been previously described. (Schepers et al., 2005).

Crystal retention and deposition was surrounded by tissue debris and fibrin. Crystal deposition and retention are not due to the lodgment of large crystals in tubular lumens, but rather as a result of debris adhesion to injured cells which stimulates additional crystallization (Menon and Resnic, 2002; Asselman et al., 2003). This mechanism explains the relatively high expression of crystal binding proteins such as osteopontin (OPN), hyaluronic acid (HA) and CD 44 (Ymate et al. 1998 and Asselman et al., 2003). X-ray analysis confirmed that the crystals are CaOx, as previously reported (Khan and Hackett, 1993).

TEM revealed damage to renal epithelial cells by the oxalate crystals even when these cells appeared normal by light microscopy. Similar observations have been made in similar studies in dogs, rats and tissue culture (MDCK) and in spontaneous oxalosis in cats.
Multiple basal infoldings of renal tubular cells resulted in coalescence and along with larger distensions are likely related to the movement of extracellular fluid in the injured kidney (Smith et al., 1990). COM matrix was organized in concentric layers of ghost crystals both inside and outside tubular cells as the crystals became tightly embedded in plasma membranes and ultimately resided inside cells (Schepers et al., 2003). The ghost crystals were arranged radially in relation to a central nidus containing electron dense material. In a previous study, CaOx ghost crystals were arranged as rosettes around a central nucleation site (Khan, 2011). Ghost crystals are presumably formed by dissolution of COM crystals and electron dense material of cellular degradation products that include nuclei, mitochondria, endoplasmic reticulum, membrane fragments and vesicles (Schepers et al., 2003 and Khan, 2011). We postulate that the electron dense matrix protects renal epithelial tissue from direct contact with the crystals. Mitochondrial collapse is significant in calcium oxalate crystal deposition because mitochondria store a considerable amount of calcium ions which are discharged into the cytosol causing further crystallization (Niimi et al., 2012).

We conclude that direct intravenous injection of KOx for 7 consecutive days induced CaOx crystal deposition in renal tissue of canids and induced obvious damage to the kidney.

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6. References


