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Potassium oxonate induces acute hyperuricemia in the tree shrew (*tupaia belangeri chinensis*)

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Abstract: Potassium oxonate, a selectively competitive uricase inhibitor, produced hyperuricemia (HUA) in rodents in a previous study. In this study, we employed the tree shrew as an animal model to study potassium oxonate-induced HUA. The effect of allopurinol (ALLO), a uric acid reducer, was also examined in this model. Potassium oxonate at doses of 5, 20, 40, 60, 80, 100, and 1,000 mg/kg was given intraperitoneally to tree shrews. The results showed that potassium oxonate can effectively increase the levels of uric acid in tree shrews at doses ranging from 40 to 100 mg/kg. Semiquantitative RT-PCR showed that the xanthine dehydrogenase/oxidase *(XDH/XO)* mRNA expression level was significantly higher in the liver tissue of tree shrews with high levels of uric acid. There were no changes in serum urea nitrogen, or serum creatinine values. ALLO can significantly decrease serum uric acid levels (*P*<0.01) and raise *XDH/XO* mRNA expression levels in the liver tissue of tree shrews with HUA. *XDH/XO* mRNA expression levels did not change in untreated tree shrews. Studies on acute toxicity in the tree shrew did not show any significantly abnormal signs. There were no adverse effects at the macroscopic level up to doses ≤100 mg/kg. Potassium oxonate induced acute HUA in tree shrews at lower doses compared with other animal models. Potassium oxonate-treated tree shrews may be a potential animal model for studying pathogenic mechanism and evaluating a new therapeutic agent for treatment of HUA in humans.
Key words: allopurinol, hyperuricemia, potassium oxonate, tree shrew, xanthine dehydrogenase/ oxidase (XDH/XO)

Introduction

Uric acid, the end product of purine metabolism in the human body, is produced mainly in the liver by xanthine dehydrogenase/oxidase (XDH/XO) and excreted through the kidney. Hyperuricemia (HUA) is characterized by an elevated level of uric acid in the blood. Gout is a disorder caused by the deposition of monosodium urate crystals in the joints and other tissues because of extracellular urate supersaturation [7]. HUA is defined as a serum urate concentration that allows urate saturation in body fluids (>7.0 mg/dl, equal to 416.5 μmol/l). The incidence of gout and HUA in humans has been increasing in recent times [13]. Currently, China has about 120

million patients with HUA, accounting for approximately 10% of the total population. Middle-aged or older men and postmenopausal women usually have a higher incidence of HUA. However, in recent years, the onset age is getting lower. HUA is also associated with the pathogenesis of hypertension and metabolic syndrome. According to clinical reports, the key causative agent, uric acid, is associated not only with an increased risk of gout, but also with an increased risk of cardiovascular disorders, nephrolithiasis, diabetes, obesity, and dyslipidemia [1, 5]. Therefore, there is an urgent need for a reasonable widely recognized, reasonable animal model for HUA studies not only for drug screenings, but also for pathogenesis research.

Potassium oxonate, a selectively competitive uricase inhibitor, blocks the effect of hepatic uricase and produces HUA in rodents [14, 16, 18, 19]. There are several limitations to the rodent (rat and mouse) HUA models, which are most commonly used at present [12, 14, 16, 18, 19]. Studies using rodent models [12, 14] have indicated a need for another animal model for studying drug sensitivity and stability of potassium oxonate. Mice and rats have a relatively low sensitivity to drugs. Furthermore, despite the fact that mice, rats, and humans are all mammals, mice and rats are taxonomically far from that of humans. Mice and rats also possess anatomical and physiological characteristics different from those of humans. Thus, the parameters derived from working with these animal models are of limited use as reference values. Therefore, establishing and developing new HUA animal models with serum uric acid metabolism that is similar to that of humans and performing an in-depth study on them is an urgent need in the field of HUA research.

The tree shrew (*Tupaia belangeri chinensis*) is a non-rodent mammal, phylogenetically located between insectivores and primates. Currently, the tree shrew is classified within its own order Scandentia. Tree shrews are small squirrel-like animals, found mainly in South Asia, Southeast Asia, and Southern China [2, 4, 17]. The tree shrew (*T. belangeri chinensis*) is a new experimental animal that is being used widely in medical and biological research, because of its unique characteristics such as its small size, ease of feeding, convenient management, low price, and its high degree of evolution. Its metabolism and gross anatomy are closer to those of humans compared with other animals such as dogs, rats, and mice [11]. Tree shrews are relatively easy to obtain-

able and simple to handle, as opposed to primates. Recent studies using whole genomic sequences have suggested that tree shrews are more closely related to primates than to rodents [4]. In an earlier study, we prepared cDNA from fresh livers of tree shrews and determined the sequence of an *XDH/XO* nucleotide fragment. Sequence alignment studies showed that the human and tree shrew nucleotide fragment sequences of *XDH/XO* were 87.04% identical (manuscript submitted). Based on these findings, we hypothesized that the tree shrew might be a potential animal model for studying the pathogenesis of HUA. In this study, we employed the tree shrew as the animal model to study potassium oxonate-induced HUA. The effect of allopurinol, a uric acid reducer, was also examined in this model. The serum urate levels, *XDH/XO* mRNA expression level in liver tissue, serum urea nitrogen, and serum creatinine (Cr) values associated with acute toxicity were investigated.

Materials and Methods

Reagents

Potassium oxonate was purchased from Sigma-Aldrich Corporation Co. (lot#STBC6418V). ALLO was purchased from Nanjing Dolai Biological Co., Ltd. Sodium carboxymethyl cellulose (CMC-Na) was purchased from Chengyue Technology Co. Uric acid (UA) assay kit, Cr and blood urea nitrogen (BUN) assay kits were purchased from Nanjing Jiancheng Biological Engineering Institute. A Transcriptor First Strand cDNA Synthesis Kit (cat# 04897030001) was purchased from Roche Co. Premix Taq™ (code No. RR003A) and RNAiso Plus (Lot#AK8504) were purchased from Takara Biotechnology (Dalian) Co., Ltd.

Animals and ethics statement

A total of two hundred 2- to 3-year-old adult male and female Chinese tree shrews (*Tupaia belangeri chinensis*) were used for this study. Body weight ranged from 110 to 140 g. The tree shrews were from the colony of the Medical Primate Research Center of the Institute of Medical Biology (Certificate No. is SCXK (Dian) K2013-0001). All animals were fed *ad libitum* with regular mixed soft food. The feed formula, which was based on the nutritional needs of the experimental tree shrews, was≥200 g crude protein,≥50 g crude fat, ≤30 g crude fiber, ≤60 g crude ash, 10–15 g calcium, and 6–8 g phosphorus, per kilogram of the diet, with a Ca/P ratio

of 1.2:1–1.4:1; the tree shrews also received fresh fruits. The animals were maintained individually in cages (100 cm length, 50 cm width, 40 cm height) at a temperature between 18 and 27°C (the difference in temperature between day and night was ≤4°C), at a relative humidity of approximately 60%, and with a 12 h light/dark cycle. The air change frequency was≥10 times/h. Other conditions included an ammonia concentration of ≤14 mg/m^3, noise level ≤60 dB (A), and minimum illuminance of 150 lx (the level of illumination in a conventional experimental animal environment is 15–20 lx). In this facility, a single corridor with buffer rooms separates the streams of people, objects, and animals from each other. The facility certificate No. for this facility is SYXK (Dian) K 2013–0001. For ethical treatment of tree shrews in compliance with current animal welfare guidelines, humane endpoints were designed to minimize animal suffering [15]. The following criteria were used to identify moribund animals: major organ failure or severe respiratory distress, labored breathing, and abnormal vocalization when handled. Animals were humanely euthanized by asphyxiation using CO_2, if these end-point signs and symptoms appeared. All animal care and experimental protocols were approved by the Animal Care and Use Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences/Peking Union Medical College. (approval No.: 2015–005).

Measuring fasting serum uric acid levels in tree shrews

Fasting blood samples were collected from the tail veins of 200 tree shrews. Serum was separated by centrifugation at 5,000 g at 10°C for 15 min. Uric acid levels were measured within 2 h of preparing the serum to avoid errors from uric acid degradation. Serum uric acid values were measured using commercially available UA assay kits, and the serum uric acid values of male and female animals were grouped by gender.

Effectiveness of potassium oxonate in inducing acute HUA in tree shrews

Tree shrews were randomly assigned to 3 groups of 6 each. The control group was injected intraperitoneally with 1% CMC-Na, whereas the others were intraperitoneally injected with a potassium oxonate suspension in 1% CMC-Na at a dose of 100 or 1,000 mg/kg. Blood samples were collected at 0, 2, 4, and 12 h after the administration. Serum was separated by centrifugation at 5,000 g at 10°C for 15 min for analysis of the levels of SUA, Cr, and BUN.

Serum uric acid levels were measured using commercially available UA assay kits. Serum Cr levels and BUN levels were also measured using assay kits.

Dose-dependent effectiveness of potassium oxonate in inducing acute HUA in tree shrews

Tree shrews were randomly assigned to 6 groups of 6 each. The control group was injected intraperitoneally with 1% CMC-Na, whereas the others were injected intraperitoneally with potassium oxonate suspension in 1% CMC-Na at doses of 5, 20, 40, 80, or 100 mg/kg. Blood samples were collected 0, 1, 2, 4, and 8 h after administration. Serum uric acid levels were determined as described above.

Confirmation with ALLO in tree shrews with acute HUA

Control animals were injected intraperitoneally with 1% CMC-Na. Experimental groups received the following intraperitoneal injections: 80 mg/kg oxonate suspension in 1% CMC-Na, 80 mg/kg oxonate suspension in 1% CMC-Na + 4 mg/kg ALLO, 80 mg/kg oxonate suspension in 1% CMC-Na + 30 mg/kg ALLO, or 30 mg/kg ALLO. ALLO was administered to the animals in the 80 mg/kg oxonate + 4 mg/kg ALLO group and 80 mg/kg oxonate + 30 mg/kg group, half an hour after oxonate injection. Blood samples were collected at 0, 1, 2, 4, and 8 h after administration. Serum uric acid levels were determined as above.

Approximately 100 mg of fresh liver tissue was collected surgically from 2 animals in each group, 2 h after treatment. The tissue samples were stored in liquid nitrogen until total RNA extraction.

RNA isolation and reverse transcription PCR to analyze the expression of XDH/XO mRNA in liver tissue

A semiquantitative RT-PCR approach was used to measure *XDH/XO* mRNA levels. Total RNA was extracted from liver tissues using RNAiso Plus (Takara, Japan), and was reverse-transcribed to cDNA, in accordance with the manufacturer's protocol. Specific transcripts were measured by semiquantitative PCR.

GAPDH mRNA was used as an internal control; *XDH/XO, GAPDH*, and the negative control were amplified on the same plate. The following *XDH/X*O, and *GAPD*H-specific primers were designed using the Primer 5.0 software: *XDH/XO* sense, 5′-GTTG-GAGGGAACATCATCACT-3′, *XDH/XO* antisense, 5′-GCAGGGTCTTTCTGTAGCCA-3′, *GAPDH* sense,

5′-CAAGAAGGTAGTGAAGCAGGC-3′, *GAPDH* antisense, 5′- TGTTGAAGTCGGAGGAGACC-3′. PCR was performed as follows: initial denayuration for 5 min at 94°C; 35 cycles of 30 s at 98°C, 30 s at 59°C, and 1 min at 72°C; and then final elongation for 10 min at 72°C. PCR products were electrophoresed on 1.5% agarose gels and visualized with a Bio-Rad ChemiDoc XRS gel documentation system, and then quantified using the Bio-Rad Quantity One 1D analysis software. Relative quantification of PCR products was performed by normalization to the level of *GAPDH* mRNA.

Acute toxicity studies

Control and treated groups consisted of 6 animals each. The treated groups received, by intraperitoneal injection, 5, 20, 40, 80, or 100 mg/kg of potassium oxonate, and the control group received 1% CMC-Na. The maximum concentration of the suspension injected was 1,000 mg/ml. Mortality, body weights, and behavior of the animals were observed and recorded daily for 15 days. Macroscopic changes in the treated group were compared with those of the control group.

Kidneys from the dead animals were fixed in 10% phosphate-buffered formalin (pH 7.1) for renal pathology detection. A conventional morphological evaluation was performed by a commercial company.

Statistical analysis

The values shown represent the mean ± SEM. Data were analyzed using a one-way ANOVA followed by the Tukey post hoc test for multiple comparison using the Prism 6 software from GraphPad Software Inc. (San Diego, CA, USA). Differences were considered significant (asterisk) when $P<0.05$.

Results

Fasting serum uric acid levels in tree shrews

The average value of serum uric acid for the 200 adult tree shrews was 175.76 ± 53.14 μmol/l. The average value for the females was 168.98 ± 53.31 μmol/l, whereas that for the males average was 185.81 ± 50.44 μmol/l.
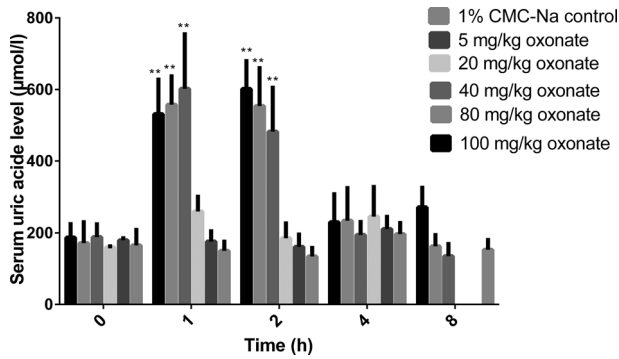
Effectiveness of potassium oxonate in inducing acute HUA in tree shrews

Figure 1A shows that intraperitoneal injection of 100 or 1,000 mg/kg potassium oxonate could significantly increased the serum uric acid levels in tree shrews,
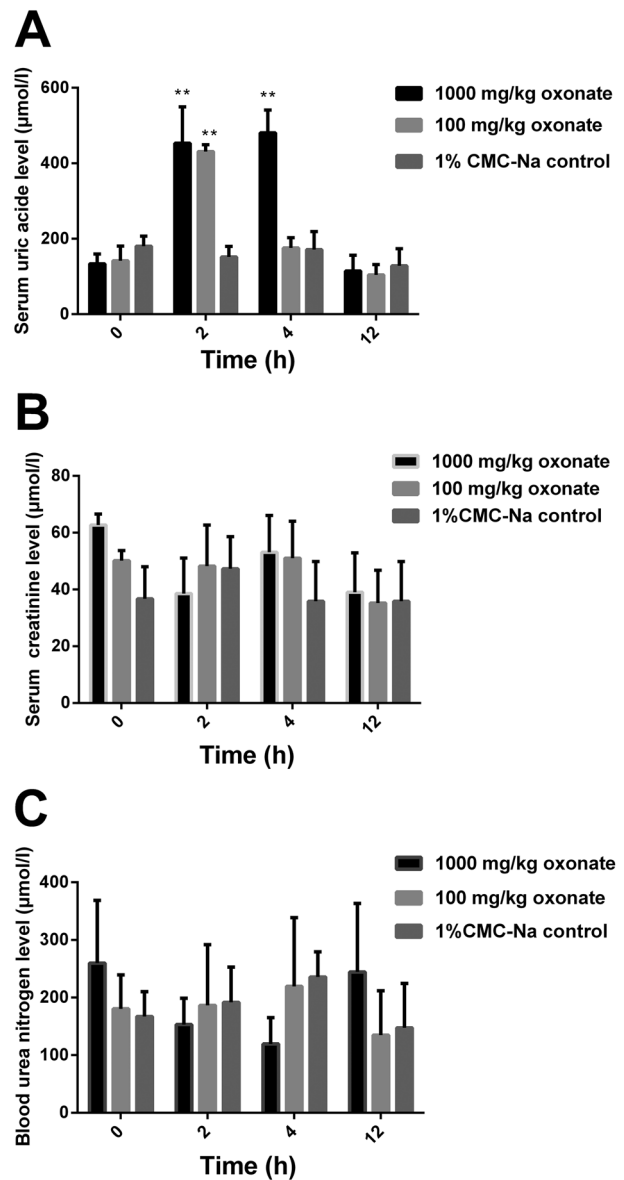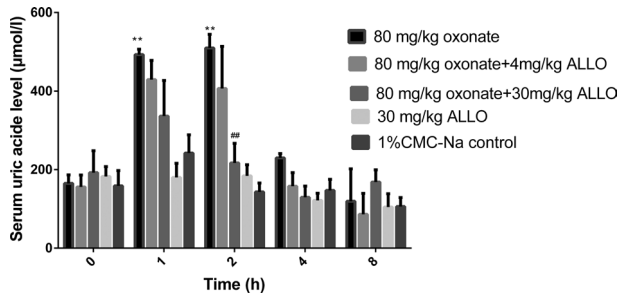

Fig. 1. Effectiveness of potassium oxonate in inducing acute hyperuricemia in tree shrews. Values are mean ± SEM values, with n=6 in each group. Significant differences were observed between the potassium oxonate and control groups (*$P<0.05$ and **$P<0.01$). (A) Serum uric acid levels, (B) Serum creatinine levels, (C) Blood urea nitrogen levels.

within 2 h. In the group treated with 1,000 mg/kg potassium oxonate, the serum uric acid level increased from 133.54 ± 26.39 μmol/l at 0 h to 453.01 ± 96.94 μmol/l in 2 h and reached 480.57 ± 60.76 μmol/l by 4 h. The serum uric acid levels of the tree shrews treated with 100 mg/kg potassium oxonate dose increased from 141.89 ± 39 μmol/l to 431.24 ± 18.36 μmol/l at 2 h after injection. The serum uric acid levels in both the groups were

Fig. 2. Dose-effect relationship of potassium oxonate in inducing acute hyperuricemia in tree shrews. Values are mean ± SEM values, with n=6 in each group. Significant differences were observed between the potassium oxonate and control groups (*$P<0.05$ and **$P<0.01$).
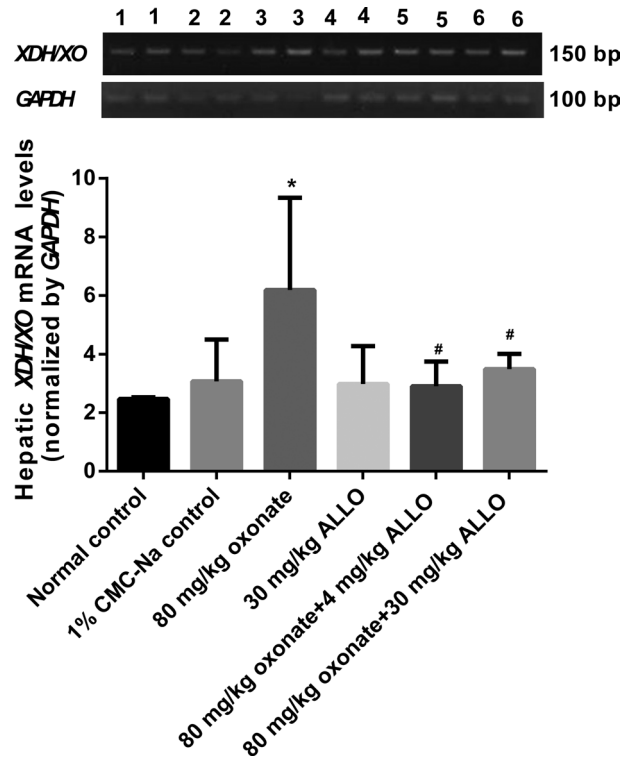

Fig. 3. Effect of ALLO on the level of serum uric acid in tree shrews with acute hyperuricemia. Values are mean ± SEM values, with n=6 in each group. Significant differences were observed compared with the control (*$P<0.05$ and **$P<0.01$). Significant differences were observed compared with the hyperuricemia tree shrew (#$P<0.05$ and ##$P<0.01$).

Fig. 4. Semiquantitative PCR to analyze the expression of *XDH/XO* mRNA in liver tissue of the tree shrew. Values are mean ± SEM values. Significant differences were observed compared with the control (*$P<0.05$ and **$P<0.01$). Significant differences were observed compared with the hyperuricemia tree shrew (#$P<0.05$ and ##$P<0.01$) (Lane 1, Normal control; 2, 1% CMC-Na control; 3, 80 mg/kg oxonate; 4, 30 mg/kg ALLO; 5, 80 mg/kg oxonate + 4 mg/kg ALLO; 6: 80 mg/kg oxonate + 30 mg/kg ALLO).

higher than the uric acid crystallization point (417 μmol/l). There were a significant differences between the serum uric acid levels in the potassium oxonate-treated groups and those in the control group (**$P<0.01$). There were no changes in the levels of serum urea nitrogen or serum Cr (Figs. 1B and C).

Dose-dependent efficacy of potassium oxonate in inducing acute HUA in tree shrews

Figure 2 shows that injecting 40, 80 or 100 mg/kg potassium oxonate resulted in a significant increase in serum uric acid levels at both 1 and 2 h after administration time points, to 480 μmol/l or more, which is significantly higher than the saturation concentration of uric acid solution at which crystallization can occur ($P<0.01$). The serum uric acid level started to decline 4 h after administration. In comparison, the serum uric acid level increased

only slightly to 259.61 ± 41.51 μmol/l at 1 h after administration in the group treated with 20 mg/kg potassium oxonate. ($P<0.01$ compared with that of the control group at the same time point). It did not reach the crystallization point. The serum uric acid level in this group dropped after 2 h. Administration of 5 mg/kg potassium oxonate had no effect on the serum uric acid level.

Confirmation with ALLO and quantitative RT-PCR to analyze of XDH/XO mRNA expression in liver tissue

As shown in Fig. 3, administering 30 mg/kg ALLO to tree shrews with HUA (80 mg/kg potassium oxonate group) effectively reduced the serum uric acid levels, whereas it had no effect on the levels of the control tree shrews. Moreover, ALLO significantly downregulated, the expression of *XDH/XO* mRNA, compared with the levels of the control group (1% CMC-Na) and non-injected tree shrew control (Fig. 4).
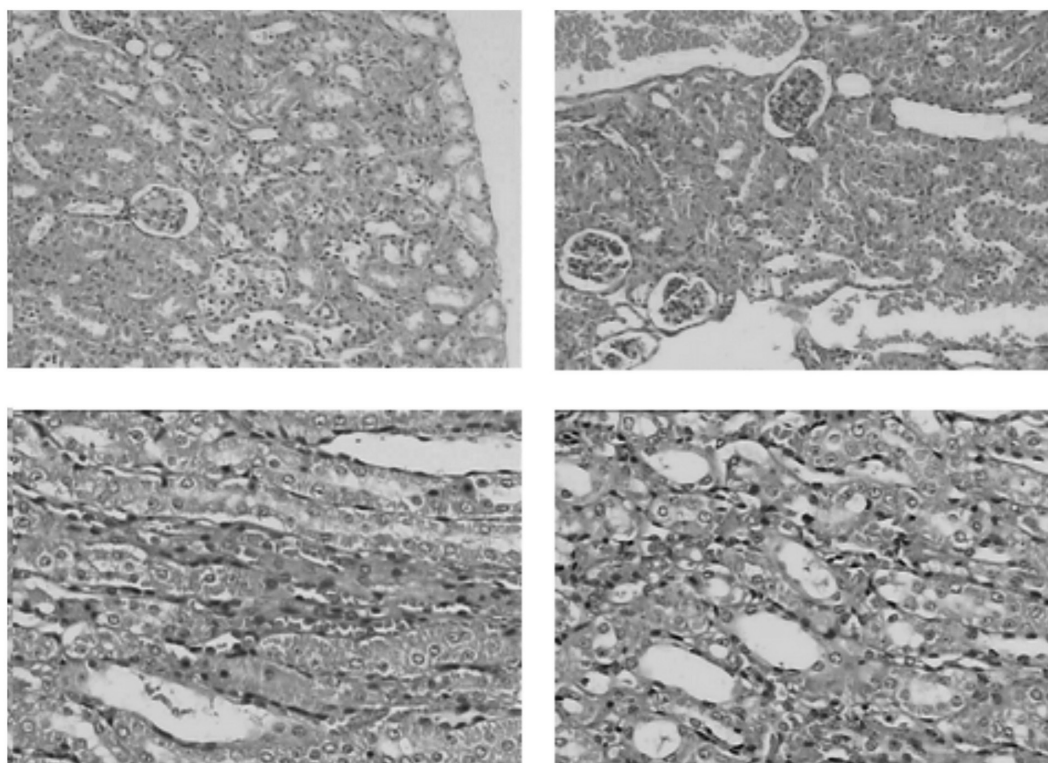
Fig. 5. Renal pathology showed renal tubules renal congestion and swelling at a dose of 1,000 mg/kg of oxonate.

Acute toxicity studies

An acute toxicity study revealed that animals injected with potassium oxonate at dosages of 5, 20, 40, 80, or 100 mg/kg did not show any gross abnormalities, behavioral changes, body weight changes, or macroscopic changes at any time of observation. No mortality was observed during the 15 days of the experiment with the exception of two animals that received the dose of 1,000 mg/kg and died at 4 and 15 h after treatment, respectively. There were no internal regions of abnormality with the exception of the kidneys, which showed necrosis. Renal pathology detected tubule congestion, and swelling (Fig. 5).

Discussion

Tree shrews are small animals that are more closely related to primates than to rodents. The aim of the present study was to investigate whether the tree shrew can be used as an animal model for potassium oxonate-induced HUA. The findings were confirmed with ALLO administration experiments and measurement of *XDH/XO* mRNA expression in liver tissue. In this study, potassium oxonate was injected intraperitoneally, because the oral and intragastric routes of administration, intraperitoneal route offered the advantages of accuracy of delivery, ease, and simplicity of administration. After several experiments, 80 mg/kg was found to be the optimal dose for this study.

Previous studies [3, 6, 14] have used potassium oxonate to induce HUA in rodent animal models for drug evaluation purposes. However, due to the complexity of uric acid metabolism *in vivo* and species differences between experimental animals, HUA induced by different routes of administration and doses of the same compound was different in different animal models. Therefore, the dose of potassium oxonate used varied in different studies. There are studies reported in the literature that used potassium oxonate doses as high as 500 mg/kg [3], 400 mg/kg [10] and 250 mg/kg [9] in mice, and 250 mg/kg [8, 14, 15] rats when administered.

In contrast, our results showed that HUA could be induced stably in the tree shrews at the comparatively low dose of 80 mg/kg, which also increased significantly the expression of *XDH/XO* mRNA in liver tissue. ALLO, a competitive inhibitor of XO, is commonly used

in the treatment of gout and metabolic syndrome caused by HUA. In this study, we used ALLO to reduce the serum uric acid level in the tree shrews. Our results showed that ALLO could reduced the serum uric acid and *XDH/XO* mRNA expression levels in tree shrews with HUA, whereas it did not decrease the serum uric acid and *XDH/XO* mRNA expression levels in normal tree shrews.

Since the tree shrew is a new type of experimental animal, it is necessary to measure the fasting serum uric acid level in healthy individuals. We measured the serum uric acid levels in 200 adult tree shrews. The average value for the 200 animals was 175.76 ± 53.14 μmol/l. The average for females was 168.98 ± 53.31 μmol/l and that for males was 185.81 ± 50.44 μmol/l, theses are similar to those in humans.

Tree shrews and rats are similar in size, but are different in their sensitivity to drugs. Preliminary experiments in our previous study that assessed the dose-dependent induction of acute hyperuricemia by potassium oxonate in Wistar rats and ICR mice suggested a difference in sensitivity to potassium oxonate between mice and rats (unpublished data). The potassium oxonate doses used in mice and rats in these experiments were similar to those reported earlier [3, 6, 9, 14]. In contrast, acute HUA was induced in tree shrews at lower doses of potassium oxonate compared with in other rodent animal models [9, 14, 18]. Thus, potassium oxonate-treated tree shrews may be a potential animal model for studying pathogenic mechanisms and developing a new therapeutic agent for the treatment of HUA. We demonstrated in this study for the first time that potassium oxonate-treated tree shrews exhibited HUA symptoms. Additionally, potassium oxonate-treated tree shrews exhibited these symptoms at a lower dosage compared with that needed in rodents. This animal model is stable and reproducible. Hence, the tree shrew promises to be a useful animal model for studying the pathogenesis of HUA.

Conflict of Interest

The authors declare that they have no conflicts.

Acknowledgments

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