Inhibitory Effect of Hydroxycitrate on Calcium Oxalate Crystal Formation in a Drosophila Model

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Abstract In this study, we aimed to compare the effects of hydroxycitrate (HCA) and potassium citrate (PC) on the prevention and treatment of calcium oxalate (CaOx) stones in a fruit fly model. Drosophila melanogaster was used as an insect model of lithogenesis. The lithogenic agent used was 0.25% ethylene glycol. For determining the preventive effects, 2% PC and 2% HCA were added along with the lithogenic agent at the start of experiment. For determining the treatment effects, the lithogenic agent was added at the start of experiment to induce crystal formation, and 2% PC and 2% HCA were added from the third week. After 3 weeks, the Malpighian tubules of Drosophila were observed under polarized light microscopy, and the results were calculated. The preventive effect on the formation of CaOx in PC group was 9.38 ± 4.42% and in HCA group was 4.51 ± 3.85%. The treatment effect of PC was 56.90 ± 20.43% and that of HCA was 39.09 ± 15.36%. HCA has both preventive and treatment effects on the formation of CaOx crystals in the Malpighian tubules of Drosophila, and the effects were better than those of PC.

Keywords: calcium oxalate, citrate, hydroxycitrate, drosophila melanogaster, Malphigian tubules


1. Introduction

Potassium citrate (PC) has been used as a standard preventive drug for patients with calcium oxalate (CaOx) stones [1,2]. PC is a classical drug for the prevention of CaOx stone formation [3,4,5] and has been extensively studied. It has been reported to possess the ability to inhibit crystal formation by chelation of calcium ions in urine [6,7]. A novel drug hydroxycitrate (HCA) has a structure similar to citrate, with a difference of a single alcohol group. It not only exerts excellent inhibitory effect on CaOx crystal nucleation, but also reduces the growth rate of CaOx crystal [8]. Therefore, HCA is a more effective inhibitor than PC. In the study by Chung et al., HCA inhibits CaOx crystallization regardless of the alkalinity of the solution. Thus, HCA may have potential as a novel drug for both prevention and dissolution of stones. However, further studies in animals and humans are required before application for clinical use.

We established a versatile model using Drosophila melanogaster (fruit fly) to investigate the formation of CaOx as well as the inhibitory effects of HCA [9,10,11]. Since an animal study of the effects of HCA on the prevention and treatment of CaOx crystal formation was warranted to confirm the findings of previous studies, we used Drosophila for investigating the dissolution effects of HCA. In this study, we aimed to investigate the preventive and treatment effects of HCA in comparison with potassium citrate on CaOx crystal formation in a Drosophila model.

2. Materials and Methods

2.1. Preparation of Flies and Stock

The lithogenesis animal was wild-type male flies, Drosophila melanogaster CS. The preparation of experiment was according to our previous published articles [9,10,11]. In brief, flies were bred in plastic vials containing standard medium for fly (agar, yeast, corn syrup, and sugar), at 25°C, 50–60% humidity, with a 12-h light–dark cycle.

2.2. Lithogenesis of Flies

This study of fly CaOx crystal formation was divided into two experimental models. The lithogenic agent was 0.25% ethylene glycol (EG) added in the fly medium (wt/vol) in each group of flies. The first experiment was
designed as comparative preventive effect of 2% PC and 2% HCA. All the agents were added since the start of experiment until the end of study. The second experiment was designed as treatment effect of 2% PC and 2% HCA. Flies were fed with 0.25% EG from the beginning and last to the end of experiment. The addition of PC and HCA started from the third week to the end of experiment. After 3 weeks, the flies (200 flies for each group) were killed under CO2 narcotization, and removed the Malpighian tubules. Dissection and processing tubules were observed under polarized light microscopy (Olympus BX51 optical microscope, Tokyo, Japan).

2.3. Survival Rate of HCA on Fly

We set up lifespan assay for HCA on a fly model according to our previous report [9,10,11]. In brief, new fly emergents were collected in foam plugs and kept horizontally. Flies were divided into two groups (n=150 in each group) in term of control and 2% HCA. We counted survivors in each vial and removed dead flies daily. Life spans of control and 2% HCA were compared and tested for significance with log-rank test.

2.4. Polarized Light Microscopy Observation

The relevant aspects were photographed and the scales were obtained. The degree of CaOx crystal formation in each group was recorded and calculated. The degree of CaOx crystal formation were defined as grade 1, 2, and 3 according to previous reports [9,10,11].

2.5. Statistical Analyses

One-way analysis of variance (ANNOVA) was applied to detect overall differences among the groups; for all multiple comparisons, Bonferroni correction was applied. Significantly different groups were compared pairwise using the Mann–Whitney U-test for crystal scores. All statistics were done using the SigmaStat software (SPSS; Systat Software, San Jose, CA).

3. Results

CaOx crystal formation induced by 0.25% EG in the prevention and treatment groups was 67.71 ± 1.47% and 70.04 ± 16.16%, respectively (Figure 1). In the first study, the preventive effects were 9.38 ± 4.42% and 4.51 ± 3.85% in PC and HCA groups, respectively (Table 1). HCA exhibited a better preventive effect than PC on CaOx crystal formation in the Malpighian tubules of Drosophila.

Table 1. Comparative results of potassium citrate and hydroxycitrate in the prevention of calcium oxalate crystal formation in the Malpighian tubules of fly

<table>
<thead>
<tr>
<th>Total Fly Number</th>
<th>CaOx Crystal Formation ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>48</td>
</tr>
<tr>
<td>0.25% EG</td>
<td>143</td>
</tr>
<tr>
<td>0.25% EG + 2% PC</td>
<td>152</td>
</tr>
<tr>
<td>0.25% EG + 2% HCA</td>
<td>139</td>
</tr>
</tbody>
</table>

*EG: ethylene glycol, PC: potassium citrate, and HCA: hydroxycitrate.

The treatment effect of PC on CaOx formation rate in the Malpighian tubules of Drosophila was 56.90 ± 20.43% (Table 2). However, HCA had a better treatment effect than PC, with a crystal formation rate of 39.09 ± 15.36%.

Table 2. Comparative results of potassium citrate and hydroxycitrate in the treatment of calcium oxalate crystal formation in the Malpighian tubules of fly. EG was added from the start of experiment until the end of study. PC and EG were added from the third week to the end of study

<table>
<thead>
<tr>
<th>Total Fly Number</th>
<th>CaOx Crystal Formation ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>133</td>
</tr>
<tr>
<td>0.25% EG</td>
<td>221</td>
</tr>
<tr>
<td>0.25% EG + 2% PC</td>
<td>247</td>
</tr>
<tr>
<td>0.25% EG + 2% HCA</td>
<td>242</td>
</tr>
</tbody>
</table>

*EG: ethylene glycol, PC: potassium citrate, and HCA: hydroxycitrate

Survival analysis of 2% HCA group was compared with blank, 0.25% EG, and 2% PC groups (Figure 2). The results showed that the 0.25% EG group demonstrated significantly lower survival than the blank groups (log
compared with the blank. 2% HCA and 2% PC revealed no statistical difference (P values less than 0.001, respectively) (Table 1). The survival curve of 2% HCA and 2% PC revealed no statistical difference compared with the blank.

Figure 2. Lifespan of control, EG, PC, and HCA-treated flies. Cumulative survival distributions by administration of different drugs (n = 150 for each group, P < 0.05 from log-rank test)

4. Discussion

We observed a marked decrease in crystal formation rate by HCA in both preventive and treatment groups. These results confirm the in vitro findings of Chung et al. that CaOx crystal formation can be treated and prevented [8]. This is the first experimental report of HCA on the treatment of urolithiasis in an insect model. For determining the preventive effects on CaOx crystal formation, both HCA and lithogenic agent were added at the start of the experiment. For determining the treatment effects, the lithogenic agent was added at the start of the experiment to induce crystal formation, and HCA was added from the third week and the effects of HCA were observed.

Chung et al. proposed two molecular inhibitors of CaOx crystallization, namely, PC and HCA in vitro. PC and HCA exhibited a mechanism different from the classical theory of crystal growth inhibition [8]. HCA not only chelated calcium to inhibit crystal growth, but was also adsorbed on crystal surfaces to induce dissolution of the crystal under specific conditions. Their hypothesis that inhibitor-crystal interactions impart localized strain to the crystal lattice, and oxalate and calcium ions are released into solution to alleviate this strain is confirmed by in situ atomic force microscopy and density functional theory studies. Chung et al. reported HCA as an alternative to citrate for the treatment of kidney stones. Although we found significant inhibition of crystal formation in the treatment group in Drosophila (invertebrate) model, this potential should be further investigated in vertebrates and in clinical trials.

HCA has inhibitory effects on food intake and has been proposed as a weight reducing agent [12-16]. Laboratory and animal studies of HCA have produced results that show its potential for modulation of lipid metabolism [17]. In animal model, HCA may reduce body weight regain after effects on large amount weight loss, which is hypothesis of inhibiting effect on lipogenesis [14,15]. However, HCA did not have any effect on metabolism parameters, and its anorectic effect was not caused by increasing hepatic fatty acid oxidation [18]. Therefore, the exact mechanism underlying the weight reducing effects of HCA remains to be elucidated. Furthermore, the results of clinical trials performed in overweight humans to determine the effects of HCA on weight reduction do not support the hypothesis of inhibitory effect on satiety, fat oxidation, energy expenditure, and body weight loss in animal study [19]. Owing to these contradictory results, the clinical use of HCA in weight reduction is limited. However, a clinical study demonstrated that HCA has no effect on weight loss or reduction of fat mass [20]. A meta-analysis published in 2010 revealed that HCA users were twice as likely to develop gastrointestinal adverse effects [21].

Direct ingestion of HCA-containing herbal products is dangerous. A case report of severe liver toxicity after consuming a HCA-containing herb has been reported [22]. This case was reviewed and dismissed because the patient had also taken aspirin and acetaminophen concomitantly [23]. Another case of dangerous hepatic toxicity requiring liver transplantation in a patient who consumed hydroxycitric acid, the active ingredient in Garcinia cambogia extract (dietary supplement) and Garcinia cambogia-containing products, was reported by Lunsford et al. [24]. A post-marketing safety should be well surveillance was claimed by Lobb due to several liver toxicity reports [25]. HCA was found to be highly toxic to the testis in male Zucker obese rats [26]. Although Stohs et al. reported that there was no evidence of HCA toxicity in 2009 [23], new cases of toxicity were reported in 2016 [24]. Therefore, toxicity studies on HCA need to be performed. Our animal study revealed no lethal effects on survival rate. However, as our study was performed in an invertebrate species, these findings cannot be directly applied to humans [27].

In survival analysis, we found that HCA did not affect the survival of EG-induced lithogenic flies. The flies treated with EG alone had a short survival curve than those treated with both EG and HCA. These data revealed that HCA may have a protective effect on EG-induced toxicity. Nevertheless, the life span of HCA plus EG-treated group was shorter than that of the control group, indicating that HCA did not reverse the toxicity of EG completely in long-term feeding. Furthermore, flies treated with HCA alone revealed a relatively shorter life span than the controls. Therefore, further research on the long-term preventive effects of stone formation by HCA (i.e. life-long) is required.

The present study has the following limitations: (1) this study used an invertebrate model; (2) crystal formation was investigated in the insect model instead of real stone formation; (3) only a single dose was administered; and (4) there was lack of evidence of HCA in the Malpighian tubules of Drosophila. However, we performed the survival analysis of HCA and found HCA to be safe. Since Chung et al. have confirmed the presence of HCA in human urine [8], HCA can be used clinically.
5. Conclusions

In this study, HCA demonstrated a more potent inhibitory effect on CaOx crystal formation than PC. HCA showed a preventative effect on CaOx on crystal formation and decreased the number of crystals in pre-treated lithogenic flies. These findings show that HCA has the potential to dissolve CaOx crystals. Since HCA was found to be safe in Drosophila, clinical trials are warranted to confirm the potential of HCA.

Acknowledgements

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References