Mechanisms of star fruit-induced acute renal failure

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Received 26 June 2007; accepted 9 January 2008

Abstract

We have previously discovered that star fruit can induce oliguric acute renal failure. To investigate the mechanisms of star fruit-associated acute oxalate nephropathy, the nephrotoxic effect of star fruit was examined in both cellular experiments and animal models. We evaluated renal function, pathological changes in kidney tissues and apoptotic effects using terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay in four groups of rats – a control group (CG), fed with tap water (1); a star fruit group (SG), fed with star fruit juice naturally containing 0.2 M oxalate (2); and oxalate groups (OxG), fed with 0.2 M (3) or 0.4 M (4) oxalate solution. The effects of both star fruit juice and oxalate on MDCK cells were also analyzed by flow cytometry. We found that the mean creatinine clearance was significantly lower in the SG, 0.2 M OxG and 0.4 M OxG. Dose-dependent apoptotic effects were evident from the TUNEL assay, and flow cytometry analysis of treated MDCK cells showed dose- and time-dependent effects. Our findings suggest that star fruit juice produces acute renal injury, not only through the obstructive effect of calcium oxalate crystals, but also by inducing apoptosis of renal epithelial cells, which may be caused by the levels of oxalate in the fruit.

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Keywords: Apoptosis; Star fruit; Oxalate; Acute renal failure

1. Introduction

In a recent study we observed that star fruit could induce oliguric acute renal failure, and the pathological findings of kidney sections revealed typical changes of acute tubular damage with diffuse oxalate deposition (Chen et al., 2001; Fang et al., 2001). Star fruit, a member of the Oxalidaceae family, is popular in many tropical countries and contains high levels of oxalate (Chen et al., 2001). Thus, acute oxalate nephropathy, although previously reported to result primarily from ethylene glycol ingestion, massive ascorbic acid administration, or methoxyflurane anesthesia (Hollenberg et al., 1972; Lawton et al., 1985; Jacobsen et al., 1988; Sarica et al., 2001; Corley et al., 2005), may be caused by the large amount of oxalate contained in star fruit. However, the cytotoxic effects of oxalate on renal epithelial cells have predominantly been investigated in chronic renal stone disease (Khan and Thamilselvan, 2000; Jonassen et al., 2003). Although many papers have described the renal morphology and urinary biochemistry following acute hyperoxaluria (Khan et al., 1982, 1989, 1992), in most of these studies, hyperoxaluria was induced by intraperitoneal administration of oxalate or by gavage of various hyperoxaluric agents, e.g., ethylene glycol (EG), which are known toxins. It remains unclear whether or not a high content of oxalate in foods taken orally could induce acute oxalate
nephropathy; in particular, the quantity of dietary oxalate appearing in the urine is still debated (Laker, 1983; Holmes et al., 2001). Although both apoptosis and necrosis have been observed in oxalate-treated cultured renal epithelial cells (Khan et al., 1999; Miller et al., 2000), the relationship between serum level of oxalate and renal function or pathological findings has never been explored. In this study, we investigate the mechanisms of star fruit-associated acute oxalate nephropathy.

2. Materials and methods

2.1. Star fruit juice and oxalate solution preparation

Sour-type star fruit were collected from a local farm and homogenized. Insoluble residues were removed by passing the mixture through a paper filter of a 0.22 μm pore size, and the transparent star fruit juice obtained was stored at −20 °C until use. The star fruit juice was subjected to electrolytes (288 Blood Gas System and 664 Fast 4 System; Ciba Corning Diagnostics Corp., Medfield, MA, USA) and pH (pH 72 pH Meter; Beckman, Fullerton, CA, USA) measurement, and the oxalate concentration was measured as described below. The natural oxalate concentration of the star fruit juice was 0.2 M, calcium concentration was less than 0.25 mM/L, potassium concentration was 30 mM/L, and the pH was 2.1. Oxalate solutions of 0.2 M and 0.4 M were prepared using sodium oxalate powder (Sigma, St. Louis, MO, USA). The final pH and concentrations of electrolytes of the culture media after adding star fruit juice and various concentrations of oxalate solution are shown in Table 1.

2.2. Cell cultures

MDCK cells were purchased from the Bioresources Collection and Research Center, Taipei, Taiwan. The cells were cultured in the minimum essential medium alpha medium (MEM) with 10% fetal calf serum (FBS) and 5% CO₂ at 37 °C. Two passages, MDCK cells at 60–70% confluency were used for the experiments described below.

2.3. Animals

This study was performed using 8–10-week-old male Sprague–Dawley rats (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan). The study protocol was approved by the Animal Care and Use Committee of Kaohsiung Veterans General Hospital. Animals were housed in individual home cages, kept on a 12 h light/dark schedule (lights on at 7:00 AM), and provided with food and water ad libitum. The room temperatures were maintained at 72 °F. The humidity was kept within the range from 30% to 70%. Rats with a mean weight of 209 ± 14 g were allocated into four groups of eight: a control group (CG), fed with tap water; a star fruit group (SG), fed with star fruit juice naturally containing 0.2 M oxalate; and two oxalate groups (OxG), fed with 0.2 M or 0.4 M oxalate solution. After overnight fasting, the appropriate food solutions were administered via a metal oral-gastric tube at a dose of 2 mL/100 g body weight. Blood samples were obtained via tail vein puncture and urine samples were collected 6 h after feeding via a metabolic cage. The urine samples were centrifuged at 2000g for 10 min to remove debris, which was then collected to investigate the presence of calcium oxalate crystals by polarized microscopy. The supernatants were used to determine the urinary pH and oxalate levels. The kidneys were immediately removed 24 h after feeding, while the rats were under deep anesthesia, and were promptly placed whole into fixative to facilitate pathological examination. Blood samples were also collected at this time. To examine the earlier pathologic changes, we also harvested the kidneys of another group of rats at 6 and 12 h (n = 3 for each time point) after feeding with star fruit.

2.4. Creatinine and oxalate measurements

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Star fruit</th>
<th>Oxalate solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/10</td>
<td>1/100</td>
<td>1/1000</td>
</tr>
<tr>
<td>Na (mEq/L)</td>
<td>136.3</td>
<td>128</td>
<td>134.7</td>
</tr>
<tr>
<td>K (mEq/L)</td>
<td>5.0</td>
<td>7.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Cl (mEq/L)</td>
<td>121</td>
<td>110</td>
<td>120</td>
</tr>
<tr>
<td>Ca (mEq/L)</td>
<td>2.6</td>
<td>–</td>
<td>2.6</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
<td>6.6</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Plasma and urine creatinine levels were determined according to the equipment manufacturer’s instructions (EXPRESS Plus System; Chiron Diagnostics, Emeryville, CA, USA). Plasma and urine oxalate levels were then measured.

To remove proteins and macromolecules for oxalate measurements, 1 mL of plasma and fresh urine were transferred into the sample reservoir of a centrifugal filter (Microcon YM-10, Cut off 10,000 D; Millipore, Bedford, MA USA). Ultrafiltration was performed at 14,000 rpm for 60 min at 4 °C. To prevent spontaneous conversion of ascorbic acid to oxalate, 5 μL of 1 M hydrochloric acid were added to the inner tube of the ultrafiltration tubes containing plasma or urine. The ultrafiltrate of blood and urine was frozen at −20 °C until analysis. Measurements of oxalate concentration in the blood and urine were performed using a modified method described by Petrarulo et al. (1994); the lower limit of quantification is 0.6 μM, with a coefficient of variation of 20%. Briefly, 750 μL of plasma and urine filtrate were mixed with 600 μL 25 mM 3-methyl-2-benzothiazolione hydrazo hydrochloride/8.2 M 3-methylaminobenzoic acid (Sigma, St. Louis, MO, USA) and 150 μL of 1000 U/mL peroxidase (horseradish peroxidase; Sigma, St. Louis, MO, USA) and incubated at room temperature for 30 min. One hundred and forty microliters of 3.3 U/mL oxalate oxidase (Sigma, St. Louis, MO, USA) was then added to 700 μL of the previous mixture and incubated for 60 min at room temperature. Absorbance at 590 nm was measured using a spectrophotometer (DU-640; Beckman, Fullerton, CA, USA).

2.5. Determination of enzymuria

Urine samples were collected before feeding and at 6, 12 and 24 h after feeding with star fruit juice for the measurement of lactate dehydrogenase (LDH), γ-glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP). The samples were processed as described previously and the supernatants were kept on ice before assay. All measurements were performed by standard laboratory methods using a Vitros 5.1 multianalyzer (Johnson–Johnson, Clinical Diagnostics Inc.).

2.6. Flow cytometry

Flow cytometry was used to examine the in vitro apoptotic effects of oxalate-containing preparations on MDCK cells. Briefly, MDCK cells
with 70% confluency were treated with various preparations for different lengths of time before harvesting using standard procedures. The trypsinized cells were washed with 1× phosphate-buffered saline (PBS) several times, followed by resuspension in 100% ethanol. After washing, the DNA of the trypsinized cells was stained with propidium iodide (0.32 g/L) for 10 min and cells were subsequently subjected to flow cytometry analysis (FACS Calibur, Becton Dickinson, Mountain View, CA, USA). The cell-cycle profiles were analyzed using WinMDI 2.6 (J. Trotter, The Scripps Research Institute; La Jolla, CA, USA).

2.7. Pathological examination of kidneys

Kidney samples were put into 10% neutral buffered formalin for fixation and were later subjected to hematoxylin–eosin staining or TUNEL assay. The TUNEL apoptosis detection kit (ApoAlert DNA Fragmentation Assay Kit; BD Biosciences Clontech, Palo Alto, CA, USA) was used for the detection of apoptosis in renal tissue according to the manufacturer’s instructions. Paraaffin sections were deparaffinized using xylene and descending grades of ethanol, fixed with 4% formaldehyde/PBS for 30 min at 4 °C, permeabilized with proteinase K at room temperature for 15 min and 0.2% Triton X-100/PBS for 15 min at 4 °C, then incubated with a mixture of nucleotides and TdT enzyme for 60 min at 37 °C. The reaction was terminated with 2× standard sodium citrate. The sections were then washed with PBS and mounted with crystal/mount (Biomeda, Foster City, CA, USA). Fluorescent nuclei were detected by visualization using a microscope equipped with fluorescein filters (IX70; Olympus, Lake Success, NY, USA). The degree of apoptosis was estimated by counting the number of TUNEL-positive cells per 100 tubular epithelial cells examined, as apoptosis occurred predominantly in this cell type.

2.8. Statistics

All continuous parameters are expressed as mean ± SEM, whereas nominal variables use proportional expression. One-way ANOVA or Fisher’s exact test was used to measure the differences between groups when appropriate, and repeated ANOVA measurements were performed for within-group analysis. The Bonferroni test for further pair-wise comparison was performed for the post hoc analyses. Results with a probability of less than 0.05 were considered significant.

3. Results

None of the rats in any group died or exhibited movement disorders prior to sacrifice during this study. Weakness and diarrhea were observed in the groups fed with star fruit juice and oxalate solution, but not in the control group.

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Star fruit juice</th>
<th>0.2 M oxalate</th>
<th>0.4 M oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity</td>
<td>–</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

The average value of 6 different fields in each sample was recorded and analyzed. –, no crystal; +, fewer than 10 crystals per low power field; ++, 10–50 crystals per low power field; ++++, more than 50 crystals per low power field.
group. The mean creatinine clearances of the CG, SG, 0.2 M OxG and 0.4 M OxG at 24 h after feeding were 1.67 ± 0.12, 0.78 ± 0.05, 0.76 ± 0.08 and 0.37 ± 0.08 ml/min, respectively (Fig. 1A), showing significantly lower values in the SG, 0.2 M OxG and 0.4 M OxG (p < 0.05). The mean plasma oxalate levels of the CG, SG, 0.2 M OxG and 0.4 M OxG at 6 h after feeding were 4.6 ± 1.5, 14.6 ± 2.4, 17.1 ± 2.7 and 25.2 ± 4.5 mM, respectively (Fig. 1B), and were higher in the experimental groups than in the CG (p < 0.05). The mean urine oxalate levels of the CG, SG, 0.2 M OxG and 0.4 M OxG at 6 h after feeding were 0.14 ± 0.07, 0.81 ± 0.12, 1.01 ± 0.21 and 1.93 ± 0.41 mM, respectively (Fig. 1C); they were higher in the experimental groups than in the CG (p < 0.05), and increased

Fig. 2. Representative rat kidney pathological sections performed 24 h after ingestion of various oxalate-containing preparations: control group in the left column, photomicrographs (A), (D) and (G); star fruit group or 0.2 M oxalate group in the middle column, (B), (E) and (H); 0.4 M oxalate group in the right column, (C), (F) and (I). Upper row, hematoxylin–eosin stain, 200×; middle row, polarized light, 40×; lower row, TUNEL assay, fluorescein, 400×.
proportionally with the amount of oxalate ingested. Compared with the control group, an increasing intensity of crystalluria was found in the SG, 0.2 M OxG and 0.4 M OxG (Table 2). The urinary cytosolic enzyme LDH was found to have significantly increased in activity at 6 h after feeding with star fruit juice and remained higher for 24 h (Fig. 1D). The activity of brush border marker enzymes, GGT (Fig. 1E) and ALP (Fig. 1F) was dramatically elevated 6 h after feeding with star fruit juice, then gradually declined but remained significantly higher than the baseline activity. In addition, the blood and urine pH of the CG, SG, 0.2 M OxG and 0.4 M OxG at 6 h after feeding were 7.40 ± 0.04, 7.38 ± 0.03, 7.39 ± 0.03 and 7.38 ± 0.02 (blood pH) and 6.5 ± 0.3, 6.0 ± 0.2, 6.1 ± 0.2 and 5.9 ± 0.3 (urine pH), respectively.

Representative rat kidney pathological sections, taken 24 h after ingestion of various oxalate-containing preparations, are shown in Fig. 2. The pathological sections of the SG or 0.2 M OxG, shown in the photomicrograph (B), displayed moderate changes in the dilatation of renal tubules and calcium oxalate crystal deposition as compared with the normal kidney structure of the CG, shown in photomicrograph (A), whereas diffuse changes can be seen in photomicrograph (C), which shows the results for the 0.4 M OxG. The intensity of calcium oxalate crystal deposition is clearly visible at a lower magnification of 40× under polarized light, as shown in photomicrographs (D), (E) and (F). Calcium oxalate crystals, observed as refractile particles, can be seen to be distributed extensively in photomicrograph (F) of the 0.4 M OxG and moderately in photomicrograph (E) of the SG or 0.2 M OxG, whereas photomicrograph (D) of the CG shows no calcium oxalate crystals. Photomicrographs (G), (H) and (I) show the pathological sections with TUNEL assay examined through a fluorescein filter. The apoptotic cells were stained yellow, and are seen to be diffusely distributed in photomicrograph (I) of the 0.4 M OxG. The distribution is less extensive in photomicrograph (H) of the SG or 0.2 M OxG, and almost nonexistent in photomicrograph (G) of the CG. A quantitative evaluation of the TUNEL-positive cells was performed (Fig. 4). The percentages of apoptotic cells in the CG, SG, 0.2 M OxG and 0.4 M OxG were 2.1 ± 1.3%, 14.3 ± 7.8%, 20.1 ± 5.2% and 56.9 ± 5.1%, respectively, indicating a dose-dependent intensity of apoptosis, increasing from the CG to the 0.4 M OxG. The earlier pathological changes in the rat kidneys after feeding with star fruit juice are displayed in Fig. 3. Note that calcium oxalate crystals are scattered in the tubular lumen and that the tubular epithelium was generally intact in the kidney sections taken at 6 h (A) after feeding. In the sections taken at 12 h (B) and
24 h (C) after feeding, foci of simplified tubules and flattened tubular epithelium with dilated lumen could be seen. In addition, apoptosis was observed to have developed in the TUNEL assay at 6 h (D) after feeding, and was seen to have progressed in the 12 h (E) and 24 h (F) sections.

Flow cytometry analysis of MDCK cells treated with vehicle, diluted star fruit juice (1/1000, 1/100 and 1/10, equal to 0.2 mM, 2 mM and 20 mM oxalate, respectively) and 0.5 mM, 1.0 mM and 2 mM oxalate solutions for 16 h, is shown in Fig. 5. These concentrations approximated the urine concentrations of oxalate that we observed 6 h after oral feeding with star fruit, 0.2 M or 0.4 M oxalate. A dose-dependent increase in the percentage of subG1-phase cells was observed after both star fruit juice and oxalate solution feeding. Furthermore, Fig. 6 shows the flow cytometry analysis of MDCK cells treated with
1/100 diluted star fruit juice (A) and 1.0 mM oxalate solution (B) at various time intervals. A time-dependent increase in the percentage of subG1-phase cells was observed with both star fruit juice and oxalate solution.

4. Discussion

Star fruit nephrotoxicity presenting as acute renal failure has been demonstrated in our previous reports (Chen et al., 2001; Fang et al., 2001). The large content of oxalate in star fruit and the pathological sections (obtained from patients and experimental animals) showing diffuse calcium oxalate deposition suggest that acute oxalate nephropathy is responsible for star fruit nephrotoxicity.

Given the morphological changes observed, such as calcium oxalate crystal deposition in dilated renal tubules, it seems reasonable to speculate that the obstruction of renal tubules by these crystals is the mechanism by which tubular damage is caused. However, tubular dilatation was much more extensive than the crystal deposition observed, especially in human cases (Chen et al., 2001), and the focal tubular deposition of crystals was not compatible with the profound loss of glomerular filtration rates – for which temporary hemodialysis might be necessary (Chen et al., 2001). Whether or not, in addition to the obstructive effect of the crystals of calcium oxalate, the oxalate alone could act as a cell toxin capable of producing acute cell damage, which exacerbates the acute loss of glomerular filtration rate, remained unresolved. Although previous studies have demonstrated the direct toxic effects of oxalate and calcium oxalate crystals (Hackett et al., 1995; Khan et al., 1999; Khan and Thamilselvan, 2000; Miller et al., 2000; Sarica et al., 2001; Jonassen et al., 2003), these studies predominantly investigated the pathogenesis of renal stone formation, a chronic disease. In this study, we found that acute oral administration of star fruit or oxalate solution could directly cause renal tubular cell apoptosis that leads clinically to acute renal shutdown.

The mode of cell death caused by oxalate may involve necrosis and apoptosis (Miller et al., 2000). Tissue injury in which either necrosis or apoptosis dominates depends on several factors, including the local concentration of oxalate, mode of exposure (acute or chronic administration) and differences in tissue susceptibility (Wyllie, 1987; Hale et al., 1996; Lieberthal and Levine, 1996). Many tissues exhibit apoptotic cell death at lower toxin concentrations and necrotic cell death at higher toxin concentrations (Wyllie, 1987). Here, a predominance of apoptosis was observed in star fruit-induced acute renal failure, as evident from the morphological changes, such as flattened tubular epithelial cells with dense nuclei on hematoxylin–eosin staining, but not cell swelling. In addition, both in vivo TUNEL assay and in vitro flow cytometry analysis showed dose-depen-

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**Fig. 4.** The percentages of apoptotic cells labeled by terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay in rat kidney pathological sections. Each group consists of eight rats. *p < 0.05 compared to control group. **p < 0.05 compared to control and star fruit groups.

**Fig. 5.** Flow cytometry analysis of MDCK cells treated with vehicle, diluted star fruit juice (1/1000, 1/100, 1/10), 0.5 mM, 1.0 mM and 2 mM oxalate solution for 16 h. The oxalate concentration of star fruit juice is 0.2 M. Each group contains 8–10 experiments. *p < 0.05 compared to control group.

**Fig. 6.** Flow cytometry analysis of MDCK cells treated with (A) 1/100 diluted star fruit juice and (B) 1.0 mM oxalate solution at various time intervals. The oxalate concentration of star fruit juice is 0.2 M. Each group contains 8–10 experiments. *p < 0.05 compared to the 2-h group.
dent effects of apoptosis that result in more apoptotic cells in groups of greater toxin concentrations. This suggests that, within the range of oxalate concentrations caused by oral administration of star fruit juice or oxalate solution to simulate the clinical condition, apoptotic cell death was the predominant presentation of the cytotoxic effects of oxalate and star fruit.

The plasma concentration of oxalate in our study was between three and five times greater than the concentration in the controls and was one-twentieth of the concentration of 0.5 mM at which apoptotic effects began to be observed in MDCK cells (Fig. 5). However, the oxalate concentration could reach a level of as high as 1 – 3 mM in the urinary compartment (Fig. 1C), which could cause significant apoptosis. As oxalate is highly concentrated in the urine, not only by being freely filtrated through glomeruli but also by active secretion from the proximal tubules (Pinto et al., 1974; Prehen et al., 1981), the urine oxalate concentration effect is the key factor that causes local concentrations of oxalate to be high enough to induce apoptosis and renal injury. Although acid could also induce cell damage, the ingested star fruit or oxalate was absorbed by the gastrointestinal tract and was not in direct contact with the renal epithelial cells. Furthermore, the arterial blood pH did not display significant metabolic acidosis and the urine pH was within the usual variation of normal diet, indicating that it was unlikely that the acid of oxalate induced apoptosis.

The mechanisms underlying the apoptotic effects of oxalate on renal epithelial cells have been partially investigated previously (Cao et al., 2001; Koul et al., 2003; Wiessner et al., 1999). Through redistributing phosphatidylserine and activating lipid signaling pathways, oxalate damages the cell membrane, causes programmed cell death and a reparative proliferation for replacement of damaged cells (Wiessner et al., 1999; Cao et al., 2001; Koul et al., 2003). Although antioxidant therapy may be useful for preventing oxalate-associated free radical injury and apoptosis, this effect was only observed in cell lines or in animal models of chronic hyperoxaluria (Thamilselvan et al., 2000, 2003; Rashed et al., 2004; Thamilselvan and Menon, 2005; Huang et al., 2006). Therefore, clinically, supportive care remains the cornerstone of treatment for star fruit-induced acute renal failure.

In conclusion, oral ingestion of star fruit juice can produce acute renal injury, not only through the obstructive effect of crystals of calcium oxalate, but also by inducing apoptosis of renal epithelial cells, which may be caused by the abundance of oxalate in star fruit.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgments

This work was supported by grants from the National Science Council (NSC 93-2314-B-075B-011) and Kaohsiung Veterans General Hospital (VGHKS91-46) to Dr. Kung-Ju Chou and (VGHKS 92-32 and VGHKS 93-30) to Dr. Hua-Chang Fang.

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