Evaluation of the nephrotoxicity and safety of low-dose aristolochic acid, extending to the use of Xixin (Asurum), by determination of methylglyoxal and D-lactate

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Abstract

**Background:** Most *Aristolochiaceae* plants are prohibited due to aristolochic acid nephropathy (AAN), except Xixin (*Asarum spp.*). Xixin contains trace amounts of aristolochic acid (AA) and is widely used in Traditional Chinese Medicine. Methylglyoxal and D-lactate are regarded as biomarkers for nephrotoxicity. Thus, this study aimed to evaluate tubulointerstitial injury and interstitial renal fibrosis by determining urinary methylglyoxal and D-lactate after withdrawal of low-dose AA in a chronic mouse model.

**Methods:** C3H/He mice in the AA group (*n* = 24/group) were given ad libitum access to distilled water containing 3 µg/mL AA (0.5 mg/kg/day) for 56 days and drinking water from days 57 to 84. The severity of tubulointerstitial injury and fibrosis were evaluated using the tubulointerstitial histological score (TIHS) and Masson’s trichrome staining. Urinary and serum methylglyoxal were determined by high-performance liquid chromatography (HPLC); urinary D-lactate were determined by column-switching HPLC.
Results: After AA withdrawal, serum methylglyoxal in the AA group increased from day 56 (429.4 ± 48.3 μg/L) to 84 (600.2 ± 99.9 μg/L), and peaked on day 70 (878.3 ± 171.8 μg/L; p < 0.05); TIHS and fibrosis exhibited similar patterns. Urinary methylglyoxal was high on day 56 (3.522 ± 1.061 μg), declined by day 70 (1.583 ± 0.437 μg) and increased by day 84 (2.390 ± 0.130 μg). Moreover, urinary D-lactate was elevated on day 56 (82.10 ± 18.80 μg) and higher from day 70 (201.10 ± 90.82 μg) to 84 (193.28 ± 61.32 μg).

Conclusions: Methylglyoxal is induced after AA-induced tubulointerstitial injury, thus methylglyoxal excretion and metabolism may be a detoxification and repair strategy. A low cumulative AA dose is the key factor that limits tubulointerstitial injury and repair. Thus, AA-containing herbs, especially Xixin, should be used at low doses for short durations (less than one month).

Keywords: Aristolochic acid (AA), D-lactate, fibrosis, methylglyoxal, nephrotoxicity

Background

Aristolochiaceae plants are traditionally used in several Traditional Chinese Medicine (TCM) formulas, such as Xiao-Qing-Long-Tang, Chuan Xiong Cha Tiao San, Ma Huang Fu Zi Xi Xin Tang, Qing Shang Juan Tong Tang and Du Hwa Jih Sheng Tang. However, as they induce aristolochic acid nephropathy (AAN), most
aristolochic acid (AA)-containing plants is prohibited, including *Aristolochia contorta* (Ma Dou Ling) [1], *Aristolochia fangchi* (Guang Fang Ji) [1], *Aristolochia manshuriensis* (Guan Mu Tong) [1], *Aristolochia contorta* (Tian Xian Teng) and *Aristolochia debilis* (Qing Mu Xiang) [1]. These species can be replaced in Traditional Chinese Medicine (TCM) by other non-*Aristolochiaceae* plants [2].

However, Xixin cannot be replaced by other non-*Aristolochiaceae* plants. All origins of Xixin, including *Asarum heterotropoides* Fr. Schmidt var. mandshuricum (Maxim.) Kitag, *Asarum crispulatum* C.Y. Cheng and C.S. Yang, *Asarum forbesii* Maxim, *A. himalaicum* Hooh. F. and Thoms. Ex Klotzsch, *Asarum sieboldii* Miq, *Asarum debile* Franch *Asarum maximum* Hemsl, *Asarum ichangense* C.Y. Cheng and C.S. Yang, and *Asarum fukienense* C.Y. Cheng and C.S. Yang, are members of the *Aristolochiaceae* family. Moreover, Xixin and its combination with other herbs are frequently used to treat a variety of conditions, including fever [3], influenza in the elderly [4], infection [5], allergy [6], caries [7], inflammation [8, 9], pain [8], rhinitis [9] and rheumatoid arthritis [10]. Consequently, while the use of AA-related products, especially Xixin, remains essential, their safety is controversial.

Methylglyoxal and D-lactate can be used to assess nephrotoxicity and play key roles in the progression of renal injury, including diabetic [11], gentamicin-induced [12], Pb-induced [13] and acute AA-induced nephropathy [14-16]. The highly reactive
dicarbonyl groups of methylglyoxal denature proteins and nucleic acids; the resulting methylglyoxal-derived compounds are called advanced glycation end products (AGEs) [17]. As a mechanism to limit the toxicity of methylglyoxal, methylglyoxal is metabolized into D-lactate via the glyoxalase system [18, 19]. Thus, D-lactate is considered to be a marker of renal damage and accumulation of methylglyoxal. On the other hand, renal injury increases both D-lactate and L-lactate, while prednisolone [18] or metformin [10] treatment alleviate histological damage and decrease D-lactate—rather than L-lactate or D/L-lactate—in AAN models. Nevertheless, the changes in methylglyoxal and D-lactate contents after long-term administration of AA have not been explored, although elevation of urinary D-lactate was observed in a previous study [20].

This study aimed to explore whether the progression of tubulointerstitial injury and interstitial renal fibrosis stop and/or reverse after discontinuing 56-day administration of low-dose AA by determining the contents of urinary methylglyoxal and D-lactate. The results of this study may help to identify the safe dose and duration of administration of AA-related products, especially Xixin.

Methods

Materials and chemicals
Animal experiments

All animal protocols were approved by the Animal Care and Use Committee of Taipei Medical University (LAC-2019-0482). Six-week-old female C3H/He mice were obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and randomly allocated to either the normal group (N) or the AA group (n = 24/group) [20]. Six mice in the same group were placed in per cage. These mice were placed in temperature-controlled (25 ± 2°C) and humidity-controlled (65 ± 5%) rooms with a 12:12 light-dark photoperiod, and had access to standardized food pellets (Fwusow Industry Co., LTD., Taichung, Taiwan). The AA group were given ad libitum access to distilled water containing 3 µg/mL AA (Sigma-Aldrich, Inc., MO, USA; AAI:AAII = 63:31; 0.5 mg/kg/day) as drinking water for 56 days. After day 56, the AA group mice received normal drinking water [20, 21]. The N group received only normal drinking water. Urine samples were collected over 12 hours on days 28, 56, 70, and 84 using a metabolic cage (Tokiwa Chemical Industries Co. Ltd., Japan). The mice were humanely sacrificed on day 28, 56, 70 or 84 (n = 6/group). Whole blood samples were collected and the kidneys were excised [20, 21].

Biochemical parameters
The Blood Urea Nitrogen Kit (Beckman Coulter, Brea, California, USA) was used to determine blood urea nitrogen (BUN). Urinary N-acetyl-β-D-glucosaminidase (NAG) activity, i.e., production of 4-methylumbelliferone (4-MU) from 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide within 15 min, was reacted in 100 mM citrate buffer (pH 4.6-5.0). The reaction was stopped by addition of 200 mM glycine buffer (pH 10.4-10.6) and fluorescence was measured at 460 nm after excitation at 370 nm [21].

Serum creatinine (Scr) was determined using a modified version of the previous protocol [14, 22]. Briefly, 20 µL blood samples were mixed with 10 µL of 1 mM cimetidine·HCl (Sigma-Aldrich, Inc., MO, USA) as an internal standard [I.S.] and 170 µL acetonitrile (MeCN) and centrifuged at 700 g for 15 min at 4°C. The supernatants (50 µL) were injected into the UV-HPLC system, which included an L-7100 Pump (Hitachi, Tokyo, Japan), L-2200 Intelligent Autosampler (Hitachi), TSKgel ODS-80Ts column (250 × 4.6 mm i.d., 5 µm; Tosoh Co., Tokyo, Japan) and an 875-UV Intelligent UV/VIS Detector (JASCO International Co., Ltd., Tokyo, Japan). The mobile phase was 36:60 (v/v) MeCN/30 mM sodium lauryl sulfate (aq):100 mM sodium dihydrogen phosphate (aq) (pH 3.0) and the flow rate was 0.8 mL/min. The fractions were monitored at 234 nm.

Histological examinations
Half of each left kidney was fixed in 10% buffered neutral formalin solution for 24 h at 4°C, embedded in paraffin, sectioned at 4-5 μm and stained with periodic acid–Schiff (PAS) (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), Masson’s trichrome (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), or Picro Sirius Red Staining Kit (CIS-Biotechnology Co., Ltd., Taichung, Taiwan) according to the instruction of manufactures [20, 21]. Sections were observed at a magnification of 200× using a G-330 light microscope (Optima, New Taipei City, Taiwan) and images were captured using a Nikon Coolpix 4500 camera (Nikon, Tokyo, Japan) [20, 21].

**Tubulointerstitial histological score**

The tubulointerstitial histological score (TIHS) was used to evaluate the severity of tubulointerstitial damage in the PAS-stained sections. Ten non-overlapping fields of view were scored for each mouse. The TIHS assesses three major items: the severity of mononuclear cell infiltration into the interstitium (0, absent; 1, few scattered cells; 2, groups of mononuclear cells; and 3, dense widespread infiltrate); the severity of degeneration in the tubular epithelium (0, no degeneration of the tubular epithelium; 1, one group or a single degenerated tubule; 2, several clusters of degenerated tubules; 3, moderate degeneration of the tubular epithelium; 4, more severe degeneration of the tubular epithelium; and 5, extremely severe degeneration of the tubular epithelium, with massive necrosis and atrophy); and the severity of interstitial fibrosis.
(0, absent; 1, mild diffuse fibrosis; 2, moderate fibrosis; and 3, severe fibrosis). The
THIS of each mouse was expressed as the sum of the three scores [14, 15, 20, 21, 23].

Semi-quantitative analysis of fibrosis

Interstitial renal fibrosis was assessed in the Masson trichrome and Picro Sirius
Red-stained sections by determining the percentage area positive for aniline blue and
red in ten non-overlapping fields of view for each mouse using ImageJ (National
Institutes of Health, MD, USA), respectively [20].

Determination of methylglyoxal

Urinary and serum methylglyoxal were determined by high-performance liquid
chromatography with fluorescence detection (FD-HPLC). The FD-HPLC system
(Hitachi, Tokyo, Japan) was composed of L-2130 Pump, L-2200 Intelligent
Autosampler and L-2480 Fluorescence Detector [13, 20].

Briefly, the serum and urine samples were fluorogenically derivatized with 6-
diamino-2,4-dihydroxypyrimidine sulfate (DDP) for 30 min at 60 °C, the reactions
were stopped with 0.01 M citric acid (pH 6.0) and 20 µL samples were loaded onto
the ODS column (250 × 4.6 mm, 5 µm particle size; Biosil Chemical Co. Ltd., Taipei,
Taiwan) at 33 °C [24]. The mobile phase was 97:3 (v/v) 0.01 M citric acid buffer (pH
6.0) and MeCN and the flow rate was 0.7 mL/min. The fractions were monitored at an
emission wavelength of 500 nm and excitation wavelength of 330 nm. Urinary
methylglyoxal content was expressed as the level of methylglyoxal × 12 h-urinary volume [20].

**Determination of D-lactate**

**Florigenic derivatization of lactate**

Urine samples (20 μL) were mixed with 10 μL of 1 mM propionic acid as an internal standard (I.S.) and 170 μL of acetonitrile (MeCN) and centrifuged at 700 g for 10 min at 4 °C. For florigenic derivatization, 100 μL of the supernatant was incubated with 100 μL of 8 mM 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ) in MeCN in 25 μL of 280 mM triphenylphosphine (TPP) and 25 μL of 280 mM 2,2’-dipyridyl disulfide (DPDS) in MeCN. After 3 h, 250 μL of 0.1% aqueous trifluoroacetic acid (TFA) was added to stop the reaction. To remove excess NBD-PZ, 100 μL of the solution was passed through a MonoSpin™ SCX cartridge (GL Science Inc., Tokyo, Japan) and the eluates were collected [20, 25].

**Isolation of D,L-lactate and D-lactate**

Urinary D-lactate were determined using a column-switching FD-HPLC system by first isolating D,L-lactate and then separating D-lactate. NBD-PZ-fluorogenic derivatized samples (20 μL) were loaded into an L-7100 Pump (Hitachi, Tokyo, Japan), L-2200 Intelligent Autosampler (Hitachi, Tokyo, Japan), Biosil ODS column (4.6 × 250 mm, 5 μm; Biotic Chemical Co., Ltd., Taipei, Taiwan) and L-2485
Fluorescence Detector (Hitachi, Tokyo, Japan) to determine D,L-lactate (total lactate) [20, 25]. Mobile phase A was 12:20:68 (v/v/v) MeCN/MeOH/H$_2$O and mobile phase B was 100% MeCN. The program was mobile phase A at 0.7 mL/min from 0 to 35 min, mobile phase A at 0.9 mL/min from 35.1 to 60 min, mobile phase B at 0.8 mL/min from 60.1 to 75 min, and mobile phase A at 0.7 mL/min from 75.1 to 90 min. The D,L-lactate fraction was switched into the second part of the column-switching FD-HPLC system using a six-valve switcher.

The second part of the FD-HPLC was L-7100 Pump (Hitachi, Tokyo, Japan), Chiralpak ADRH column (150 × 4.6 mm, 5 μm particle size; Daicel Co., Osaka, Japan) and L-2485 Fluorescence Detector (Hitachi, Tokyo, Japan). D-lactate and L-lactate were separated using 60:40 (v/v) MeCN/H$_2$O as the mobile phase at a flow rate of 0.3 mL/min. The NBD-PZ derivatives were detected using an emission wavelength of 547 nm and excitation wavelength of 491 nm in both parts of the two-dimensional FD-HPLC system. The D,L-lactate and D-lactate levels were based on the areas of the corresponding peaks (D-7500 Integrator; Hitachi, Tokyo, Japan). Thus, the actual amount of D,L-lactate was D,L-lactate level × 12-h urinary volume; the actual amount of D-lactate was D-lactate level × 12-h urinary volume [20].

**Western blotting of glyoxalase 1**
Briefly, kidney homogenates were prepared and protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The samples (10 μg protein per lane) were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto Immun-Blot PVDF Membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) [21]. The antibodies used were anti-glyoxalase 1 antibody (GLO1; GTX105792; GeneTex, Irvine, CA, USA; 1:1000), anti-β‐actin antibody (20536-1-AP; Proteintech, Rosemont, IL, USA; 1:2000) and goat anti-rabbit IgG (H+L) HRP conjugate antibody (SA00001-2; Proteintech, Rosemont, IL, USA; 1:4000). TOOLSensitive ECL Kit (Tools Biotechnology co. Ltd., New Taipei City, Taiwan) was used detect the bands. The intensity of the bands were measured using Image J (National Institute of Health, Bethesda, Maryland, USA) and the levels of GLO1 were normalized to β‐actin and expressed relative to the N group.

**Statistical analysis**

All values were expressed as means ± standard deviation. The differences between the N and AA groups were assessed using the Student’s t-test at each time point. Repeated measures ANOVA was used to compare the mean values of the variables in the same group on Day 56, 70, and 84. P-values less than 0.05 were
defined as significant. SPSS for Windows 19th version (IBM Co., New York, NY, USA) was used to analyze all data.

Results

Biochemical parameters

All biochemical parameters (BUN, Scr, and NAG) were significantly elevated in the AA group compared to the N group on days 28, 56, 70 and 84 (Table 1). There were no significant differences in the BUN and Scr contents of the AA group on day 56 (24.13 ± 1.21 mg/dL and 0.283 ± 0.041 mg/dL), day 70 (25.83 ± 1.17 mg/dL and 0.317 ± 0.075 mg/dL), and day 84 (24.99 ± 0.98 mg/dL and 0.300 ± 0.063 mg/dL). In the AA group, urinary NAG activity was higher on day 56 (2.079 ± 0.089 U/L) and day 70 (2.297 ± 0.266 U/L) than on day 84 (1.895 ± 0.110 U/L).

Histological examination

PAS stained kidney sections (Fig. 1) indicated exposure to AA induced interstitial renal fibrosis, with the most severe fibrosis observed on day 70 (Fig. 1f). The PAS staining sections revealed severe renal damage in the AA group, including cellular infiltration, epithelial damage and interstitial renal fibrosis. There was a slightly cell infiltration and tubular atrophy on day 28, but no significant difference in TIHS between AA (0.8 ± 0.6) and N (0.2 ± 0.4) groups (Fig. 1a,1b,1f). The AA group
had significantly higher TIHS than the N group on day 56 (AA: $3.6 \pm 0.7$ vs. N: $0.2 \pm 0.4$; $p < 0.01$), day 70 (AA: $6.3 \pm 0.8$ vs. N: $0.3 \pm 0.7$; $p < 0.01$), and day 84 (AA: $4.6 \pm 0.5$ vs. N: $0.3 \pm 0.7$; $p < 0.01$) (Fig. 1f). The TIHS of the AA group increased between day 28 and day 70 and then reduced by day 84 (Fig. 1f).

Masson’s trichrome staining was performed to semi-quantitatively analyze collagen deposition (Fig. 2). There was no significant difference in collagen deposition between AA ($3.6 \pm 0.2\%$) and N ($3.5 \pm 0.4\%$) groups on day 28 (Fig. 2a,b,i). Collagen deposition was significantly higher in the AA group than the N group on day 56 (AA: $14.1 \pm 2.3\%$ vs. N: $3.6 \pm 0.5\%$; $p < 0.01$), day 70 (AA: $28.5 \pm 3.5\%$ vs. N: $3.8 \pm 0.4\%$; $p < 0.01$), and day 84 (AA: $20.6 \pm 1.6\%$ vs. N: $4.0 \pm 1.0\%$; $p < 0.01$) (Fig. 2i). In the AA group, higher levels of collagen deposition were detected on day 70 (Fig. 2f) than on days 56 (Fig. 2d) and 84 (Fig. 2h). Moreover, collagen deposition was also assessed by Picro Sirius Red staining (Fig. 3), and the findings were similar to Masson’s trichrome staining. There was no significant difference in collagen deposition between AA ($5.86 \pm 1.38\%$) and N ($5.72 \pm 1.20\%$) groups on day 28 (Fig. 3a,b,i). The collagen deposition was significantly higher in the AA group than the N from day 56 (AA: $11.3 \pm 1.2\%$ vs. N: $6.5 \pm 1.3\%$; $p < 0.05$) to day 84 (AA: $15.7 \pm 1.5\%$ vs. N: $6.4 \pm 1.5\%$; $p < 0.05$) (Fig. 3i). The highest levels of collagen deposition in the AA group on day 70 (Fig. 3f), and the collagen deposition
significantly decreased from day 70 (21.9 ± 1.6%) to day 84 (15.7 ± 1.5%) (p < 0.05) (Fig. 3i).

**Methylglyoxal in serum and urine**

The AA group had a higher serum methylglyoxal level than the N group on day 56 (AA: 429.4 ± 48.3 μg/L vs. N: 311.9 ± 29.2 μg/L; p < 0.05), day 70 (AA: 878.3 ± 171.8 μg/L vs. N: 373.1 ± 52.9 μg/L; p < 0.01), and day 84 (AA: 600.2 ± 99.9 μg/L vs. N: 386.6 ± 61.5 μg/L; p < 0.05) (Fig. 4d). In the AA group, the serum methylglyoxal level content significantly increased between day 56 and day 70 and declined between day 70 and day 84 (Fig. 4d).

The AA group had higher urine methylglyoxal contents than the N group on day 56 (AA: 3.522 ± 1.061 μg vs. N: 1.408 ± 0.135 μg; p < 0.05) and day 84 (AA: 2.390 ± 0.130 μg vs. N: 1.630 ± 0.081 μg; p < 0.05) (Fig. 4h). There was no significant difference in the urinary methylglyoxal contents of the AA (1.583 ± 0.437 μg) and N groups (1.386 ± 0.255 μg) on day 70 (Fig. 4h). The urinary methylglyoxal content of the AA group significantly decreased between day 56 and 70 and significantly increased between day 70 and 84 (Fig. 4h).

**Urinary D,L-lactate and D-lactate contents**

The AA group had significantly higher urinary D,L-lactate contents than the N group on day 56 (AA: 994.1 ± 161.0 μg vs. N: 151.9 ± 71.1 μg; p < 0.001), day 70
(AA: 1598.9 ± 396.0 μg vs. N: 125.8 ± 57.4 μg; *p* < 0.001), and day 84 (AA: 1447.0 ± 531.4 μg vs. N: 141.5 ± 48.6 μg; *p* < 0.001) (Fig. 5g). There was no significant change
in the urinary D,L-lactate content of the N group from day 56 to 84. However, the
urinary D/L-lactate content of the AA group significantly increased between day 56
and day 70 (*p* < 0.05) and remained high at day 84 (Fig. 5g).

The urinary D-lactate contents of the AA group were significantly higher than
those of the N group on day 56 (AA: 82.10 ± 18.80 μg vs. N: 5.88 ± 3.40 μg; *p* <
0.001), day 70 (AA: 201.09 ± 90.82 μg vs. N: 5.78 ± 2.77 μg; *p* < 0.001), and day 84
(AA: 193.28 ± 61.32 μg vs. N: 7.15 ± 3.28 μg; *p* < 0.001) (Fig. 5h). The urinary D-
lactate amount of the N group did not significantly change between day 56 and 84.
However, the urinary D-lactate amount of the AA group significantly increased
between day 56 to 70 (*p* < 0.05) and remained high at day 84 (Fig. 5h).

**Expression of GLO1**

The relative levels of GLO1 in the kidney of the AA group on day 56 (180.9 ±
12.8%), day 70 (150.8 ± 14.9%), and day 84 (167.2 ± 13.3%) were significantly
higher than the N group (100.0 ± 9.0%; *p* < 0.05) (Fig. 6b).

**Discussion**
The cumulative dose of AA administered to the mice in this study was approximately 0.56 mg (equivalent to 136.22 mg in humans), which is similar to the human study by Vanhaelen et al. (about 130 mg) [26]. The concentration of AA in Xixin depends on the origin (A. heterotropoides, A. crispulatum, A. forbesii, A. himalaicum, A. sieboldii, A. debile, A. maximum, A. ichangense, A. fukienense) [27], the portion of the plant used (roots, rhizomes, petioles, leaves) [28], and the extraction solvent (water, methanol) [28], thus the concentration of AA in Xixin ranges from trace to 3376.9 ppm [27, 28]. In patients receiving Xixin containing the highest concentrations of AA, intake of 38.5 g Xixin crude herbs can lead to interstitial renal fibrosis or urothelial carcinoma. Moreover, most TCM doctors use concentrated granules rather than the crude herbs. Additionally, while the regulations states that only the roots of Xixin should be used due to their lower AA content, whole plants are still available in markets [28]. Thus, it is necessary to determine the AA concentrations of Xixin crude herbs and concentrated granule samples.

Several animal studies have revealed that higher doses or longer durations of AA administration lead to severe AAN. In a rabbit model, renal hypocellular interstitial fibrosis was induced by intraperitoneal injection of AA (0.1 mg/kg/day) five times a week for more than 17 months [29], a much longer duration than the current study (56 days). Interstitial fibrosis was also induced in rats by subcutaneous administration of
AA (10 mg/kg/day) for five weeks; however, the changes after withdrawal of AA were not observed [30, 31]. In the current study, slight elevation of biochemical parameters were observed but rare fibrosis by day 28, compared to those on day 56 and 70. Moreover, while another acute mouse model used a much higher dose (10 mg/kg/day) than the current study (0.5 mg/kg/day), tubular necrosis—but rarely interstitial renal fibrosis—were observed after intravenous administration of AA for five days [14, 16]. Consequently, administration of a low cumulative dose (i.e., a low dose for a short period) of Xixin and monitoring NAG and renal function are essential to avoid exacerbation of tubulointerstitial injury.

Methylglyoxal and D-lactate are regarded as biomarkers for nephropathy and diabetes, thus most previous studies focused on how methylglyoxal is induced endogenously, how methylglyoxal reacts with proteins and nucleic acids to produce AGEs [27], and testing drugs that may lower methylglyoxal contents. Therefore, urinary excretion of methylglyoxal and D-lactate are considered to reflect excessive production of methylglyoxal due to renal injury and inflammation. Based on the biochemical parameters and renal biopsies in this study, cell infiltration and renal injury such as tubule degeneration and moderate fibrosis truly existed in the injured kidney after administration of AA for 56 days. In a previous study, inflammation markers, such as F4/80 and tumor necrosis factor-α (TNF-α), were induced in the
damaged kidney after long-term administration of AA [21]. On the other hand, methylglyoxal is produced after injury or inflammation and is also a pro-inflammatory factor. Methylglyoxal promotes inflammation via upregulating the nuclear factor-kappa B (NF-κB) signaling pathway [28]. Furthermore, AGEs, methylglyoxal-derivatized adducts, react with receptors for advanced glycation end products (RAGE) to further evoke inflammation [32].

Another essential finding of this study was that the level of serum methylglyoxal peaked on day 70, while the levels of MG excreted in urine were lower at this time point than at day 70. These findings indirectly reflect accumulation of methylglyoxal in the injured kidney. In agreement with these observations, severe renal injury and interstitial fibrosis were detected at day 70. As previously described, accumulation of methylglyoxal is harmful and aggravates fibrosis in a variety of pathologies [33-35].

The potential mechanisms include activation of transient receptor potential ankyrin 1 (TRPA1) [35], promotion of the cell cycle [35], differentiation of fibroblasts [33-36], induction of the epithelial-mesenchymal transition (EMT) via the TGF-β/Snail axis [34], and inhibition of the binding step of collagen phagocytosis [37]. Moreover, Kottmann et al. proposed that lactic acid may activate TGF-β and lead to accumulation of hypoxia-inducible factor 1-α (HIF-1α)—which promotes
myofibroblast differentiation—in idiopathic pulmonary fibrosis [38]. However, the
effects of methylglyoxal on interstitial renal fibrosis are still unknown.

Previous studies only assessed urinary methylglyoxal and D-lactate under injury
conditions, such as oxidative stress [39], inflammation [40], necrosis [14] or fibrosis [20]). However, we found that urinary methylglyoxal and D-lactate remained elevated at 28 days after AA withdrawal (day 84), when the severity of fibrosis and tubulointerstitial injury had reduced to mild. These changes in urinary methylglyoxal and D-lactate may be related to renal repair and detoxification. Generally, injured tissues use glycolysis to produce energy for repair [41], thus methylglyoxal might be released. Lan et al. detected increased levels of byproducts of glycolysis (lactate and pyruvate) in the kidney during reperfusion after acute ischemia-reperfusion injury [42]. Thus, methylglyoxal may be excreted into urine and metabolized into D-lactate by GLO1 as a mechanism of detoxification [18]. Indeed, most studies did not assess the pathological alterations after long-term withdrawal of AA. However, our assessment of the changes up to 28 days after withdrawal of AA (day 84) indicate repair is a time-consuming process. The association between repair and methylglyoxal warrants more detailed investigation in further research. Moreover—as a limitation of this study—although AA-DNA adducts are a biomarker for AA exposure and urothelial cell carcinoma [43], AA-DNA adducts were not determined in this study. In
the future, AA-DNA adducts should be detected to evaluate the severity of
tubulointerstitial injury.

Conclusion

Methylglyoxal is produced and exacerbates kidney injury (inflammation and
fibrosis), and excretion and metabolism of methylglyoxal may represent a strategy of
detoxification after injury. After withdrawal of AA, tubulointerstitial injury became
mild, due to the low cumulative dose of AA. Thus, AA-containing herbs such as Xixin
should be used at low doses for short durations (i.e., less than one month) and the
renal function of the patients should be monitored.

List of abbreviations

AA: aristolochic acid; AAI: aristolochic acid I; AA II: aristolochic acid II; AAN:
aristolochic acid nephropathy; AGEs: advanced glycation end products; BUN: blood
urea nitrogen; DDP: 2,20-dipyridyl disulfide; DPDS: 6-diamino-2,4-
dihydroxypyrimidine sulfate; EMT: epithelial-mesenchymal transition;
FD-HPLC: high-performance liquid chromatography and fluorescence detection;
GLO1: glyoxalase 1; HIF-1α: hypoxia-inducible factor 1-α; MeCN: acetonitrile; 4-
MU: 4-methylumbelliferone; NAG: N-acetyl-β-D-glucosaminidase; NBD-PZ: 4-nitro-
7-piperazino-2,1,3-benoxadiazole; NF-κB: nuclear factor-kappa B; OAT: organic anion transporter; PAS: periodic acid–Schiff; PTEC: proximal tubular epithelial cells; RAGE: receptors for advanced glycation end products; Scr: serum creatinine; SSAO: semicarbazide-sensitive amine oxidase; TFA: trifluoroacetic acid; THIS: tubulointerstitial histological score; TNF-α: tumor necrosis factor-α; TPP: triphenylphosphine; TRPA1: transient receptor potential ankyrin 1.

Declarations

Ethics approval and consent to participate
All animal protocols were approved by the Animal Care and Use Committee of Taipei Medical University (LAC-2019-0482).

Consent for publication
Not applicable.

Availability of data and materials
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Funding
We are grateful to the financial support from the Ministry of Science and Technology, Taiwan, R.O.C. (NSC 97-2320-B-038-007-MY3).

Authors’ contributions

Study design and conception of animal experiment: Shih-Ming Chen and Chia-En Lin; data collection and data analysis: Chia-En Lin, Po-Yeh Lin, Wen-Chi Yang, Tzu-Yao Lin, and Chien-Ming Chen; animal experiment: Chia-En Lin, Po-Yeh Lin, Wen-Chi Yang, Yu-Shen Huang, Tzu-Yao Lin; data interpretation: Jen-Ai Lee and Chia-En Lin; manuscript drafting: Chia-En Lin; semi-quantitative analysis for collagen deposition: Hung-Shing Chen; critical revising the manuscript: Shih-Ming Chen and Jen-Ai Lee. All authors reviewed and approved the manuscript.

Acknowledgements

We appreciate it very much that Prof Shiro Ueda provided recommendation to our animal experiments and metabolic cages for urine collection. The graphical abstract was created with BioRender.com. We are grateful to the financial support from the Ministry of Science and Technology (NSC 97-2320-B-038-007-MY3). We acknowledge UNIVERSAL LINK CO., LTD. who provided professional writing services or materials.

Footnotes

Table 1. Biochemical parameters of the normal (N) and aristolochic acid (AA) group
BUN: blood urea nitrogen; Scr: serum creatinine; NAG: N-acetyl-β-D-glucosaminidase; * p < 0.05 vs. N group at the same time point, Student’s t-test; # p < 0.05 vs. AA group on day 56; † p < 0.05 vs. AA group on day 70, repeated measures ANOVA

Fig. 1 Periodic acid-Schiff (PAS) stained kidney sections and tubular interstitial histological scores (TIHS)

(a-i) Representative images of PAS-stained kidney sections from the N group on day 28 (a), 56 (c), 70 (e), and 84 (g) and AA group on days 28 (b), 56 (d), 70 (f), and 84 (h). The arrow indicates interstitial renal fibrosis. The stars indicate cellular infiltration. (i) Tubular interstitial histological scores (THIS). N group, normal group; AA group, aristolochic acid group. ** p < 0.01 vs. N group at the same time point, Student’s t-test; # p < 0.05 vs. AA group on day 56. † p < 0.05 vs. AA group on day 70, repeated measures ANOVA.

Fig. 2 Masson’s trichrome stained kidney sections and semi-quantitative analysis of collagen deposition

The fraction of interstitial fibrosis was assessed as the percentage of aniline blue-stained area. (a-i) Representative images of Masson’s trichrome-stained kidney sections from the N group on day 28 (a), 56 (c), day 70 (e), and day 84 (g) and AA group on day 28 (b), 56 (d), day 70 (f), and day 84 (h). The arrow indicates collagen
deposition. (i) Semiquantitative analysis of collagen deposition. N group, normal

AA group, aristolochic acid group. ** p < 0.01 vs. N group at the same time point, Student’s t-test; # p < 0.05 vs. AA group on day 56. † p < 0.05 vs. AA group on day 70, repeated measures ANOVA.

Fig. 3 Picro Sirius Red stained kidney sections and semi-quantitative analysis of collagen deposition

The fraction of interstitial fibrosis was assessed as the percentage of red-stained area.

(a-f) Representative images of Picro Sirius Red-stained kidney sections from the N group on day 28 (a), 56 (c), day 70 (e), and day 84 (g) and AA group on day 28 (b), 56 (d), day 70 (f), and day 84 (h). The arrow indicates collagen deposition. (i)

Semiquantitative analysis of collagen deposition. N group, normal group; AA group, aristolochic acid group. * p < 0.05, ** p < 0.01 vs. N group at the same time point, Student’s t-test; # p < 0.05 vs. AA group on day 56. † p < 0.05 vs. AA group on day 70, repeated measures ANOVA.

Fig. 4 Chromatograms of analysis and contents of methylglyoxal in serum and urine

HPLC chromatograms for serum methylglyoxal level on day 56 (a), day 70 (b), and day 84 (c). The corresponding peaks of methylglyoxal derivative was labeled in HPLC chromatograms. (d) Serum methylglyoxal content. The white bars indicate the N group and the black bars indicate the AA group. N group, normal group; AA group,
aristolochic acid group. HPLC chromatograms for urinary methylglyoxal on day 56 (e), day 70 (f), and day 84 (g). The corresponding peaks of methylglyoxal derivative was labeled in HPLC chromatograms. (h) Urinary methylglyoxal content, calculated as methylglyoxal level × 12-h urinary volume. N group, normal group; AA group, aristolochic acid group. * p < 0.05 and ** p < 0.01 vs. N group at the same time point, Student’s t-test; # p < 0.05 vs. AA group on day 56. † p < 0.05 vs. AA group on day 70, repeated measures ANOVA.

Fig. 5 Chromatographs of analysis and contents of lactate (D,L-lactate and D-lactate) in urine

(a, c, e) HPLC chromatographs for the D,L-lactate on day 56 (a), day 70 (c), and day 84 (e). (b, d, f) HPLC chromatographs for the D-lactate on day 56 (a), day 70 (b), and day 84 (c). The corresponding peaks of D,L-lactate and D-lactate derivative and internal standard (I.S.) was labeled in HPLC chromatograms. (g, h) Quantification of D,L-lactate (g) and D-lactate (h) amount. Urinary D,L-lactate amount, calculated as D,L-lactate level × 12-h urinary volume; urinary D-lactate amount, calculated as D-lactate level × 12-h urinary volume. N group, normal group; AA group, aristolochic acid group; I.S., internal standard. *** p < 0.001 vs. N group at the same time point, Student’s t-test; # p < 0.05 vs. the AA group on day 56, repeated measures ANOVA.

Fig. 6 Western blot analysis and relative levels of glyoxalase 1(GLO1) in the kidney
homogenates

(a) Representative western blot of GLO-1; β-actin was used as an internal control. (b) Semi-quantitative analysis of GLO1 expression. AA56, AA group on day 56; AA70, AA group on day 70; AA84, AA group on day 84; N group, normal group; AA group, aristolochic acid group. * p < 0.05 vs. N group, ANOVA.

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