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# Increased renal semicarbazide-sensitive amine oxidase activity and methylglyoxal levels in aristolochic acid-induced nephrotoxicity

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ABSTRACT

*Aims:* Aristolochic acid (AA) nephrotoxicity is related to accumulation of methylglyoxal (MGO) and $N^{\varepsilon}$-(carboxymethyl)lysine (CML) in the mouse kidney. We studied the activity of renal semicarbazide-sensitive amine oxidase (SSAO), a key enzyme involved in MGO generation, in AA-treated mice, and investigated nephroprotective effects produced by metformin, a MGO scavenger.

*Methods:* Mice were orally administered water or metformin for 15 days (12 or 24 mg $kg^{-1}$ $day^{-1}$), and injected AA (5 mg $kg^{-1}$ $day^{-1}$) intraperitoneally for 8 days starting on day 8. Renal function was studied, and histopathological examination, determination of renal SSAO activity, and measurement of MGO levels were performed.

*Key findings:* Compared to control mice, AA-injected mice showed significant renal damage and approximately 2.7-fold greater renal SSAO activity ($p < 0.05$). Further, compared to control treatment, administration of 12 mg/kg metformin inhibited formation of renal lesions, and significantly decreased renal MGO levels ($37.33 \pm 9.78$ vs. $5.89 \pm 2.64$ μg/mg of protein, respectively, $p < 0.01$). In the AA-treated mice, metformin also inhibited the accumulation of CML in renal tubules, but did not affect SSAO activity.

*Significance:* This study is the first to show elevated renal SSAO activity in AA-treated mice, which could be involved in MGO accumulation. Moreover, MGO scavenging by metformin reduces AA nephrotoxicity. These findings suggest that reducing MGO accumulation produces nephroprotection, revealing new therapeutic strategies for the management. SSAO is a key enzyme involved in MGO generation, and consequently, inhibition of renal SSAO activity is worth investigating in AA nephrotoxicity and other renal pathologies further.



## Introduction

Aristolochic acid (AA), a compound found in plants of the genus *Aristolochia*, causes nephrotoxicity (AA-induced nephrotoxicity, or AAN) characterized by initial damage to the proximal renal tubule, with later development of interstitial fibrosis and urothelial carcinoma {Debelle et al., 2002}. The U.S. Food and Drug Administration has recommended cessation of the use of botanical products containing AA {Schwetz, 2001}. However, products containing AA remain available online, and some herbal remedies have been found to be contaminated with AA, which has led to cases of AAN {Gold and Slone, 2003; Li et al., 2011}. There is no established optimal clinical recommendation for AAN therapy. Steroid therapy slows the progression of renal failure that characteristically accompanies progressive interstitial fibrosis in AAN {Vanherweghem et al., 1996}. However, steroid therapy is not effective in all cases of AAN, and long-term administration of steroids is associated with major side effects. Currently, the top priorities in the management of AAN are to control the progression of nephropathy and to prepare for renal replacement therapy {Gokmen et al., 2013}. Therefore, it is important to develop a clinically effective and safe treatment for AAN.

Accumulation of methylglyoxal (MGO) and $N^{\varepsilon}$-(carboxymethyl)lysine (CML) in kidney tissue has been demonstrated in a mouse model of AAN Li et al., 2012. MGO is an endogenous, highly-reactive, and cytotoxic aldehyde compound {Inoue and Kimura, 1995}. It can react with and modify DNA and other biomolecules, which alters their structures and functions, and results in the formation of advanced glycation end-products (AGEs), such as CML {Brownlee, 1994}. We speculated that renal MGO and CML were related to damage produced by AA, but the causes of AA-induced MGO generation and the effect of MGO reduction on AAN have not been investigated.

Semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.21) is involved in a MGO generation pathway that converts aminoacetone into MGO {Inoue and Kimura, 1995}. SSAO is found in several tissues, with particularly high activity in kidney {Lewinsohn, 1984; Lyles and Singh, 1985}. SSAO activates profibrogenic cytokines {Wong et al., 2013} that are involved in the progression of renal fibrosis that is characteristic of AAN. The activity of renal SSAO may be closely related to the production of MGO and the pathological progression of AAN, but this hypothesis has not been verified.

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The MGO inhibitor metformin was used to investigate whether a decrease in MGO levels mitigates the severity of AAN. Metformin (*N*,*N*-dimethylimidodicarbonimidic diamide) is generally accepted as the first-line treatment for type 2 diabetes mellitus. In addition, metformin is a MGO scavenger and an inhibitor of the formation of AGEs. The drug has been shown to reduce systemic levels of MGO and AGEs in patients with type 2 diabetes mellitus, and to prevent diabetic complications {Beisswenger et al., 1999; Yamagishi et al., 2008; Ruggiero-Lopez et al., 1999; Kiho et al., 2005 #6;Rahbar and Figarola, 2003}. Metformin has also been shown to ameliorate gentamicin-induced renal toxicity in rats by decreasing renal MGO levels {Amini et al., 2012; Li et al., 2013}. The safety, widespread clinical use, and proven efficacy of metformin make it an ideal MGO inhibitor for evaluation in animals with AAN.

This study investigated the relationship between increased renal MGO levels and SSAO activity in renal tissue by using a mouse model of AAN. Furthermore, the MGO inhibitor metformin was administered to confirm that reduction in MGO levels is nephroprotective in AAN mice, and to investigate the effect of metformin on renal SSAO activity.

## Materials and methods

### *Animal experiments*

All animal experiments were approved by the Animal Care and Use Committee of Taipei Medical University, Taiwan. Thirty-two C57BL/6 mice (7-week-old males) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). These mice were housed in temperature-controlled (25 ± 2 °C) and humidity-controlled (65% ± 5%) facility with a 12/12 light/dark photoperiod and access to standardized food pellets (TMI, USA) and tap water ad libitum. After 1 week of acclimatization, the mice were divided randomly into 4 groups (each group consisted of 8 mice).

Control group: mice treated with water (P.O) and saline (i.p.)

AA group: mice treated with water (P.O) and AA (i.p.)
AAML group: mice treated with low dose metformin (P.O) and AA (i.p.)
AAMH group: mice treated with high dose metformin (P.O) and AA (i.p.)

The control group was orally administered 0.1 mL water for 15 days (days 1–15) and intraperitoneally injected with 0.15 mL saline for 8 days (day 8–15). The AA group was orally administered 0.1 mL water for 15 days and intraperitoneally injected with 0.15 mL AA (days 8–15; 5 mg $kg^{-1}$ $day^{-1}$). The AAML and AAMH groups were treated with 0.1 mL metformin at doses of 12 and 24 mg $kg^{-1}$ $day^{-1}$, respectively, for 15 days, and injected with 0.15 mL AA for 8 days (days 8–15; 5 mg $kg^{-1}$ $day^{-1}$). At the end of the treatment period, the mice were placed in rodent metabolic cages with free access to food for 12-h urine collection. At the end of the experiment, the mice were sacrificed under pentobarbital (50 mg/kg), and a blood sample was collected by intracardiac puncture. A thorough necropsy was performed on all mice, and the kidneys were dissected for histopathological evaluation, immunohistochemistry, determination of SSAO activity, and quantification of MGO levels.

### *Preparation of urine, blood, and kidney samples*

Sediments was removed from the urine samples by centrifugation (700 × *g* at 4 °C for 5 min), and the supernatant was stored at −80 °C until analysis. The blood samples were centrifuged (700 × *g*, 15 min) and the serum was stored at −20 °C. The kidneys were embedded in paraffin, sectioned (thickness, 3–5 μm), and stained with periodic acid-Schiff stain for immunohistochemistry.

### *Serum and urinary creatinine assays*

Creatinine levels in the serum and urinary samples were measured using high-performance liquid chromatography (HPLC), as described previously {Lee et al., 2005; Marsilio et al., 1999}. The measured concentrations were expressed in milligrams per deciliter, and 0.1 mmol/L cimetidine in 0.01 mol/L HCl aqueous solution was used as the internal standard. A Capcell Pak C18 column (250 mm × 4.6 mm, ID; particle size, 5 μm; Shiseido, Tokyo, Japan) was used, and absorbance was monitored at 234 nm. The mobile phase was composed of acetonitrile and 100 mmol/L of PBS containing 30 mmol/L sodium lauryl sulfate adjusted to pH 3.0 with hydrochloric acid (36/60, v/v). The flow rate was 0.8 mL/min.

### *Measurement of the glomerular filtration rate*

Creatinine clearance ($C_{Cr}$), an index of the glomerular filtration rate (GFR), was calculated using the following equation adapted from Hsu and co-workers {Hsu et al., 2008}:

$$CCr = \frac{Ucr \times UV}{Scr} \times \frac{100}{BW} \times \frac{1}{720}$$

Where $C_{Cr}$ is creatinine clearance (mL $min^{-1}$ $kg^{-1}$ BW); Ucr, urinary creatinine concentration (mg/dL); UV, is urine volume (mL); Scr, serum creatinine (mg/dL); BW, body weight (g); and 720, the period in which urine was collected (21 h, expressed in min).

### *Determination of urinary protein levels and N-acetyl-β-D-glucosaminidase activity*

Urinary protein levels were quantified using a Bio-Rad protein assay kit with bovine serum albumin as the standard, according to the manufacturer's instructions. Urinary *N*-acetyl-β-D-glucosaminidase (NAG, EC 3.2.1.30) activity was determined using the method described by Leaback and Walker {Leaback and Walker, 1961}. NAG reacted with the 4-methylumbelliferyl-*N*-acetyl-β-glucosaminide, and fluorescence was measured and used to quantify NAG concentration.

### *Determination of blood urea nitrogen concentration*

The concentration of blood urea nitrogen (BUN) was determined using a urease assay kit (Sigma-Aldrich, St Louis, MO), according to the manufacturer's instructions. Urea was hydrolyzed by urease to ammonia and carbonate. In the presence of glutamic dehydrogenase, ammonia reacts with ketoglutaric acid with the concurrent oxidation of NADH to NAD. BUN activity is directly proportional to the rate of conversion of NADH to NAD, and it is monitored at 340 nm {Kaltwasser and Schlegel, 1966}.

### *Urinary microalbumin*

Microalbumin levels were measured using a commercial kit (Good Biotech Corp., Taiwan) based on immunoturbidimetric methods, with procedures performed according to the manufacturer's instructions. The samples were conditioned with a Tris buffer before incubation with an anti-albumin antibody for 10 min. The samples were then analyzed at 405 nm.

### *Histopathological examination*

A histopathological evaluation was performed on the kidneys of the mice according to the guidelines proposed previously {Sato et al., 2004}. The histopathological severity of the lesions was rated from one to five (1 = minimal; 2 = slight; 3 = moderate; 4 = moderately severe; 5 = severe/high). The sum of scores for each sample, reflecting tubular