Sex-Dependent Effects of Aristolochic Acid on Kidney Injury in Mice

ABSTRACT

Aristolochic acid (AA) is widely recognized for its nephrotoxic effects; however, the role of sex in AA-induced renal injury remains ambiguous. The present investigation aimed to elucidate the sex-based disparities in renal dysfunction and tubular damage resulting from AA exposure.Male and female murine models underwent bilateral orchiectomy and ovariectomy, respectively. Fourteen days post-gonadectomy, the subjects were administered AA (10 mg/kg body weight/day) via intraperitoneal injection for two consecutive days, followed by euthanasia seven days subsequent to the initial injection. Parameters such as body weight, renal functionality, and tubular morphology were meticulously evaluated. A comparative analysis between male and female non-gonadectomized subjects revealed that AA-induced weight reduction was significantly more pronounced in male mice. Additionally, functional and structural impairments in male kidneys were significantly exacerbated following AA administration, whereas the kidneys of AA-treated female mice exhibited either negligible or mild injuries. Ovariectomy did not influence AA-induced nephrotoxic acute kidney injury in the female cohort. In contrast, orchiectomy resulted in a significant attenuation of weight loss, renal dysfunction, and tubular damage in the context of AA-induced nephrotoxicity among male mice. This research has conclusively established that the presence of testes contributes to the development of AA-induced nephrotoxic acute kidney injury.

Keywords:Aristolochic acid; nephrotoxicity, kidney injury; sex difference; gonadectomy, ovariectomy, renal dysfunction; tubular damage.

3. Research Papers and Short Notes should follow the structure of Abstract, Introduction, Methodology, Results and Discussion, Conclusion, Acknowledgements, Competing Interests, Authors' Contributions, Consent (where applicable), Ethical approval (where applicable), and References plus figures and/or tables.)

1. INTRODUCTION

Sociocultural gender represents an idealized construct that is systematically actualized over time[1]; nonetheless, biological sex constitutes a critical variable that is unequivocally associated with the prevalence and advancement of numerous diseases [2], such as hypertension [3], chronic kidney disease (CKD) [4], and acute kidney injury (AKI) [5]. The observed sex disparities in non-

reproductive anatomical structures are generally ascribed to gonadal hormones or sex chromosomes [6]. Notably, the female sex exhibits a protective effect against ischemic AKI in both rodent models and hospitalized individuals necessitating renal replacement therapy [7]. While it is recognized that chemotherapeutic drugs like doxorubicin and cisplatin elicit reduced nephrotoxic effects in female rodents relative to their male counterparts[8,9], the role of sex in the context of nephrotoxic AKI remains inadequately understood.

Aristolochic acid (AA) is a highly potent nephrotoxin extracted from the flora of the *Aristolochiaceae* family, yet it has been utilized for therapeutic applications for over 2500 years across various nations. AA serves as a contributing factor in the clinical manifestations termed Chinese herb nephropathy (CHN) and Balkan endemic nephropathy (BEN), which are linked with chronic kidney disease (CKD) and urothelial cancers[10,11]. The sex ratio observed in BEN is approximately 1:1, unlike the female dominance seen in CHN, attributed to the context of a weight loss herbal treatment [12]. The incidence of AKI among individuals afflicted with AA nephropathy is notably elevated, correlating with renal dysfunction, tubular atrophy, infiltration of interstitial inflammatory cells, and eventual progression to end-stage renal failure [13-15]. Presently, there exists a limited number of investigations addressing sex differences in renal function and tubular architecture as a response to AA-induced nephrotoxic AKI. This study aims to evaluate the influence of sex on nephropathy induced by AA.

2. MATERIAL AND METHODS

2.1 Animal Preparation

Eight- to ten-week-old C57BL/6 mice were acquired from Orient Bio (Seongnam, Gyeonggi, Republic of Korea). Mice were housed under a 12-hour light/dark cycle at 22 ± 2 °C with 55 ± 5% humidity and were provided unrestricted access to both water and standard mouse diet chow. Mice received an intraperitoneal injection of AA (10 mg/kg body weight/day; Sigma-Aldrich, St. Louis, MO, USA; product no. A9451) in 0.9% saline (control) for two consecutive days, followed by euthanasia seven days post-initial injection. Male and female mice underwent bilateral orchiectomy and ovariectomy, respectively [5,16], fourteen days prior to the initial injection. Sham-operated mice experienced identical surgical procedures without the gonadectomy. Prior to surgery and euthanasia, mice were anesthetized with a pentobarbital sodium (ENTOBAR; 60 mg/kg body weight; Hanlim Pharm, Seoul, Republic of Korea) intraperitoneal injection, as previously documented [17-30].

2.2. Body Weight

The mass of the subjects was recorded on a daily from day 0 to day 7 subsequent to the initial administration,using an electronic digital scale with a precision of 0.1 g (Ohaus, Parsippany, NJ, USA; product no. CS200).

2.3. Kidney Function

Following euthanasia induced by pentobarbital sodium (Hanlim Pharm), blood specimens were acquired via cardiac puncture utilizing heparinized syringes immediately prior to termination. The specimens underwent centrifugation at 9000 rpm for a duration of 10 minutes in a refrigerated Fresco 17 microcentrifuge (Thermo Fisher Scientific, Waltham, MA, USA; product no. 75002402). The resultant supernatants were employed as plasma to quantify creatinine levels using an enhanced Jaffe method, facilitated by the QuantiChrom creatinine assay kit (BioAssay Systems, Hayward, CA, USA; product no. DICT-500). The absorbance of the plasma samples was determined at a wavelength of 510 nm utilizing a SpectraMax i3x multi-mode microplate reader (Molecular Devices, San Jose, CA, USA, situated in the Bio-Health Materials Core-Facility, Jeju National University), in accordance with previously established protocols[16-19,23,26-32].

2.4. Tubular Injury Score

The kidneys were preserved in 4% paraformaldehyde (Tech and Innovation, Chuncheon, Gangwon, Republic of Korea; product no. BPP-9016) for subsequent periodic acid-Schiff (PAS) staining. The assessment of tubular injury was quantified as the percentage of affected tubules exhibiting cast formation, tubular dilation, and cellular sloughing, evaluated in a blinded manner under a Nikon Eclipse Ni microscope (Nikon, Tokyo, Japan), in accordance with previously established methodologies[16-18,23,25-32]. Counts of total and damaged tubules were conducted across five randomly selected fields at ×400 magnification within the kidney cortex: outer stripe of the outer medulla (OSOM), inner stripe of the outer medulla (ISOM), and inner medulla, all stained with PAS.

2.5. Statistical Analysis

All statistical evaluations of the dataset were conducted utilizing SigmaPlot 14.0 software (Systat Software Inc., San Jose, CA, USA), as previously delineated[17,30,33-36]. The assessment of normal distribution was carried out through a Shapiro-Wilk normality test. In instances where the data did not conform to a normal distribution, a logarithmic transformation was employed. Data that exhibited a normal distribution were analyzed using a two-way analysis of variance (ANOVA), followed by a Holm-Sidak post hoc analysis. In contrast, data that displayed a non-normal distribution were analyzed using the Kruskal-Wallis test, accompanied by the Student-Newman-Keuls post hoc analysis. A *P*-value of < 0.05 was deemed to be statistically significant.

3. RESULTS

3.1. Comparative Analysis of Body Weight, Renal Function, and Tubular Injury in Male and Female Mice

The investigation of sex differences in nephrotoxicity induced by AA involved an assessment of body weight reduction in adult male and female mice at 6 days following two daily administrations of AA or a control solution. Mice subjected to

AA injections exhibited a significant reduction in body weight in comparison to the control group (Table 1). When analyzing the male and female mice, the body weight loss attributed to AA was notably more pronounced in the male cohort than in the female cohort (Table 1). To ascertain renal dysfunction, plasma levels of creatinine were quantified as an index of the glomerular filtration rate. In control mice, plasma creatinine levels were recorded at less than 0.3 mg/dL (Table 1). Post AA administration, male mice experienced a substantial elevation in plasma creatinine levels, whereas the increase observed in female mice was relatively modest (Table 1).

Table 1.Changes in body weight and plasma creatinine concentration

Condition	Body weight change (g)	Plasma creatinine (mg/dl)
Control in male	0.37±0.15	0.24±0.02
Control in female	0.68±0.09	0.26±0.01
AA in male	−5.92±0.71 **	2.78±0.36 ***
AA in female	−1.88±0.17 ***$^{\#}$	0.38±0.05 *$^{\#\#}$

*Significant from control, * P < 0.05; ** P < 0.01; *** P < 0.001. Significant from male, $^{\#}$ P < 0.01; $^{\#\#}$ P < 0.001. Data are presented as means ± standard error of the mean for six mice*

To evaluate structural impairment of the kidneys, renal tissue was categorized into the cortex, OSOM, ISOM, and inner medulla, with subsequent quantification based on tubular injury parameters, including cast formation, tubular dilation, and cellular sloughing. Correlating with the observed increase in renal dysfunction, male mice exhibited heightened tubular injury scores across the cortex, OSOM, ISOM, and inner medulla compared to females post AA exposure (Table 2). Among the renal regions in male mice, the tubular injury score for the inner medulla demonstrated reduced susceptibility to AA when juxtaposed with the scores from the cortex, OSOM, and ISOM (Table 2). Notably, female mice did not exhibit significant tubular injury in any renal regions following AA treatment when contrasted with the control female group (Table 2). In control renal tissues, no substantial discrepancies were detected in body weight alterations, renal function, or tubular structural integrity between male and female mice.

Table 2.Tubular injury score

Cdt	Cortex	OSOM	ISOM	Inner medulla
CM	2.3 ± 0.4	4.1 ± 0.8	4.7 ± 0.5	0.9 ± 0.2
CF	2.3 ± 0.3	4.8 ± 1.2	7.2 ± 0.3	0.9 ± 0.2
AM	87.4 ± 0.8 *	90.8 ± 0.8 *	88.7 ± 1.7 *	47.8 ± 2.6 *
AF	3.2 ± 0.9 $^{\#}$	4.4 ± 0.7 $^{\#}$	8.6 ± 0.9 $^{\#}$	1.7 ± 0.3 $^{\#}$

*Significant from control, * P < 0.001.Significant from male, $^{\#}$ P < 0.001. Data are presented as means ± standard error of the mean for six mice. Cdt, condition; CM, control in male; CF, control in female; AM, AA in male; AF, AA in female*

3.2. Impact of Ovariectomy on AA-induced Nephrotoxicity

In order to ascertain the impact of ovaries on the safeguarding of renal functionality and tubular integrity from AA-induced nephrotoxicity, bilateral ovariectomy was executed in female murine subjects, followed by an examination of body weight reduction, renal impairment, and structural injuries resulting from AA administration. Despite the observation that female mice exhibited considerable body weight reduction and an increase in plasma creatinine levels post-AA injection, no notable differences were detected in these metrics between ovariectomized and intact renal tissues (Table 3).

Table 3.Changes in body weight and plasma creatinine concentration

Condition	Body weight change (g)	Plasma creatinine (mg/dl)
CIF	0.83 ± 0.25	0.26 ± 0.02
COF	0.63 ± 0.10	0.27 ± 0.02
AIF	−1.32 ± 0.28 **	0.37 ± 0.04 *
AOF	−0.98 ± 0.19 **	0.38 ± 0.02 *

*Significant from control, * P < 0.01; ** P < 0.001. Data are presented as means ± standard error of the mean for six mice. CIF, control in intact female; COF, control in ovariectomized female; AIF, AA in intact female; AOF, AA in ovariectomized female*

The scores for tubular damage in the cortex, OSOM, ISOM, and inner medulla remained largely unchanged due to AA exposure and/or ovariectomy (Table 4).

Table 4.Tubular injury score

Cdt	Cortex	OSOM	ISOM	Inner medulla
CIF	2.2 ± 0.3	3.8 ± 0.7	4.2 ± 0.3	1.0 ± 0.2
COF	2.3 ± 0.3	3.9 ± 0.5	3.8 ± 0.4	1.1 ± 0.2
AIF	3.2 ± 0.7	4.1 ± 0.5	4.9 ± 0.3	1.2 ± 0.2
AOF	3.4 ± 0.5	4.2 ± 0.7	4.2 ± 0.4	1.2 ± 0.3

Data are presented as means ± standard error of the mean for six mice. Cdt, condition; CIF, control in intact female; COF, control in ovariectomized female; AIF, AA in intact female; AOF, AA in ovariectomized female

3.2. Effects of Orchiectomy on Renal Function, Body Weight, and Tubular Injury in Male Mice

In order to ascertain whether testes contribute to functional and structural impairment subsequent to an AA injection, a bilateral orchiectomy was conducted on male mice, followed by an evaluation of body weight loss, renal dysfunction, and structural damage resulting from AA exposure. Male mice exhibited a considerable reduction in body weight post-AA injection when juxtaposed with control mice (Table 5). A comparison between orchiectomized and intact mice revealed that the body weight loss induced by AA was significantly diminished in orchiectomized subjects relative to intact mice (Table 5). Following AA administration, the plasma creatinine concentration experienced a notable increase in intact mice; however, the extent of this increase was significantly attenuated in orchiectomized mice (Table 5).

Table 5.Changes in body weight and plasma creatinine concentration

Condition	Body weight change (g)	Plasma creatinine (mg/dl)
CIM	0.52 ± 0.15	0.26 ± 0.04
COM	1.12 ± 0.85	0.28 ± 0.02
AIM	−6.08 ± 0.62 *	2.59 ± 0.38 *
AOM	−4.32 ± 0.36*,#	1.02 ± 0.13*,##

*Significant from control, * P < 0.001. Significant from male, # P < 0.01; ## P < 0.001. Data are presented as means ± standard error of the mean for six mice. CIM, control in intact male; COM, control in orchiectomized male; AIM, AA in intact male; AOM, AA in orchiectomized male*

In alignment with improved kidney function, orchiectomized mice demonstrated significantly lower tubular injury scores in the cortex, OSOM, ISOM, and inner medulla compared to those of intact mice after AA injection (Table 6). In the control kidneys, no significant differences were detected in body weight changes, renal function, or tubular morphology between orchiectomized and intact mice (Table 6).

Table 6.Tubular injury score

Cdt	Cortex	OSOM	ISOM	Inner medulla
CIM	2.4 ± 0.3	2.7 ± 0.5	3.2 ± 0.2	1.1 ± 0.1
COM	3.1 ± 0.5	2.1 ± 0.5	2.8 ± 0.4	1.0 ± 0.1
AIM	82.6 ± 0.8 **	90.2 ± 0.8 **	81.7 ± 1.2 **	48.4 ± 2.5 **
AOM	32.6 ± 1.9 **,##	64.2 ± 2.1*,#	44.2 ± 2.6 **,##	9.9 ± 0.7 **,##

*Significant from control, * P < 0.001; ** P < 0.001. Significant from male, # P < 0.01; ## P < 0.001. Data are presented as means ± standard error of the mean for six mice. Cdt, condition; CIM, control in intact male; COM, control in orchiectomized male; AIM, AA in intact male; AOM, AA in orchiectomized male*

4. DISCUSSION

Numerous facets of physiology and pathology exhibit significant variation across genders, yet a substantial number of animal experiments predominantly utilize a single sex, often male. Additionally, clinical investigations frequently neglect to incorporate sex as a variable [2,37]. Consequently, this oversight in accounting for sex as a biological variable has engendered a considerable knowledge deficit. The present study delivers critical novel insights concerning sex-based disparities in response to AA-induced nephrotoxicity. These findings indicate, firstly, that AA administration in male mice significantly exacerbates weight loss, renal impairment, and tubular damage compared to controls, while the female sex mitigates these effects. Secondly, ovariectomy does not alter renal function or tubular morphology after AA exposure. Thirdly, orchiectomy confers protection against both functional and structural injuries associated with AA-induced nephrotoxicity, as well as against weight loss.

The kidney represents an intricate organ comprised of diverse forms of tubular epithelial cells, typically categorized into segments based on their morphology and anatomical positioning. Consequently, under conditions of AKI, these segments manifest a wide range of pathological reactions [38,39]. Ischemic AKI primarily impacts the S3 segment of the proximal tubule situated within the kidney's OSOM[16-19,23,26,34,40], while septic AKI predominantly inflicts severe damage upon the S1 to S3 segments of the proximal tubule within both the kidney cortex and OSOM[41,42]. The findings from the majority of animal and clinical investigations concerning AA-induced nephrotoxicity have been restricted to the cortical region [14,43]. In this study, AA-induced tubular injuries were observed extending from the cortex into the outer medulla and further into the inner medulla in male kidneys, although such damage was not evident in female kidneys. Notably, the inner medulla exhibited a reduced vulnerability to AA when compared to other regions within the male kidney.

AA-linked pathological syndromes, particularly BEN, are correlated with local polymorphisms in cytochrome 450[44]. Additionally, AA influences the cytochrome 450 metabolic pathway during the onset of renal injury and oncogenesis[45,46]. Among the cytochrome P450 enzymes, CYP19A1, commonly referred to as aromatase, facilitates the conversion of androgen to estrogen [47] and can significantly influence estrogen concentrations in males. Moreover, prior research has indicated that the estrogen steroid hormone confers protection against AA-induced nephrotoxicity [23,48] and that its receptor serves as a target protein related to AA. Nonylphenol, recognized as an environmental estrogen, exhibits distinct toxicological impacts across sexes through sex-specific induction of cytochrome P450 [49,50]. Nonetheless, the intricate molecular mechanisms underlying gonadal hormones and sex chromosomes in the context of AA-induced nephrotoxicity remain to be examined.

It is widely acknowledged that the incidence of cardiovascular diseases is heightened in male and postmenopausal female patients [51], with this increase being critically linked to the depletion of estrogen [52,53]. Certain renal pathologies also exhibit marked gender disparities regarding their occurrence, development, and therapeutic responses. Empirical investigations, both clinical and preclinical, have indicated that the male sex demonstrates significantly greater vulnerability to ischemic AKI compared to the female sex [16,54]. Moreover, orchiectomy has been shown to diminish renal vulnerability to ischemic AKI, while ovariectomy appears to have no discernible impact on this susceptibility [5,55]. In alignment with earlier findings concerning ischemic AKI, the present study revealed that nephrotoxicity induced by AA was substantially pronounced in male mice, in contrast to female mice. Additionally, upon subjecting both sexes to gonadectomy, a notable reduction in kidney injury was observed in male mice, whereas no alteration was recorded in female mice. The overall mortality associated with AA-induced acute toxicity was significantly higher in male mice compared to their female counterparts [56,57]. Both prior and current findings indicate that the presence of testes, rather than the absence of ovaries, is pivotal in elucidating the sex differences in renal susceptibility to AA-induced nephrotoxicity. Caution should be exercised when extrapolating

these animal study results to human subjects. Prior investigations have established the existence of species-dependent variances in toxicity profiles between rodents and humans [58,59], which represents a limitation of the present study.

5. CONCLUSION

The present investigation has demonstrated that female murine subjects exhibit a reduced vulnerability to AA-induced nephrotoxicity, and that the surgical removal of the testes significantly mitigates both functional and structural renal impairments attributable to AA. This observation offers an innovative perspective on the sexually dimorphic traits relevant to the development of protective strategies aimed at preventing nephrotoxicity and AKI.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

All animal experiments were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of Jeju National University under approval no. 2021-0045 on 13 July 2021.

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