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Leonurine ameliorates adriamycin-induced podocyte injury via suppression of oxidative stress

Xi Liu#, Wei Cao#, Jia Qi, Qing Li, Min Zhao, Zhuyun Chen, Jingfeng Zhu, Zhimin Huang, Lin Wu, Bo Zhang, Yanggang Yuan, and Changying Xing*

Department of Nephrology, the First Affiliated Hospital of Nanjing Medical University, Nanjing Medical University, Nanjing, China; Department of Pharmacy, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China; Department of Nephrology, Affiliated Nanjing Drum Tower Hospital, Nanjing University School of Medicine, Nanjing, China

ABSTRACT
Leonurine, a major bioactive component from Herba Leonuri, shows therapeutic potential in several diseases, including diabetes, cardiovascular disease, bovine mastitis and depression. In kidney, it was reported that leonurine was performing a protective effect in both acute kidney injury and renal fibrosis mice models. The aim of this study is to investigate the effect of leonurine in podocyte injury. In the mice model of adriamycin (ADR)-induced nephropathy, the application of leonurine significantly prevented early kidney damage, macrophage infiltration and proteinuria. Meanwhile, leonurine suppressed ADR-induced podocyte injury and reactive oxygen species (ROS) production. Consistent to in vivo results, leonurine prevented ADR-induced podocyte injury and ROS production in cultured human podocytes. All these results suggested that leonurine might suppress ADR-induced podocyte injury via inhibiting oxidative stress. Leonurine might be a novel therapeutic drug for prevention of glomerular diseases.

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KEYWORDS
Adriamycin; leonurine; oxidative stress; podocyte injury

Introduction
Proteinuria is considered a major healthcare problem affecting several hundred million people worldwide [1]. Podocytes are the specialised visceral epithelial cells that cover the outer layer of the glomerular basement membrane [2]. Injury to podocytes is considered a major contributor to proteinuria in a variety of glomerular diseases [3]. Although dramatic advances have occurred in the understanding of the pathogenesis of podocyte injury in recent years [4], the efficacy of strategies currently available for the prevention of proteinuria kidney disease is still unsatisfactory.

Herba Leonuri is a traditional Chinese herbal medicine which is usually used for the treatment of patients with uterine fibroid [5]. Recently, leonurine, a natural alkaloid extract of Herba Leonuri, has been documented as having anti-inflammatory and antioxidation properties. We previously found that leonurine ameliorated age-dependent impairment of angiogenesis possibly through attenuation of the mitochondrial oxidative stress and the membrane potential collapses [6]. In kidney, it was reported that leonurine was performing a protective effect in both acute kidney injury and renal fibrosis mice models [7,8]. However, it remains unclear whether leonurine alleviates podocyte injury via its antioxidant properties.

In this study, we identify for the first time that leonurine protected against adriamycin (ADR)-induced podocyte injury through suppressing oxidative stress both in vivo and in vitro. A better understanding of the function of leonurine in the podocyte injury will provide unexpected opportunities for developing new therapies for glomerular diseases.

Materials and methods
Reagents and antibodies
Adriamycin, leonurine and dihydroethidium (DHE) were purchased from Sigma Aldrich (St. Louis, MO). Anti-nephrin, and anti-podocin antibodies were obtained from Abcam (Cambridge, MA). Anti-PGC-1α was obtained from Santa Cruz (Santa Cruz, CA). Anti-MnSOD were obtained from Proteintech (Chicago, IL). Anti-GAPDH was obtained from Sanying biotechnology (Wuhan, China). All secondary antibodies for immunoblot analysis were from Zhongshan Golden Bridge

CONTACT Changying Xing cyxing62@126.com; Yanggang Yuan ygyuan@njmu.edu.cn Department of Nephrology, the First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital, 300 Guangzhou Road, Nanjing, Jiangsu Province, China
Senior authors have contributed equally to this work. #L.X. and C.W. contributed equally to this work.
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Biotechnology (Beijing, China). 2′, 7′-dichlorofluorescein diacetate (DCFDA) was from Invitrogen (Carlsbad, CA).

**Animals**

Eight-week-old male Balb/c mice were housed in stainless steel cages with glass water bottles at a regulated 12-h light-dark cycle. ADR-induced nephropathy was induced in six mice by injecting once intravenously ADR at 10 mg/kg body weight (BW), while control mice received saline. Leonurine groups received intraperitoneal injections of 15 mg/kg leonurine once every day for 2 weeks. Urine was collected using metabolic cages over a 24-h period on day 14 after treatment with ADR. Mice were sacrificed at day 15, and kidneys were harvested for various analyses. All the procedures for animal studies were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

**Cell culture**

Conditionally immortalised human podocytes were kindly provided by Moin A. Saleem (University of Bristol, Bristol, UK) and cultured and differentiated as described previously [9]. Briefly, podocytes were propagated on type I collagen-coated flasks at the permissive temperature (33°C–34°C) with 10% foetal bovine serum and insulin-transferrin-selenite supplement. To allow for differentiation, podocytes were thermoshifted to 37°C for 10–14 days. All experimental groups were treated with ADR (1 μg/ml) for different period, and leonurine (20 μmol/L) was added 1 h before ADR. The control group was treated with 0.1% DMSO.

**Renal histology and electron microscopy**

Histological and electron microscopic examinations were performed according to procedures described previously [10]. Kidney sections 2 μm thick were stained with PAS for histological examination by light microscopy. PAS positive glomerular mesangial matrix area was quantified and the mesangial matrix index was expressed as mesangial matrix area/tuft area. For electron microscopy, renal cortical tissues were cut into small pieces (~1 mm³) and fixed in 2.5% glutaraldehyde. Transmission electron micrographs were obtained using an electron microscope.

**Twenty-four-hour urinary albumin excretion**

Urine albumin concentrations were determined using a mouse albumin ELISA kit (Abcam, Cambridge, MA), according to the manufacturer’s guidelines.

**Western blots**

The procedure and protocol for the Western blot analysis was as previously reported [11]. Briefly, 30 mg of total protein was loaded to run 10% SDS-PAGE, and the protein was transferred to PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated with the indicated primary antibodies as anti-nephrin (1:200), anti-podocin (1:1000), anti-PGC-1α (1:200), anti-MnSOD (1:1000), and anti-GAPDH (1:5000), respectively. Subsequently, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour in room temperature. Immunoreactive bands were visualised by enhanced chemiluminescence (ECL, Thermo Fisher Scientific, Rockford, IL). Densitometric analysis of the images was performed using Quantity One Software (Bio-Rad, Hercules, CA).

**Immunohistochemistry**

Immunohistochemistry analysis of podocin in the renal tissue was performed on 2-μm paraffin-embedded renal sections. After fixing and blocking, the sections were then incubated overnight at 4°C with primary anti-podocin antibody. After washing with PBS, the secondary antibody was applied, and the signal was visualised using the standard avidin-biotin-peroxidase complex method (Santa Cruz Biotechnology).

**Immunofluorescence**

Immunofluorescence was performed on frozen sections. Macrophages were labelled with F4/80 monoclonal antibody, FITC conjugate from Invitrogen for immunofluorescence. Positive signals were examined using a confocal microscope. Macrophages infiltrating the interstitium were counted and expressed as the number of macrophages per high-power field.

**Measurement of ROS generation**

For in vivo examination, the frozen kidney sections were incubated with 10 μM DHE for 30 min at 37°C. Results were visualised using a confocal microscope (Carl Zeiss, Germany). For in vitro study, intracellular ROS production was detected by DCFDA (10 μM) staining as previously described [12]. To determine mitochondrial ROS, podocytes were incubated with 5 mM MitoSOX Red (Life Technologies, Eugene, OR) for 10 min at 37°C before direct fluorescence measurement with a microplate Reader from Eppendorf.
**F-actin immunofluorescence staining**

After treatment, rhodamine-phalloidin was used for visualisation of F-actin following the manufacturer’s protocol (Molecular Probes). Nuclear staining was performed using 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen, UK). The staining was visualised with a confocal microscope (Carl Zeiss, Germany). Ten images from each sample were acquired randomly. The percentage of cells with cytoskeletal remodelling was counted for each field was evaluated according to a previous study [13].

**Apoptosis**

In situ detection of cell death was performed using a commercially available TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling) kit (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer’s protocols. For *in vitro*, annexin V-FITC Apoptosis Detection Kit (BD Biosciences) was applied to evaluate podocyte apoptosis following the manufacturer’s protocols. After treatment, podocytes were resuspended with FITC-conjugated annexin V and propidium iodide (PI) for 10 min, and immediately analysed with a flow cytometer. Percent of total apoptotic cells is calculated from Q2+Q4.

**Measurement of podocytes oxygen consumption rates (OCR)**

A Seahorse Bioscience XFe96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA) was used to measure podocytes oxygen consumption. Podocytes were seeded in XFe96 cell culture microplates at a density of 2 × 10⁴. After differentiation and treatment, Cell Mito Stress Test Kit (Seahorse Bioscience) was used to measure OCR according to the instruction supplied. We used the ATP synthase inhibitor oligomycin (1 μM), uncoupling agent FCCP (2 μM), and complex III inhibitor antimycin (0.5 μM)/complex I inhibitor rotenone (0.5 μM). Basal OCR was calculated as OCR before oligomycin minus OCR after antimycin. Maximal OCR was calculated as OCR after FCCP minus OCR after antimycin. ATP-linked OCR was calculated as OCR before oligomycin minus OCR after oligomycin.

**Statistical analysis**

Values were presented as means ± standard error of mean (SEM). Statistical analysis was performed by one way ANOVA and Bonferroni tests by using SPSS for Windows version 22.0. Differences were considered significant at p < 0.05.

**Results**

Leonurine inhibited renal structural changes and albuminuria in adriamycin mice

To assess the possible renoprotective effect of leonurine in the murine ADR nephropathy model, kidney sections were stained with PAS to evaluate the extent of kidney pathological injury. As shown in Figure 1(A,B), PAS staining of renal sections at 2 weeks after ADR treatment showed that glomeruli developed mesangial matrix expansion, and this histological damage was ameliorated after leonurine treatment. Consistent with these histological changes, immunofluorescence staining revealed that macrophage infiltration was significantly ameliorated by leonurine treatment (Figure 1(C,D)). Meanwhile, the application of leonurine significantly prevented the production of proteinuria (Figure 1(E)). These results indicated that leonurine exerted a renoprotective effect against nephrotoxicity induced by ADR.

**Leonurine suppressed podocyte injury in adriamycin mice**

Since podocyte injury is an early and predominant pathologic hallmark in ADR model [14], we examined several indicators of podocyte injury to verify the role of leonurine in this model. As shown in Figure 2(A), electron microscopy revealed that ADR induced foot process effacement, and leonurine therapy significantly attenuates this lesion, wherein podocyte foot processes were mostly preserved by leonurine treatment. Also, leonurine significantly prevented ADR-induced reduction in the expression of slit diaphragm associated proteins nephrin (Figure 2(B)) and podocin (Figure 2(C)). Moreover, podocytes depletion by apoptosis is a typical phenomenon in the ADR nephropathy model [15]. Thus, the TUNEL assay was performed to determine whether leonurine could modulate podocyte apoptosis in the ADR model. As shown in Figure 3, podocyte apoptosis was obviously increased in ADR mice compared to control group, and leonurine treatment decreased ADR induced apoptosis. These data indicated that leonurine suppressed ADR-induced podocyte injury.

**Leonurine inhibited oxidative stress in adriamycin mice**

Given that the toxic effect of ADR on podocytes was closely associated with oxidative stress [16], we next investigated whether the renoprotective effect of leonurine depended on its antioxidant activity. As shown
**Figure 1.** Effect of leonurine on adriamycin-induced kidney injury and albuminuria in vivo. (A) Representative histological photomicrograph of glomeruli by PAS staining (×400). (B) Quantification of mesangial matrix index. (C) Immunofluorescence staining for macrophages. (D) Quantification results for the numbers of macrophages (F4/80-positive cells). (E) Urinary albumin levels. Data expressed as means ± SEM (n = 6 for each group). *p < .05 versus control group, #p < .05 versus ADR group. ADR: adriamycin; Leo: leonurine.

**Figure 2.** Effect of leonurine on adriamycin-induced podocyte injury in vivo. (A) Electron microscopy of kidney specimens procured from mice on day 15. Podocyte injury was featured by extensive foot process effacement. (B) Western blot analysis of nephrin. Left: representative immunoblots. Right: densitometric analysis. (C) Representative immunohistochemistry staining with antibody against podocin (Magnification ×400). Data expressed as means ± SEM (n = 6 for each group). *p < .05 versus control group, #p < .05 versus ADR group. ADR: adriamycin; Leo: leonurine.

**Figure 3.** Effect of leonurine on adriamycin-induced podocyte apoptosis in vivo. (A) Images of TUNEL staining in the kidney glomeruli. Magnification ×400. (B) Apoptosis rates calculated by TUNEL-positive podocytes per glomerular cross-section. Data expressed as means ± SEM (n = 6 for each group). *p < .05 versus control group, #p < .05 versus ADR group. ADR: adriamycin; Leo: leonurine.
in Figure 4(A,B), ADR mice had increased levels of DHE in glomeruli compared with control mice. As PGC-1α regulates the expression of several ROS scavenging enzymes to countering ROS generation and manganese superoxide dismutase (MnSOD) is an important ROS-detoxifying mitochondrial protein, we examined the effects of leonurine on PGC-1α and MnSOD expression. As shown in Figure 4(C), leonurine treatment suppressed ADR-induced PGC-1α and MnSOD reduction. Leonurine treatment suppressed the increases in DHE fluorescence in ADR mice. These results indicated that the renoprotective mechanisms of leonurine might be attributed to the inhibition of oxidative stress via affecting PGC-1α expression.

Leonurine inhibited adriamycin-induced oxidative stress in vivo

We further investigated the beneficial effect of leonurine in cultured human podocytes cell line. It is reported that derangements of actin cytoskeleton is a critical pathologic manifestation among several pathophysiologic mechanisms of podocyte injury [17]. Here, we found that pre-treatment with leonurine prevented the actin cytoskeletal derangement induced in cultured podocytes by ADR (Figure 5(A,B)). Similarly, leonurine treatment inhibited ADR-induced nephrin reduction (Figure 5(C)). Moreover, leonurine treatment suppressed ADR-induced podocyte apoptosis (Figure 6). These data indicated leonurine suppressed ADR-induced podocyte injury in vitro.

Leonurine inhibited adriamycin-induced oxidative stress in vitro

Consistent with in vivo findings, we tested the effect of leonurine on ADR-induced ROS production and PGC-1α reduction in cultured podocytes. As shown in Figure 7, ROS production induced the modification of carboxy-H2DCFDA that fluoresced green as observed by a microscope. Pretreatment of podocytes with leonurine inhibited ADR-enhanced total cellular ROS levels. We also measured the ROS generation by MitoSOX, a mitochondrial superoxide indicator. As shown in Figure 7(C), leonurine inhibited ADR-induced mitochondrial ROS production. Furthermore, leonurine restored the expression of PGC-1α and MnSOD after ADR treatment in cultured podocytes (Figure 7(D)). In order to examine whether leonurine improved mitochondrial function via regulating PGC-1α in the podocytes, the oxygen consumption rate (OCR) was examined using Flux analyser. As shown in Figure 7(E,F), significant reduction of basal OCR, maximal OCR and ATP-linked OCR were seen in the ADR treatment cells. Leonurine could alleviate
ADR-induced mitochondrial damage. All these data suggested that leonurine also performed a significantly antioxidative function in ADR-induced podocyte apoptosis in vitro, which might be related to its regulation on PGC-1α expression and mitochondrial function.

Discussion

ADR is a commonly used anticancer drug. However, its clinical use is limited by its side effects in normal tissues, including the heart and kidney [18]. The experimental model of proteinuric renal disease induced by ADR is a well-established and highly-reproducible nephropathy model. This model can provide useful systems for testing new therapeutic strategies [19]. In current study, we found leonurine could prevent ADR induced podocyte injury both in vivo and in vitro. Based on these data, leonurine has been suggested as possible therapeutic agents for reducing or preventing podocyte injury.

In China, traditional Chinese medicine has been widely used for improving kidney function in patients with chronic kidney disease [20]. Leonurine is a unique single compound existed in Herba Leonuri, which is commonly used in gynecological diseases [21]. Previous study suggested that leonurine exerted therapeutic effects in several diseases, including diabetes, cardiovascular disease, bovine mastitis and depression [22]. In this investigation, our study results confirmed that leonurine could effectively reduce 24 h proteinuria in ADR mice, effectively improve renal pathological damage.

Terminal differentiated podocytes have a very limited capacity for division and hereby they are lacking the ability to regeneration after a toxic injury [23]. Persistence of podocyte injury results in podocyte death and loss, leading to progressive kidney damage and ultimately kidney failure [24]. Apoptosis is a major mechanism by which podocytes die in diabetes and progressive glomerular disease [25]. Previous study has confirmed that cell apoptosis was
observed in ADR induced podocyte injury [26]. Our present results showed that leonurine prevented ADR induced podocyte apoptosis both in vitro and in vivo. However, there still a debate regarding podocyte apoptosis as a cause of podocyte loss since no reliable evidence of apoptosis is detected by electron microscopy in vivo [27]. Other types of podocyte death such as anoikis, necroptosis, autophagic cell death or mitotic catastrophe need to be elucidated in future studies [28].

The actin cytoskeleton is a dynamic structure providing cells with architectural support and functional flexibility [29]. It is involved in various important cellular processes, such as cell growth, differentiation, division, membrane organisation and motility [30]. Podocytes lean on an intact actin cytoskeleton due to its complex cell architecture [31]. Dysregulation of the actin cytoskeleton disorder participates in the occurrence and development of proteinuria across a spectrum of chronic kidney diseases [32]. Our previous also showed that the disruption of the actin cytoskeleton impaired the adhesion ability of podocytes [33]. Here, the results of our experiments showed that leonurine treatment restored the proper organisation of actin cytoskeleton in podocytes. It is noteworthy that previous studies established the concept of balanced cytoskeletal dynamics as a prerequisite for podocyte function since increased cytoskeletal dynamics were also causative correlate for podocyte disease [34]. Furthermore, it was demonstrated that mutations affecting slit diaphragm proteins, like nephrin, caused glomerular disease through rearrangement of the actin cytoskeleton and disruption of the filtration barrier [35]. In present study, leonurine also could restore the expression of slit diaphragm proteins nephrin and podocin. Further studies are needed to identify a clear role for leonurine in the modulating the actin cytoskeleton and the foot processes in podocytes.
ROS are produced by living organisms as a result of normal cellular metabolism as well as environmental factors [36]. Oxidative stress is viewed as an imbalance between the prooxidants and antioxidants homeostasis which leads to an increased production of ROS [37]. Numerous lines of evidence supported that oxidative stress plays a pivotal role in mediating podocyte injury and proteinuria and these toxic ROS might be originated from NADPH oxidase or mitochondria [38]. Previous work has linked ADR to ROS generation as a mediator of podocyte injury [39]. Our data showed that leonurine could inhibit ADR induced ROS production both \textit{in vivo} and \textit{in vitro}. This result is consistent with the previous study that leonurine was reported to show antioxidant capacities [40]. Moreover, previous study showed that ADR significantly reduced the PGC-1 expression in aDR-treated rat kidneys [41]. Here, we found that leonurine could restore PGC-1$\alpha$ and its target gene MnSOD expression both \textit{in vivo} and \textit{in vitro}. Leonurine inhibited ADR-induced mitochondrial ROS production and alleviated ADR-induced mitochondrial damage. Thus, the antioxidant activity of leonurine might exert antioxidant activity via the regulation of PGC-1$\alpha$-mitochondria axis.

Collectively, our study has demonstrated a protective effect of leonurine on ADR-induced podocyte injury through inhibiting oxidative stress. Leonurine might be a promising therapeutic drug for prevention of oxidative podocyte injury.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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