

Original Research Article

Trifolirhizin relieves renal injury in a diabetic nephropathy model by inducing autophagy and inhibiting oxidative stress through the regulation of PI3K/AKT/mTOR pathway

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Abstract

Purpose: To evaluate the effects of trifolirhizin on diabetic nephropathy (DN), and the mechanism of action.

Methods: Male db/db mice (8 weeks, $n = 24$) and age-matched control mice ($n = 6$) were obtained. The mice were further divided into four groups and administered increasing doses of trifolirhizin (0, 12.5, 25 and 50 mg/kg). Histological analysis of renal tissues were performed by H & E staining. Blood urea nitrogen (BUN) and creatinine were determined using enzyme-linked immunosorbent assay (ELISA). Immunoblot and TUNEL assay were performed to investigate the effect of trifolirhizin on autophagy and apoptosis, while ELISA and dihydroethidium (DHE) staining were conducted to evaluate reactive oxygen species (ROS), malondialdehyde (MDA) and superoxide dismutase (SOD) levels. The effect of trifolirhizin on PI3K/AKT/mTOR pathway was determined using Immunoblot assays.

Results: Trifolirhizin alleviated renal injury in diabetic mice, and also activate autophagy and inhibited apoptosis in the renal tissues in diabetic mice ($p < 0.001$). In addition, trifolirhizin inhibited the oxidative stress response in the renal tissue in diabetic mice ($p < 0.001$). Trifolirhizin further inhibited PI3K/AKT/mTOR pathway and therefore relieved renal injury in the diabetic nephropathy model ($p < 0.001$).

Conclusion: Trifolirhizin alleviates renal injury in diabetic mice, activates autophagy, and inhibits apoptosis in renal tissue of diabetic mice. Therefore, trifolirhizin is a promising a promising drug for the treatment of DN.

Keywords: Diabetic nephropathy, Trifolirhizin, Autophagy, Oxidative stress, PI3K/AKT/mTOR pathway

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INTRODUCTION

The personal and financial costs of diabetes in the elderly population have become a significant burden [1,2]. Renal impairment is common in

elderly patients with diabetes, especially type 2 diabetes [3]. This kidney damage is caused by a multifactorial pathogen [4]. Diabetic nephropathy (DN) is a microvascular complication of diabetes, characterized by persistent clinical proteinuria

and reduced glomerular filtration rate (GFR), which often lead to end-stage renal failure (ESRF) and seriously affects the quality of life of older adults [5]. Autophagy is like a double-edged sword, and its over-activation or inhibition is the cause of renal tissue injury [6]. Inadequate podocyte autophagy has been observed in patients with diabetes mellitus and proteinuria [7]. Impaired autophagy activity leads to the pathogenesis of DN, and the recovery of autophagy activity may be a promising therapeutic target for DN [8]. In addition, apoptosis and oxidative stress are important biological mechanisms involved in the pathogenesis of DN. Therefore, the inhibition of oxidative stress and apoptosis is also a way to treat DN. Trifolirhizin is one of the active components in *Sophora flavescens*, and exerts anti-inflammatory, anti-apoptotic effects [9]. For example, Trifolirhizin inhibits IPS-induced pro-inflammatory cytokines including tumor necrosis factor alpha (TNF α) and interleukin-6 (IL-6) [10]. Trifolirhizin prevents airway over-reaction in mice with allergic asthma and inhibit airway smooth muscle (ASM) contraction caused by tracheal ring induced by acetylcholine (ACh) [11]. In addition, trifolirhizin has been reported to exert anti-tumor effects, by inhibiting the progression of colorectal cancer, gastric cancer and other malignant tumors [12]. However, there are few reports about trifolirhizin in diabetic nephropathy.

Studies have shown that trifolirhizin inhibits the PI3K/AKT/mTOR pathway [13]. Inhibition of AKT/mTOR pathway, on the one hand, enhances autophagy, and on the other hand, alleviates DN renal injury caused by high glucose [14]. Trifolirhizin could inhibit the PI3K/AKT/mTOR pathway, suppress oxidative stress and apoptosis, and alleviate renal injury in diabetic nephropathy by enhancing autophagy. The objective of this study, therefore, was to investigate the role of trifolirhizin in DN.

EXPERIMENTAL

Animals

The design was approved by the Medical Ethics Committee of Suzhou Ninth People's Hospital (approval no. KY2021-047-01), and all experiments were performed following the relevant guidelines and regulations for animal studies [15]. Male db/db mice (BKC.Cg.m +/- Leprdb/J) (8 weeks, n = 24) and age-matched control mice (n = 6) were obtained from Model Animal Centre of Nanjing University, Nanjing, China. The mice were maintained in a sterile environment with a temperature of 25 \pm 2 $^{\circ}$ C, and kept on a 12/12-hour light/dark cycle. The db/db

mice were divided into db/db, db/db + Trifolirhizin (12.5 mg/kg), db/db + Trifolirhizin (25 mg/kg), db/db + Trifolirhizin (50 mg/kg) groups. After 3 weeks, the mice were sacrificed by cervical dislocation. The kidneys were excised, and stored at - 80 $^{\circ}$ C for further experiments. The other kidney was fixed in 4 % paraformaldehyde and embedded in paraffin for histological evaluation.

H&E staining

After the kidney samples were fixed in 4 % paraformaldehyde and embedded with paraffin, they were then sliced into 5 μ m thick sections which were used for histological analysis. The sections were stained with H&E for histological analysis, and images were acquired using a light microscope (Olympus, Japan).

TUNEL assay

Cell apoptosis was determined using a TUNEL assay kit (Roche Diagnostics) following its protocols. The tissues were fixed with 10 % PFA and embedded with paraffin. After deparaffinization and rehydration, renal tissue sections were incubated with 3 % H₂O₂ for 5 min, rinsed using PBS, and incubated with TUNEL reaction solution for 1 h at 37 $^{\circ}$ C. In the dark, nuclei were counterstained with DAPI and images were captured under a fluorescence microscope.

Western blotting

Tissues were collected using RIPA buffer (Beyotime Biotechnology, Shanghai, China). After centrifugation, BCA protein assay kit (Beyotime Biotechnology) was used for the determination of protein concentration. Then proteins were separated using 10 % SDS-PAGE, transferred onto polyvinylidene difluoride membranes. After incubating with 5 % BSA in Tris-buffered saline for 1 h, the membranes were incubated with LC3/II (1:1000), Beclin1 (1:1000), p62 (1:1000), p-PI3K (1:1000), PI3K (1:1000), p-AKT (1:1000), AKT (1:1000), p-mTOR (1:1000), mTOR (1:1000), and anti- β -actin (1:10000; all from Abcam) at 4 $^{\circ}$ C overnight. The membranes were resolved in HRP-conjugated secondary antibodies at a 1:1000 for 2 h after they were washed with TBST for 15 min. The signals were visualized using ECL kit.

Determination of malondialdehyde (MDA) and superoxide dismutase (SOD) activities

Kidney tissues were lysed with the protein concentration as described by the protocols of

the ELISA kit, and then measured using BCA Protein Assay Reagent Kit. The activities of SOD, and MDA were detected using an SOD assay kit (SenBejia Biotech, China), and Lipid Peroxidation MDA Assay Kit (SenBejia Biotech, China) according to the manufacturers' instructions.

Assessment of reactive oxygen species (ROS)

The SOD level in the renal tissues were measured with the oxidative fluorescent dye dihydroethidium (DHE). Cryosections (10 μ m) were stained with DHE (5 μ mol/L) for 30 min at 37 °C in the dark. Representative images were captured with a fluorescent microscope.

Statistical analysis

Quantitative data were analyzed statistically using Student's *t*-test, and graphs were generated using GraphPad Prism 6.0 software (San Diego, CA, USA). *P*-value less than 0.05 was considered statistical significance.

RESULTS

Trifolirhizin alleviated renal injury

To reveal the potential role of trifolirhizin on renal injury in diabetic mouse model, the parameters in different groups were analyzed (Table 1). The body weight and kidney weight were significantly higher in db/db mice, and Trifolirhizin lowered both the body and renal weight of diabetic mice. Moreover, the diabetic mice displayed higher urine and fasting blood glucose. Trifolirhizin suppressed the DN-induced increases of urine and glucose levels. The renal tissues of db/db mice showed altered histopathological features, including glomerular hypertrophy, tubular basement membrane thickening, and mesangial matrix expansion. Trifolirhizin treatment relieved these alterations (Figure 1 A). The blood urea nitrogen (BUN) and serum creatinine were enhanced in diabetic model, but trifolirhizin reduced the elevation of BUN and creatinine

levels (Figures 1 B). These data suggested that Trifolirhizin effectively attenuated renal injury.

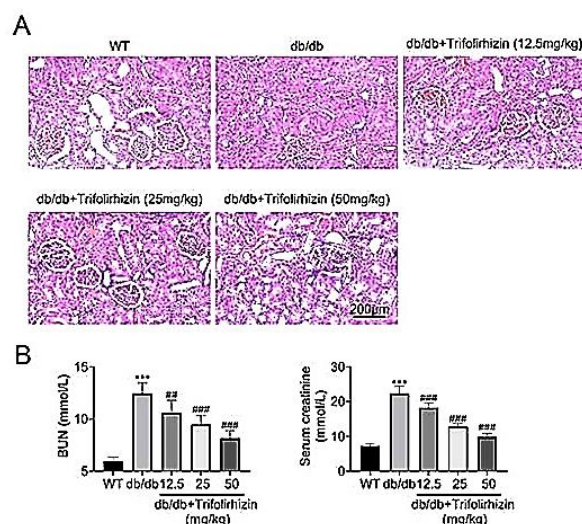


Figure 1: Trifolirhizin alleviated renal injury in diabetic mice. (A) The histological features of renal tissues in WT, db/db, db/db + 12.5 mg/kg Trifolirhizin, db/db + 25 mg/kg Trifolirhizin, db/db + 50 mg/kg Trifolirhizin groups were analyzed by HE staining. (B) ELISA results showing the levels of BUN and creatinine from WT, db/db, db/db + 12.5 mg/kg Trifolirhizin, db/db + 25 mg/kg Trifolirhizin, db/db + 50 mg/kg Trifolirhizin groups. ****P* < 0.001, vs WT group, #*p* < 0.01, ###*p* < 0.001, vs db/db

Trifolirhizin activated autophagy and inhibited apoptosis

The effect of trifolirhizin on autophagy and apoptosis in diabetic mice were evaluated using Immunoblot and TUNEL assay. ob/ob mice showed inhibited autophagy as indicated by reduced levels of LC3II, Beclin1 and increased level of p62. Trifolirhizin significantly reversed these alterations (Figure 2 A). The apoptosis in diabetic renal tissues were collected, while Trifolirhizin treatment significantly suppressed cell apoptosis in diabetic renal tissues (Figure 2 B). Therefore, trifolirhizin could activate autophagy and inhibit apoptosis in diabetic renal tissues.

Table 2: Effect of Trifolirhizin on basic biochemical parameters in db/db mice

Parameter	WT	db/db	db/db+Trifolirhizin (12.5mg/kg)	db/db+Trifolirhizin (25mg/kg)	db/db+Trifolirhizin (50mg/kg)
Body weight (g)	27.08±1.64	51.11±2.92*	43.85±3.34#	35.64±1.13#	30.45±3.81#
Kidney weight (g)	0.388±0.037	0.502±0.028*	0.456±0.019 #	0.405±0.016#	0.386±0.021#
Urine amount (mL/day)	14.38±1.66	76.15±7.46*	61.84±3.88#	48.64±2.99#	29.43±3.88#
Fasting blood glucose (mg/dL)	94.52±5.67	304.25±8.30*	215.54±7.30#	153.88±4.62#	106.42±4.17#

Values are expressed as mean ± standard error (n = 6); **p* < 0.05 vs. WT; #*p* < 0.05 vs. db/db

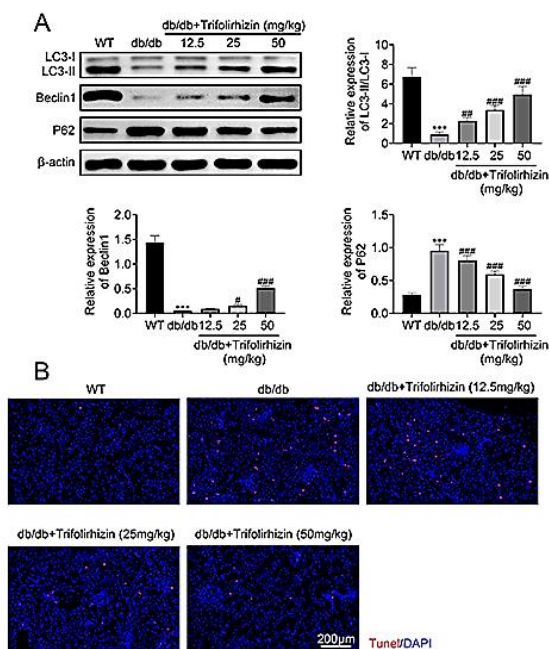


Figure 2: Trifolirhizin activated autophagy and inhibited apoptosis in the renal tissue of diabetic mice (A) The LC3I/II, Beclin1 and p62 levels in renal tissues (B) TUNEL staining of renal tissue. ****p* < 0.001, vs WT group, ##*p* < 0.01, ###*p* < 0.001, vs db/db

Trifolirhizin inhibited oxidative stress response of renal tissue in diabetic mice

To measure the antioxidant effect of trifolirhizin, the SOD and MDA levels were analyzed. Compared with the control group, SOD was reduced and MDA levels were increased in diabetic mice. However, trifolirhizin reversed the SOD and MDA levels in diabetic mice (Figure 3 A). The EOS level in each group was also determined. da/db mice displayed increased ROS, as revealed by DHE staining (Figures 3 B and C). Trifolirhizin suppressed the increase of ROS in diabetic mice. Furthermore, LDH level was upregulated in diabetic mice. Trifolirhizin treatment decreased the LDH level in db/db mice (Figure 3 D). Taken together, Trifolirhizin exert anti-oxidative stress in diabetic model.

Trifolirhizin inhibited the PI3K/AKT/mTOR pathway

Diabetic mice displayed enhanced p-PI3K, p-AKT and p-mTOR, suggesting the activation of PI3K/AKT/mTOR pathway (Figure 4). However, trifolirhizin treatment significantly reduced the phosphorylation levels of PI3K, AKT and mTOR in the renal tissues of diabetic mice (Figure 4). Therefore, these findings suggest that trifolirhizin inhibited the PI3K/AKT/mTOR pathway and

therefore relieved renal injury in the diabetic nephropathy model.

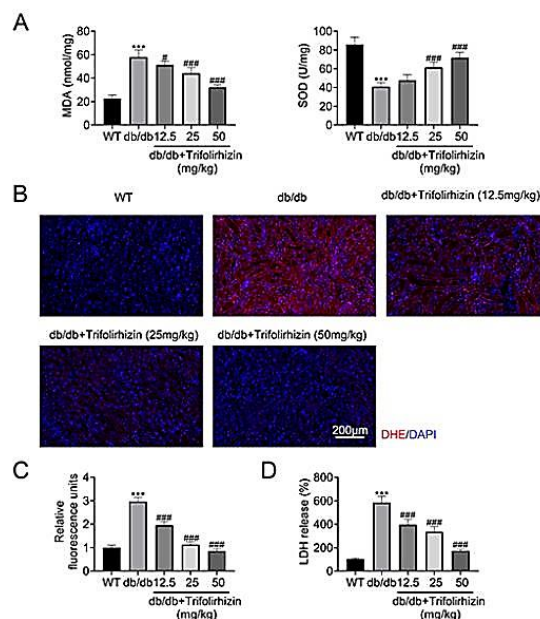


Figure 3: Trifolirhizin inhibit the oxidative stress response of renal tissue in diabetic mice. (A) MDA (nmol/mg), and SOD (U/mg) levels. (B, C) The ROS levels. (D) The LDH release. ****p* < 0.001, vs WT group, ##*p* < 0.01, ###*p* < 0.001, vs db/db

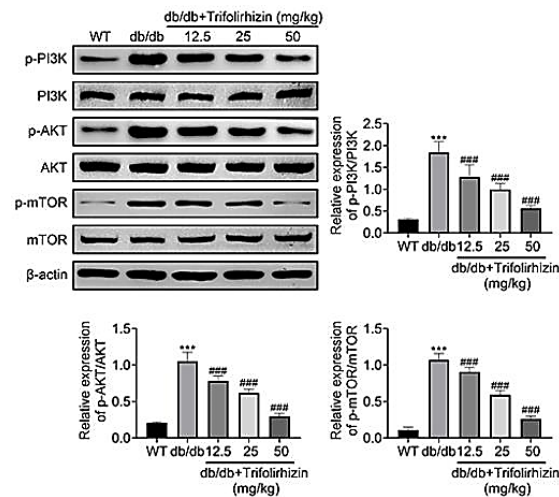


Figure 4: Trifolirhizin inhibited the PI3K/AKT/mTOR pathway and therefore relieved renal injury. Immunoblot assay showed the protein levels and phosphorylation levels of PI3K, AKT and mTOR in WT, db/db, db/db with 12.5 mg/kg Trifolirhizin, db/db with 25 mg/kg Trifolirhizin, db/db with 50 mg/kg Trifolirhizin groups. ##*P* < 0.01. ****p* < 0.001, vs WT group; ##*p* < 0.01, ###*p* < 0.001, vs db/db

DISCUSSION

Diabetic nephropathy (DN) is a common complication of diabetes, and has become the

main cause of end-stage renal disease (ESRD), but its exact pathogenesis remains unclear [16]. At present, in clinical practice the progression of DN is delayed by controlling blood glucose and blood pressure, but the progression to ESRD cannot be completely prevented. Therefore, investigating the exact pathogenesis of DN is a key link to effectively intervening in its progress, and the development of new DRUGS to treat DN is also very critical at present [17]. Traditional Chinese medicine play an increasingly important role in the treatment of diabetes. For example, the root extract of *sophora flavoris* has been shown to improve glucose tolerance, reduce hyperglycemia and restore insulin levels in diabetic mice [18]. In this study, it was revealed that trifolirhizin relieved renal injury in a DN model by inducing autophagy and inhibiting oxidative stress. Trifolirhizin therefore might serve as a promising drug for DN.

Autophagy is a double-edged sword, and its overactivation or inhibition may cause of renal tissue injury [19]. Inadequate podocyte autophagy has been observed in patients with diabetes mellitus and proteinuria [20], and impaired autophagy leads to the pathogenesis of DN. The recovery of autophagy activity may be a promising therapeutic target for DN [21]. In this study, the effect of this drug on DN were caused by the regulation of autophagy, which was confirmed through the immunoblot assays.

Oxidative stress is an important biological mechanism involved in the pathogenesis of DN. In fact, the multiple biological activities of Trifolirhizin have been revealed in different types of diseases [22-24]. Trifolirhizin could contribute to autophagy-dependent apoptosis in colon cancer by targeting the AMPK/mTOR pathway [14]. Trifolirhizin also induced autophagy-dependent apoptosis in a DN model. In addition, Trifolirhizin inhibited acetylcholine induced airway smooth muscle contraction, and as well inhibited the expression of LPS-induced pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-alpha) and interleukin-6 (IL-6) [22]. It also prevented airway overreaction in mice with allergic asthma, and inhibited smooth airway muscle (ASM) contraction [25]. These studies confirmed Trifolirhizin could serve as a promising drug in different types of diseases.

In this study, Trifolirhizin mediated the PI3K/AKT/mTOR pathway in the DN model. In fact, the inhibition of PI3K/AKT/mTOR pathway can promote autophagy on the one hand, and alleviate DN renal injury caused by high glucose on the other hand. Therefore, it was hypothesized that the autophagy regulation of

sandalwood in DN was related to the PI3K/AKT/mTOR pathway. Several proteins or drugs affected the progression of DN via this pathway. Trifolirhizin mediated the PI3K/AKT/mTOR pathway, and therefore relieved renal injury in a diabetic nephropathy model by inducing autophagy and inhibiting oxidative stress.

CONCLUSION

Trifolirhizin alleviates renal injury in diabetic mice, activates autophagy, and inhibits apoptosis in renal tissue of diabetic mice. In addition, Trifolirhizin can inhibit the oxidative stress response, as well as the PI3K/AKT/mTOR pathway, thus providing relief to the renal injury in the DN in rat model.

DECLARATIONS

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Ethical approval

This study was approved by the Medical Ethics Committee of Suzhou Ninth People's Hospital, China (approval no. KY2021-047-01).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors contributed to the study conception and design. Material preparation and the experiments were performed by Yunyi Xin and Xiao Xu; data collection and analysis were performed by Xufeng Yang and Yufan Chen; the first draft of the manuscript was written by Danning Zhu, and all authors commented on previous versions of

the manuscript. All authors read and approved the final manuscript.

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