# Icariin alleviates renal inflammation and tubulointerstitial fibrosis via Nrf2-mediated attenuation of mitochondrial damage

Nannan Ding[1], Shanyue Sun[1], Shuting Zhou[2], Zhimei Lv[1], and Rong Wang[1]

[1]Shandong Provincial Hospital
[2]Shandong Provincial Qianfoshan Hospital

April 10, 2024

**Abstract**

Tubulointerstitial fibrosis is an inevitable consequence of all progressive chronic kidney disease (CKD) and contributes to a substantial health burden worldwide. Icariin, an active flavonoid glycoside obtained from *Epimedium* species, exerts potential antifibrotic effect. The study aimed to explore the protective effects of icariin against tubulointerstitial fibrosis in unilateral ureteral obstruction (UUO)-induced CKD mice and TGF-β1-treated HK-2 cells, and furthermore, to elucidate the underlying mechanisms. The results demonstrated that icariin significantly improved renal function, alleviated tubular injuries, and reduced fibrotic lesions in UUO mice. Furthermore, icariin suppressed renal inflammation, reduced oxidative stress as evidenced by elevated SOD activity and decreased MDA level. Additionally, TOMM20 immunofluorescence staining and transmission electron microscope revealed that mitochondrial mass and morphology of tubular epithelial cells in UUO mice was improved by icariin. In HK-2 cells treated with TGF-β1, icariin markedly decreased profibrotic proteins expression, inhibited inflammatory factors, and protected mitochondria along with improving mitochondrial morphology, reducing reactive oxygen species (ROS) and mitochondrial ROS (mtROS) overproduction, and preserving membrane potential. Further investigations demonstrated that icariin could activate Nrf2/HO-1 pathway both *in vivo* and *in vitro*, whereas inhibition of Nrf2 by ML385 counteracted the protective effects of icariin on TGF-β1-induced HK-2 cells. In conclusion, icariin protects against renal inflammation and tubulointerstitial fibrosis at least partly through Nrf2-mediated attenuation of mitochondrial dysfunction, which suggests that icariin could be developed as a promising therapeutic candidate for the treatment of CKD.

**Title: Icariin alleviates renal inflammation and tubulointerstitial fibrosis via Nrf2-mediated attenuation of mitochondrial damage**

Nannan Ding[1], Shanyue Sun[2], Shuting Zhou[3], Zhimei Lv[1]*, Rong Wang[1]*

1. Department of Nephrology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, China.

2. Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, China.

3. Department of Pathology, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Jinan, China.

*Correspondence: Zhimei Lv and Rong Wang are joint corresponding authors. E-mail: lvzhimei@email.sdu.edu.cn & wangrong_sd@126.com.

**Running title:** The protective mechanism of icariin in CKD

**Significance statement**

Tubulointerstitial fibrosis is an unavoidable consequence of advancing chronic kidney disease (CKD) and imposes a substantial global health burden. However, few effective therapeutic agents are available at

present. The present study investigated the ameliorative effects of icariin on tubulointerstitial fibrosis (TIF) both *in vivo* and*in vitro* , and demonstrated that icariin inhibited inflammation and oxidative stress, at least partially, through improving Nrf2 activity and subsequent mitochondrial function, and eventually mitigated TIF and preserved renal function, suggesting that icariin could be developed as a promising therapeutic candidate for the treatment of CKD.

**Icariin alleviates renal inflammation and tubulointerstitial fibrosis via Nrf2-mediated attenuation of mitochondrial damage**

**Abstract**

Tubulointerstitial fibrosis is an inevitable consequence of all progressive chronic kidney disease (CKD) and contributes to a substantial health burden worldwide. Icariin, an active flavonoid glycoside obtained from *Epimedium* species, exerts potential antifibrotic effect. The study aimed to explore the protective effects of icariin against tubulointerstitial fibrosis in unilateral ureteral obstruction (UUO)-induced CKD mice and TGF-β1-treated HK-2 cells, and furthermore, to elucidate the underlying mechanisms. The results demonstrated that icariin significantly improved renal function, alleviated tubular injuries, and reduced fibrotic lesions in UUO mice. Furthermore, icariin suppressed renal inflammation, reduced oxidative stress as evidenced by elevated SOD activity and decreased MDA level. Additionally, TOMM20 immunofluorescence staining and transmission electron microscope revealed that mitochondrial mass and morphology of tubular epithelial cells in UUO mice was improved by icariin. In HK-2 cells treated with TGF-β1, icariin markedly decreased profibrotic proteins expression, inhibited inflammatory factors, and protected mitochondria along with improving mitochondrial morphology, reducing reactive oxygen species (ROS) and mitochondrial ROS (mtROS) overproduction, and preserving membrane potential. Further investigations demonstrated that icariin could activate Nrf2/HO-1 pathway both *in vivo* and *in vitro* , whereas inhibition of Nrf2 by ML385 counteracted the protective effects of icariin on TGF-β1-induced HK-2 cells. In conclusion, icariin protects against renal inflammation and tubulointerstitial fibrosis at least partly through Nrf2-mediated attenuation of mitochondrial dysfunction, which suggests that icariin could be developed as a promising therapeutic candidate for the treatment of CKD.

**Keywords:** renal tubulointerstitial fibrosis, icariin, oxidative stress, mitochondrial dysfunction, Nrf2 pathway

## Introduction

In recent decades, chronic kidney disease (CKD) has garnered widespread concern due to its high prevalence, severe complications and tremendous healthcare costs. It is estimated to affect 10% to 15% of the global population[1]. Regardless of the various etiologies of CKD, renal tubulointerstitial fibrosis (TIF) is considered as the final shared pathway of CKD and the most reliable indicator of renal survival[2]. Importantly, recent researches have revealed that tubulointerstitial fibrosis serves not just as the histological feature of CKD, but also as a catalyst of CKD advancement to end-stage renal disease (ESRD)[3,4]. Therefore, prevention and treatment of TIF can delay the development of CKD. Unfortunately, to date, there are limited effective therapeutic drugs that can inhibit or reverse TIF.

Despite the pathogenesis of renal tubulointerstitial fibrosis being complicated, emerging evidences from clinical and experimental studies have shown that oxidative stress and inflammation are closely involved in the initiation and advancement of TIF[5]. Mitochondria serve as the primary generator of reactive oxygen species (ROS) and take the center stage in orchestrating oxidative stress and subsequent inflammation response[6,7]. Mitochondrial damage leads to ROS accumulation, mitochondrial fragmentation, and membrane potential depolarization, resulting in deregulated inflammatory responses and secretion of profibrotic cytokines, and eventually contributes to the fibrotic remodeling observed in TIF[8]. In particular, renal tubular epithelial cells (RTECs) possess abundant mitochondria, rendering them more susceptible to mitochondrial dysfunction. Of note, increasing studies have indicated that RTECs serve as both targets and active contributors in kidney injury, as they have the ability to secrete numerous inflammatory factors, profibrotic molecules and extracellular matrix in kidney tissues[9]. Therefore, targeting mitochondria homeostasis may function as an

efficacious approach for preventing and treating renal fibrosis.

Recently, natural products have emerged as promising source of novel drugs owing to their distinctive advantages, such as multi-target activities and low adverse effects. Icariin is a pleiotropic flavonoid extracted from *Epimedium* ( in Chinses: Yin yang huo ), a renowned traditional Chinese medicinal formula that has undergone extensive clinical validation over the years as a highly effective remedy for patients suffering from kidney or bone diseases. It has attracted much attention in modern pharmacological research because of its multiple properties, including anti-inflammatory, antioxidant and antifibrotic activities. For example, icariin has been reported to reduce live fibrosis in various mouse models by inhibiting epithelial-mesenchymal transition[10] or autophagy[11]. Furthermore, icariin ameliorated STZ-induced diabetic nephropathy by suppressing NF-ϰB signaling pathway[12]. Intriguingly, recent studies have connected its therapeutic effect with its potential to maintain mitochondrial homeostasis[13-15]. However, prior studies have provided suggestive but restricted evidence regarding the potential role of icariin against tubulointerstitial fibrosis in CKD. In particular, there is a paucity of data on the protective effects of icariin on mitochondrial abnormalities in CKD.

Therefore, the current study was designed to investigate the ameliorative effects of icariin on renal inflammation, oxidative stress and tubulointerstitial fibrosis induced by unilateral ureteral obstruction (UUO). We particularly focused on the protective effects of icariin on mitochondrial homeostasis. Considering that nuclear factor erythroid 2-related factor 2 (Nrf2) /heme oxygenase-1 (HO-1) are extensively involved in the regulation of oxidative stress and mitochondrial homeostasis, we further investigated the influence of icariin on Nrf2-related signaling pathway both *in vivo* and*in vitro* , which aimed at gaining fresh perspectives on the underlying protective mechanism of icariin against renal fibrosis.

## Materials and methods

### Materials and reagents

Icariin (purity[?] 98%, I107343) was obtained from Aladdin. Recombinant TGF-β1 protein (100-21C) was obtained from PeproTech. ML385 (SML1833) was purchased from Sigma-Aldrich. Collagen I (72026, 1:1000), Nrf2 (12721, 1:1000) and F4/80 (70076, 1:500) antibodies were purchased from Cell Signaling Technology. TOMM20 (ab186735, 1:250) antibody and Alexa Fluor 594-conjugated secondary antibody (ab150076, 1:500) were purchased from Abcam. HO-1 (10701-1-AP, 1:3000), Lamin B1 (12987-1-AP, 1:5000), α-SMA (67735-1-Ig, 1:20000) and β-tubulin (10094-1-AP, 1:5000) antibodies were purchased from Proteintech. Nuclear and cytoplasmic protein extraction kit (P0027) was purchased from Beyotime.

### Animal studies

All animal experiments were conducted following approval from the Animal Care and Use Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University (NO.2023-020) and in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6J mice underwent UUO or sham operation as previously described[16]. In brief, the UUO group underwent ligation of left ureter with 4-0 silk under general anesthesia, while the sham operation followed a similar procedure but without the ureteral ligation. The mice were randomized into three groups (n = 6 in each group): (1) Sham, mice that underwent sham surgery, (2) UUO + vehicle, mice with UUO that treated with vehicle, (3) UUO + icariin, mice with UUO that treated with icariin (50 mg/kg/day) by gavage for 14 consecutive days. The dose of icariin administration was chosen based on published literatures[14,17] and preliminary experiments.

### Biochemical analysis

The levels of serum creatinine (Cr) and blood urea nitrogen (BUN) were examined by an automatic biochemical analyzer using commercial kits.

### Histopathological analysis

Kidney tissues were immersed in 4% paraformaldehyde solution for fixation, followed by dehydration and embedding in paraffin. Subsequently, the tissues were sectioned into 4 μm slices and subjected to H&E staining for morphological analysis or Masson's trichrome staining for fibrotic analysis. Tubular injury was characterized by the presence of sloughed tubules, formation of casts, tubular dilation, degeneration, and atrophy. The severity of the injury was assessed using a scoring system based on the proportion of tubular involvement (0, no injury; 1, < 25%; 2, 25%–50%; 3, 50%–75%, and 4, > 75%). Renal fibrosis was quantitatively assessed by analyzing the percentage of stained area in randomly chosen fields (400×) using Image J software. All samples were examined in a blinded fashion.

**Immunofluorescence and immunohistochemistry**

Immunofluorescent and immunohistochemistry analyses were conducted on paraffin-embedded kidney tissues sections. After standard deparaffinization, rehydration, and blocking steps, the sections were incubated with a primary antibody overnight at 4°C. Subsequently, they were washed with PBS and exposed to either fluorescence-conjugated or HRP-conjugated secondary antibodies for 1.5 hours at room temperature. Images were captured by either fluorescent or optical microscope.

**Transmission electron microscope (TEM)**

The TEM was used to visualize the ultrastructural characteristics of the mitochondria. The kidney tissues were fixed in fresh 2.5% glutaraldehyde solution at 4°C for 2 hours, followed by dehydration, embedding, and sectioning before observing the mitochondrial ultrastructure. The percentage of swollen mitochondria was determined as previously described[18].

**Malondialdehyde (MDA) content and superoxide dismutase (SOD)activity assays**

Kidney tissues were collected, homogenized, and then centrifuged at 10,000× g for 30 minutes. The resultant supernatant was subjected to MDA assay kit analysis following the manufacturer's protocol (A003-1-2, Jiancheng). The activity of SOD enzyme was also evaluated following the user's recommendations (A001-3-2, Jiancheng).

**Cell culture and viability assay**

The human renal proximal tubular epithelial (HK-2) cells were cultured in DMEM/F12 medium with 10% fetal bovine serum (FBS) under 37°C, 5% CO2 conditions. The cells were subjected to serum deprivation for 12 hours, followed by treatment with TGF-β1 (10 ng/ml) in the presence or absence of icariin (50μM) for 24 hours. For authenticate reverse, the cells were pretreated with ML385 (Nrf-2 inhibitor, 5μM) for 1 hour, before being incubated with icariin and TGF-β1 for the specified duration.

The viability of HK-2 cells was assessed using a CCK-8 assay. The cells were plated in 96-well plates and exposed to icariin (10, 25, 50, 100, 200 and 400 μM) for 24 hours, followed by incubation with CCK-8 solution at 37°C for 2 hours. The absorbance at 450 nm was then determined using a microplate reader.

**Protein extraction and western blot analysis**

Total or nuclear proteins were extracted following the respective protocols and then separated by SDS-PAGE gel, subsequently transferred to PVDF membrane (Merck Millipore, Germany). After blocking with 5% BSA for 1 hour, the membranes were subjected to overnight incubation with primary antibodies at 4°C, and subsequently incubated with HRP-secondary antibodies for 2 hours at room temperature. The ECL system was utilized to identify specific protein bands, and the grayscale intensity of the bands was subsequently quantified by Image J software.

**RNA extraction and quantitative real-time PCR**

The total RNA was extracted from tissues or cells using Trizol reagent (Takara, Japan) and then reverse transcribed into cDNA using reverse transcription kits (Takara, Japan). Amplification was conducted using SYBR Green real-time quantitative PCR system. Ct values were used to determine the relative expression level of target mRNAs that were normalized to β-actin. Primers were designed as follows:

GACGTGGAACTGGCAGAAGAG (forward) and TTGGTGGTTTGTGAGTGTGAG (reverse) for TNF-α·

GCAACTGTTCCTGAACTCAACT (forward) and ATCTTTTGGGGTCCGTCAACT (reverse) for IL-1β·

TCCATCTGCCCTTCAGGAACA (forward) and GGAAGGCAGTGGCTGTCAAC (reverse) for IL-6;

TGACGTGGACATCCGCAAAG (forward) and CTGGAAGGTGGACAGCGAGG (reverse) for β-actin.

**Measurement of ROS and mitochondrial ROS (mtROS)**

To observe intracellular ROS or mtROS, HK-2 cells were stained by DCFH-DA (S0033S, Beyotime) or MitoSOX Red (40778ES50, Yeasean) fluorescent probes for 30 minutes at 37 °C. After staining, the cells were washed and examined under a fluorescence microscope.

**Measurement of mitochondrial membrane potential (MMP)**

The mitochondrial membrane potential of HK-2 cells was assessed by JC-1 staining following the protocol of the detection kit (C2006, Beyotime). The fluorescence intensity was detected by fluorescence microscope. At elevated membrane potential (polarized mitochondria), JC-1 exists as an aggregated state and emits red fluorescence. Conversely, at decreased membrane potential (depolarized mitochondria), JC-1 exists as a monomeric state and emits green fluorescence. The ratio of red to green fluorescence intensity was used to represent the mitochondrial membrane potential.

**MitoTracker Red Staining**

The mitochondria in HK2 cells was visualized using MitoTracker Red dye (40741ES50, Yeasean) following the manufacturer's protocol, and the nuclei were visualized using Hoechst staining (C1028, Beyotime). All images were viewed under a confocal microscope. The length of mitochondria was determined by measuring 30 randomly selected cells in each experiment using Image J software.

**RNA Sequencing**

RNA was extracted from kidney tissues using the Trizol reagent and then transferred to Hangzhou KAITAI Biotechnology Co. for library construction and sequencing. Gene expression was quantified using the FPKM value. Genes meeting the criteria of $p<0.05$ and log2(fold change) $>1$ were differentially expressed genes. Gene Ontology (GO) analysis was conducted with a public online database.

**Statistical analysis**

Data are expressed as mean ± SEM and analyzed using GraphPad Prism. Comparisons between two groups were performed using the two-tailed unpaired Student's t-tests or Mann-Whitney U tests. One-way ANOVA test was used for comparisons among multiple groups. $P < 0.05$ was considered statistically significant.

**Results:**

**Icariin ameliorated renal function and pathological lesions in UUO mice.**

To explore the effect of icariin against tubulointerstitial fibrosis, an experimental UUO-induced fibrosis mouse model was employed, followed by the treatment of icariin (50 mg/kg/day) for 14 days post-surgery. Body weight was assessed on day 1 and again before sacrifice. As depicted in Figure 1C, icariin reduced the weight loss induced by UUO. Compared with the sham-operated group, UUO mice displayed a notable increase in the levels of serum BUN and Cr, while icariin effectively reduced BUN and Cr levels (Figure 1D,E). In terms of histopathological features (Figure 1F-H), H&E staining revealed that UUO mice developed typical pathological lesions, including extensive renal tubular atrophy or expansion, interstitial cell proliferation and inflammatory cells infiltration. Masson's staining revealed a heavy deposition of collagen in the obstructed kidneys of UUO mice. In parallel with histological changes, western blot further confirmed more severe fibrosis and renal injury in UUO mice, as demonstrated by remarkably enhanced expression of a-SMA and collagen I compared with the sham-operated mice (Figure 1I). After icariin treatment, renal tubular damage and extracellular matrix deposition were remarkedly alleviated, which were consistent with the changes in

serum markers mentioned above. Collectively, these results indicated that icariin exerts a protective effect in TIF induced by UUO.

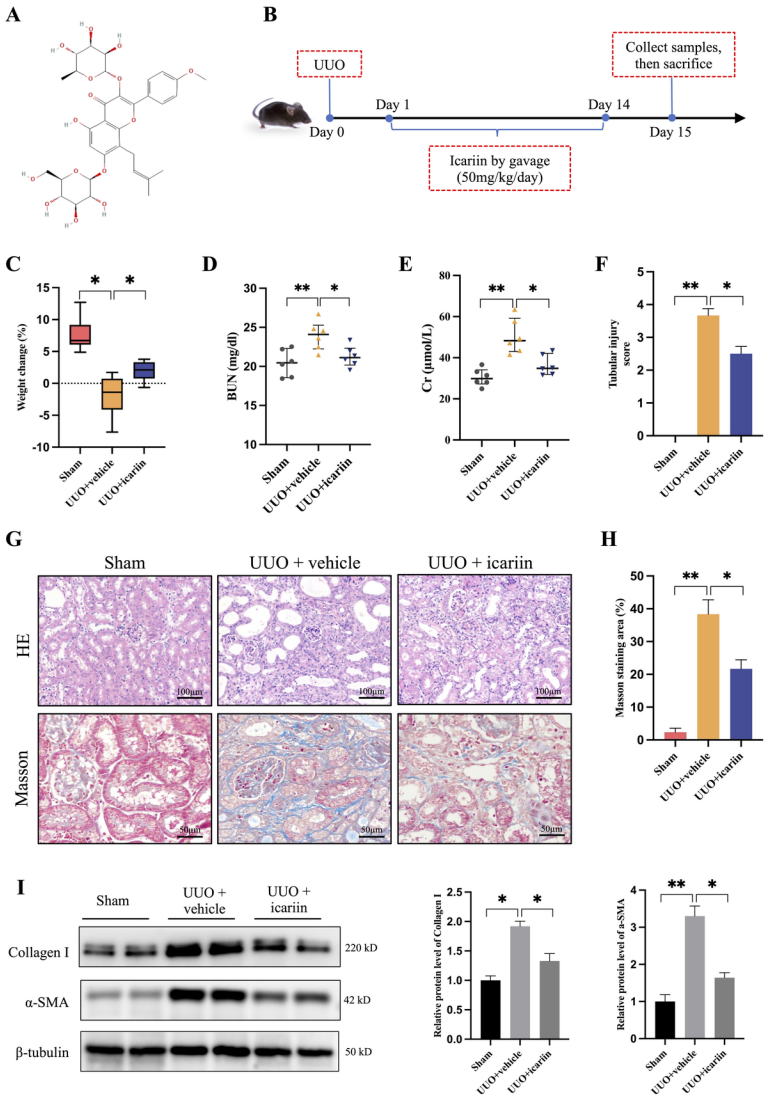



**Figure 1 Icariin attenuates renal function and histological damage in UUO mice.** (A) The chemical structure of icariin. (B) Experiment outline. (C) Weight changes after 14 days of UUO surgery. (D-E) Serum BUN and Cr levels in different group. (G) Representative images of H&E and Masson's staining, and quantitative assessment of renal injury (F) and fibrosis (H). (I) Western blot analysis of collagen I and α-SMA expression in the kidney from different groups. BUN, blood urea nitrogen; Cr, creatinine. * $p < 0.05$, ** $p < 0.01$.

**Icariin alleviated inflammatory responses and oxidative stress in UUO mice.**

To explore the underlying mechanism of icariin therapy, transcriptome sequencing was applied. According to results of the Gene Ontology (GO) term analysis between the sham and UUO mice, the differentially expressed genes exhibited strong enrichment in inflammatory response and oxidoreductase activity (Figure 2A). Considering that inflammation and oxidative stress are well-documented contributors in the development of interstitial fibrosis, the present research centered on the impact of icariin on pivotal genes within the enriched gene sets related to inflammatory response and oxidoreductase activity. As shown in Figure 2B, icariin reduced inflammatory response-related gene expression profiles. In addition, the mRNA expression of inflammatory factors IL-1β, IL-6 and TNF-α in kidneys were examined by quantitative real-time PCR to further validate the sequencing results. As expected, all these factors demonstrated a significant rise in the obstructed kidneys of UUO group, but were notably reduced following icariin treatment (Figure 2D). Moreover, we analyzed inflammatory cell infiltration of renal tissue by immunostaining of F4/80 and found significantly fewer F4/80-positive macrophages in icariin-treated mice than in sham group (Figure 2G).

The heatmap of oxidoreductase-related gene levels revealed that icariin reversed UUO-induced alterations of important oxidoreductase and improved redox balance (Figure 2C). Next, we investigated the levels of MDA, a lipid peroxidation product that is associated with pathological changes in reaction to oxidative stress, and the activity of endogenous SOD in kidney tissue (Figure 2E,F). As a result, we observed significantly increased MDA content and reduced activity of SOD in the obstructed kidneys of UUO mice. Interestingly, treatment with icariin reduced the MDA content and restored the SOD activity, suggesting that the oxidative stress was effectively alleviated by icariin. Taken together, these data demonstrate that icariin can relieve inflammation and oxidative stress, thereby delaying TIF in UUO mice.

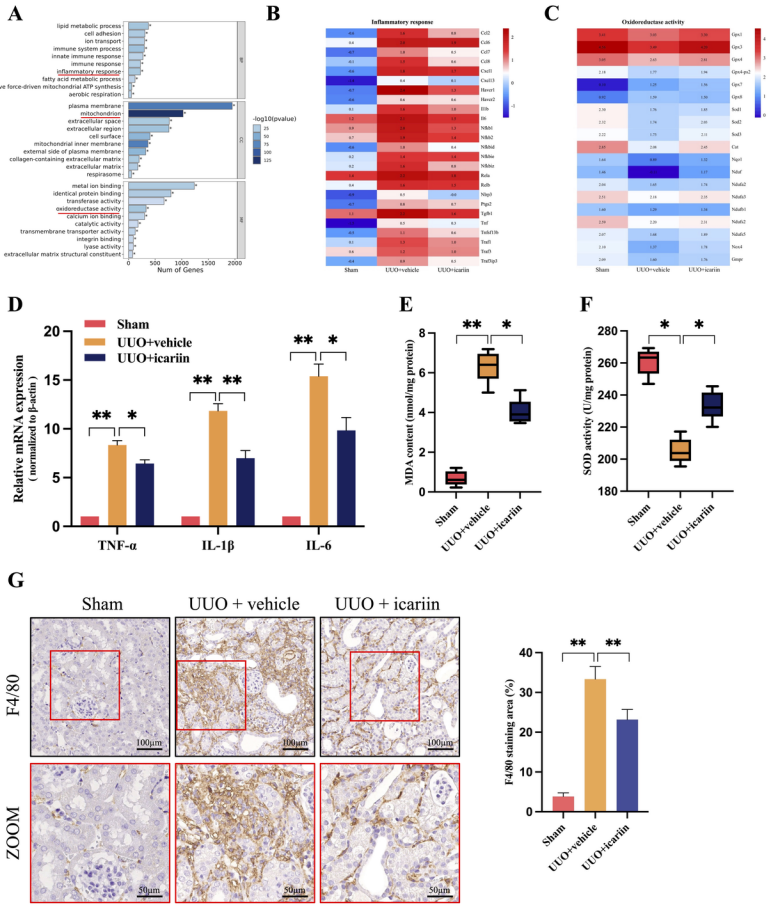



**Figure 2 Icariin alleviated inflammatory responses and oxidative stress in UUO mice.** (A) Gene Ontology (GO) enrichment analysis of differentially expressed genes between the sham and UUO mice. (B) Heatmap of inflammatory response-related genes expression profiles of different groups based on the RNA-seq data set. The quantitative value=$\log_{10}$ FPKM. (C) Heatmap of key oxidoreductase-related gene expression of different groups based on the RNA-seq data set. The quantitative value=$\log_{10}$ FPKM. (D) Quantitative real-time PCR analysis of TNF-α, IL-1β and IL-6 mRNA levels. (E) MDA levels in the kidney. (F) SOD activity in the kidney. (G) Representative IHC images and quantification of F4/80 expression in the kidney from different groups. * $p < 0.05$, ** $p < 0.01$.

**Icariin restored mitochondrial homeostasis in UUO mice.**

Emerging discoveries demonstrate that mitochondrial damage is a critical step to trigger inflammation and oxidative stress in fibrotic diseases[19,20]. Our RNA-seq data also indicated that mitochondrion-related genes were significantly differentially expressed between the sham and UUO mice (Figure 2A). To further investigate whether icariin attenuated UUO-induced oxidative stress and inflammation by maintaining mitochondrial homeostasis, we then detected mitochondrial changes. The immunofluorescence staining of TOMM20, an outer mitochondrial membrane marker, revealed a significant reduction of mitochondria in kidneys from UUO mice (Figure 3A). Additionally, TEM observed evident mitochondrial damages, characterized by pronounced swelling, cristae loss, and mitochondrial membrane rupture in UUO mice (Figure 3B). However, treatment with icariin effectively alleviated these changes, indicating that icariin can improve UUO-induced mitochondrial abnormalities.

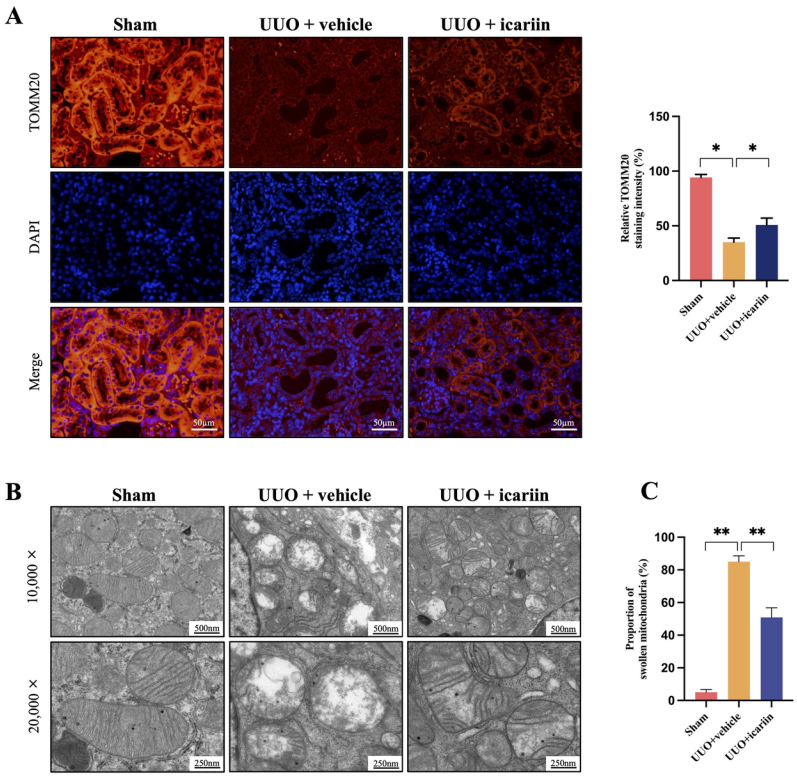
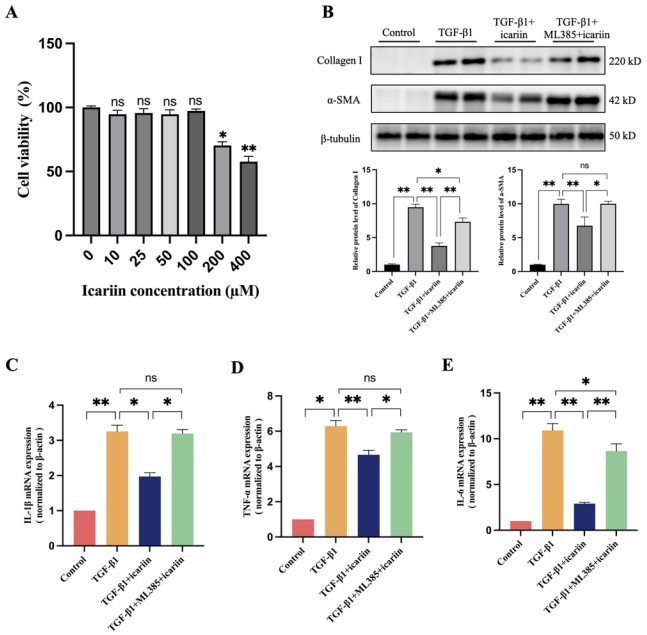


**Figure 3 Icariin restored mitochondrial homeostasis in UUO mice.** (A) Representative immunofluorescence staining and quantitative of TOMM20 showed that icariin increased mitochondrial mass. (B) Representative transmission electron microscope images of mitochondria in renal tubular epithelial cells. (C) Quantification of the percentage of damaged mitochondria. * $p < 0.05$, ** $p < 0.01$.

### Icariin inhibited profibrotic phenotype and inflammatory response in TGF-β1-exposed HK-2 cells

To further validate the protective role of icariin in tubulointerstitial fibrosis, TGF-β1-treated HK-2 cells were used to detect the effects of icariin *in vitro* . Firstly, we assessed the cytotoxic effect of icariin on HK-2 cells and observed that the cytotoxic concentration exceeded 200μM (Figure 4A). Based on published literatures and preliminary experiments, we determined that the effective concentration of icariin was 50μM. Then, we measured whether icariin (50μM) could inhibit profibrotic effects of TGF-β1 in HK-2 cells. Similar to the findings in the *in vivo* model, icariin dramatically inhibited profibrotic phenotype of TGF-β1-treated HK-2 cells, as demonstrated by the reduction of collagen I and α-SMA expression levels (Figure 4B). Additionally, quantitative real-time PCR of IL-1β, TNF-α and IL-6 demonstrated that icariin reduced the mRNA expression of inflammatory factors in HK-2 cells (Figure 4C-E). These results validated the anti-inflammatory and antifibrosis properties of icariin *in vitro* .

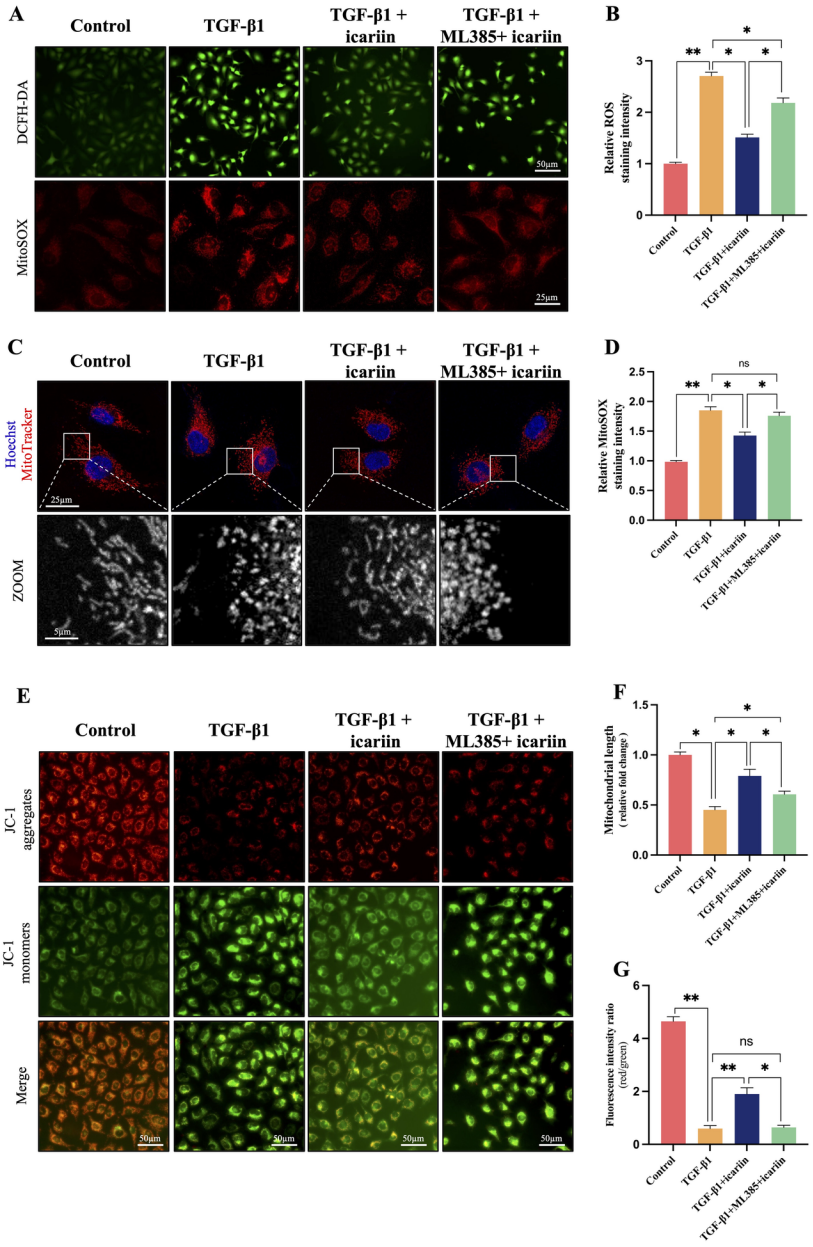



**Φιγυρε 4 Ισαριιν αττενυατεδ προφιβροτις πηενοτψπε ανδ ινφλαμματιον ρεσπονσε οφ HK-2 ςελλς ινδυςεδ βψ ΤΓΦ-β1** . (A) Effect of icariin on the viability of HK-2 cells. ns: not significant compared to the blank control. * $p < 0.05$ and ** $p < 0.01$ compared to the blank control. (B) Western blot analysis of collagen I and α-SMA in HK-2 cells. (C-E) Quantitative real-time PCR analysis of IL-1β, TNF-α and IL-6 in HK-2 cells. ns: not significant. * $p < 0.05$, ** $p < 0.01$ .

### Ισαριιν αττενυατεδ οξιδατιε στρεσς ανδ μιτοςηονδριαλ ινθυρψ ιν ΤΓΦ-β1-εξποσεδ HK-2 ςελλς

As ROS accumulation is the hallmark of oxidative stress, we firstly evaluated the total ROS level in HK-2 cells using DCFH-DA fluorescence probe. Our results revealed that TGF-β1 significantly stimulated the

generation of intracellular ROS, which was reduced by icariin (Figure 5A,B). MitoSOX is an innovative fluorescent dye specifically targeted to mitochondria, and it can serve as a specific indicator of mitochondrial ROS (mtROS) level. Through MitoSOX staining, it was observed that icariin suppressed mtROS overproduction triggered by TGF-β1 (Figure 5A,D). In addition, the application of MitoTracker Red dye revealed that TGF-β1 treatment led to a decrease in mitochondrial length and provoked mitochondrial fragmentation in HK-2 cells (Figure 5C,F); Meanwhile, TGF-β1 stimulation led to mitochondrial depolarization, as evidenced by the reduced ratio of JC-1 aggregates to monomers (Figure 5E,G). These alterations were effectively reversed by icariin treatment, suggesting that icariin improved TGF-β1-induced mitochondrial damage in HK-2 cells.




Φιγυρε 5 Ισαριιν αττενυατεδ οξιδατιε στρεσς ανδ μιτοχηονδριαλ ινθυρψ ιν ΤΓΦ-β1-

**εξποσεδ HK-2 ςελλς.** (A, B, D) Representative images and quantitative data of DCFH-DA staining and MitoSOX staining. (C, F) Representative confocal microscope images of mitochondria stained by MitoTracker Red and relative mitochondrial length in different groups of HK-2 cells. The results were normalized to the mitochondrial length of control HK-2 cells. (E) Representative images of mitochondrial membrane potential (MMP) assay using JC-1 staining. (G) Fluorescence intensity ratio of JC-1 aggregates to monomers. ns: not significant. * $p < 0.05$, ** $p < 0.01$.

**Nrf2 signaling is important for the protective effect of icariin**

Considering that Nrf2/HO-1 plays a vital role in inhibiting oxidative stress and maintaining mitochondrial homeostasis, we further detected the protein levels of Nrf2 and HO-1 in response to icariin both *in vivo* and *in vitro* . Figure 6A illustrated the results of western blot analysis, which indicated a significant reduction in protein levels of Nrf2 and HO-1 in the obstructed kidneys from UUO group. Whereas, icariin treatment rescued Nrf2 and HO-1 expression. Additionally, in HK-2 cells subjected to various treatments, western blot analysis of nuclear and cytoplasmic cell extracts showed that icariin treatment markedly promoted nuclear translocation of Nrf2 and elevated protein expression of HO-1 (Figure 6B). To further examine the role of Nrf2 activation in mediating the protective effects of icariin, we pretreated TGF-β1-induced HK-2 cells with ML385, a Nrf2 inhibitor, and subsequently re-evaluated the profibrotic molecules, inflammatory factors, and mitochondrial injury markers of HK-2 cells again. Consequently, these protective effects of icariin on TGF-β1-treated HK-2 cells were significantly counteracted by ML385 pretreatment (Figure 4B-E and Figure 5). Taken together, these observations provided strong evidence that icariin ameliorated renal interstitial fibrosis and inflammation, at least in part, through Nrf2-dependent attenuation of mitochondrial function.

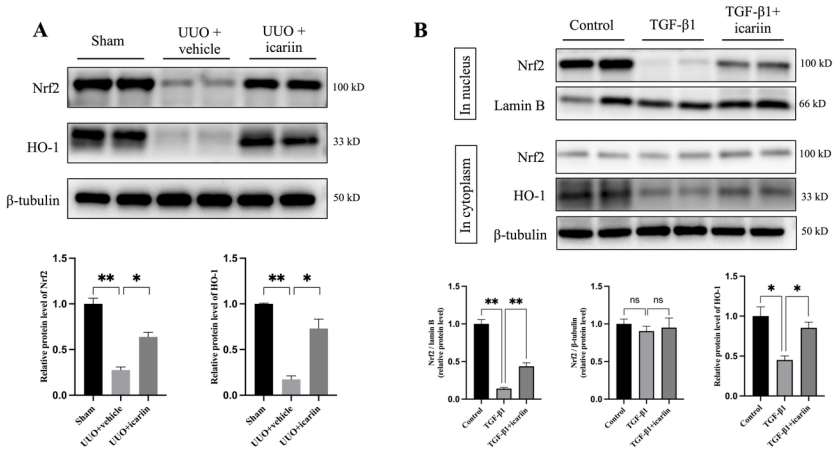



**Figure 6 Icariin activates the NRF2 signaling pathway both*in vivo* and *in vitro*.** (A) Western blot analysis of Nrf2 and HO-1 expression in the kidney from different groups. (B) Western blot analysis of nuclear and cytoplasmic cell extracts from HK-2 cells with different treatments. ns: not significant. * $p < 0.05$, ** $p < 0.01$.

## Discussion

Renal tubulointerstitial fibrosis (TIF) is a detrimental progression that inevitably results in renal function deterioration and structural changes, eventually culminating in ESRD. Nevertheless, the clinical management of TIF remains challenging because of the limited availability of effective prevention and treatment options. Novel therapeutic agents against TIF are urgently needed to delay the progression of CKD. In the current

study, we confirmed icariin's protective effect in UUO-induced CKD mouse model and in TGF-β1-treated HK-2 cells. UUO resulted in tubular atrophy, inflammation, oxidative stress and collagen deposition, whereas icariin markedly ameliorated these changes. In particular, icariin significantly improved mitochondrial mass and morphology of tubular epithelial cell in UUO mice. In TGF-β1-treated HK-2 cells, icariin markedly decreased the expression of profibrotic proteins and inflammatory factors, and protected mitochondria along with improving mitochondrial morphology, reducing mtROS accumulation, and preserving membrane potential. Further investigations uncovered that icariin activated Nrf2/HO-1 pathway both*in vivo* and *in vitro* , whereas inhibition of Nrf2 by ML385 counteracted icariin's protective effects on TGF-β1-treated HK-2 cells. Thus, icariin might exert anti-inflammation and antifibrotic effects, at least partially, through improving Nrf2 activity and subsequent mitochondrial function, and eventually mitigated TIF and preserved renal function (Figure 7).

*Epimedium* is a renowned traditional Chinese medicine that has been used for many centuries as a potent treatment for individuals with kidney ailments. Icariin has been demonstrated to be the primary bioactive component from *Epimedium* species. Modern pharmacological researches have showed that icariin exhibits considerable therapeutic capacities including neuroprotective[21], cardiovascular protective[22], anti-cancer[23], as well as improving immune system and reproductive function. Interestingly, recent researches have indicated that icariin could be beneficial in preventing or improving various fibrotic diseases, such as hepatic fibrosis[11], pulmonary fibrosis[24] and myocardial fibrosis[25]. Additionally, a prior research has ever reported the protective effect against kidney fibrosis in mouse model[26]. However, to fully harness the therapeutic benefits of icariin for CKD patients, a deeper comprehension of the underlying mechanisms is essential. In UUO-induced CKD mice, our results showed that icariin effectively reduced BUN and Cr levels. On the other hand, the renal histopathological lesions and elevated fibrotic proteins in UUO group were also markedly mitigated after icariin treatment. As mounting studies have shown that RTECs not only bear the brunt of different insults, but also play a crucial role in TIF by crosstalk with other cells and trans-differentiation into fibrotic phenotype[9,27], TGF-β1-treated HK-2 cells were used to verify the antifibrotic effect of icariin *in vitro* . Consistently, our *in vitro* experiments identified that icariin inhibited the profibrotic molecules expression, including a-SMA and collagen I. All these results highlight the pharmacological effect of icariin in inhibiting tubulointerstitial fibrosis and protecting renal function.

Oxidative stress and inflammation serve as interconnected players that underlie various causes of TIF and consistently manifest throughout the progression of CKD[5]. The transcriptomic data also indicated a pronounced enrichment in inflammatory response, mitochondrion, and oxidoreductase activity among the differentially expressed genes in UUO mice as compared to sham mice. Oxidative stress is a condition characterized by an imbalance between the production of ROS and their elimination by antioxidant defense systems, including SOD, glutathione peroxidase (GSH-Px) and catalase, as well as numerous non-enzymatic ROS scavengers. Excessive ROS can trigger renal fibrosis and inflammatory response, leading to substantial tissue lesion through promoting lipid peroxidation, DNA damage, and protein modifications, as well as the activation of profibrotic factors[28,29]. Icariin has been documented to safeguard vascular endothelial cells against oxidative stress by enhancing the activity of SOD and GSH-Px[30]. Additionally, icariin has also been shown to shield cardiac cells from oxidative damage through scavenging ROS and stimulating the ERK pathway[31]. In this study, we confirmed oxidative stress in UUO mice by demonstrating the accumulation of MDA and a decline in the activity of endogenous SOD, which were attenuated after icariin treatment. Accordingly, in TGF-β1-induced HK-2 cells, icariin can significantly inhibit intracellular ROS and mtROS overproduction, and protect HK-2 cells from oxidative stress. These results reinforce the effective anti-oxidation ability of icariin in CKD models.

Oxidative stress triggers deregulated inflammatory responses; while uncontrolled inflammatory responses, in turn, acts as a crucial mediator in the initiation and advancement of TIF, amplifying the deleterious consequences of oxidative stress in a "vicious cycle". Accumulating studies have implicated anti-inflammatory capacity of icariin in different diseases[32,33]. Of importance, icariin mitigated renal inflammation in murine lupus nephritis by suppressing NF-ϰB activation[34]. In our experiments, icariin treatment markedly reduced macrophages filtration in renal interstitium of UUO mice and downregulated levels of IL-6, TNF-α and IL-1β

inflammatory factors both *in vivo* and *in vitro* . Therefore, we inferred that icariin might confer protection against renal TIF by inhibiting inflammation and oxidative stress.

Consolidative proposals suggest that mitochondria, apart from being a powerhouse, also serve as central hubs that regulate cellular redox state and pro-inflammation signaling[6,35]. Mitochondria serve as the primary generator of intracellular ROS; meanwhile, excessive ROS and other damage-associated molecular patterns (DAMPs) originating from mitochondria can stimulate the expression of pro-inflammatory genes, leading to the activation of NF-ϰB signaling and NLRP3 inflammasome. Growing number of researches have proved the involvement of mitochondrial dysfunction in the development of kidney injury and impaired repair following injury[8]. Relieving mitochondrial dysfunction through administration of mitochondria-targeted antioxidants has been shown to attenuate kidney injury in animal models of UUO-induced CKD and diabetic kidney disease[36,37]. In the present investigation, we observed significant impairment in both the quantity and structure of mitochondria in kidneys from UUO mice. Consistent with prior research findings, the defective mitochondria exhibited prominent signs of matrix swelling, cristae depletion, and disruption of mitochondrial membranes. Interestingly, icariin effectively alleviated UUO-induced mitochondrial abnormalities. Consistently, in HK-2 cells treated with TGF-β1, icariin remarkedly protected mitochondrial function along with improving mitochondrial morphology, reducing mtROS accumulation, and preserving membrane potential. Collectively, our results expand the understanding of the renoprotection properties of icariin by maintaining mitochondrial homeostasis.

Mechanistically, we discover that the Nrf2/HO-1 signaling pathway is crucial in connecting icariin to its renoprotection effect. Nrf2 is a central redox-sensitive transcription factor that actively regulates pathways for antioxidant defense. Under normal physiological conditions, Nrf2 is kept at a low level in the cytoplasm by its inhibitor protein, Kelch-like ECH-associated protein 1 (Keap1), which facilitates the degradation of Nrf2 through a ubiquitin-proteosome pathway[38]. Upon oxidative stress, Keap1 undergoes modified that render it incapable of promoting Nrf2 degradation, leading to the accumulation of Nrf2 protein and its translocation to the nucleus. Once in the nucleus, Nrf2 binds to the antioxidant response element (ARE) and activates downstream antioxidant genes, such as HO-1, NAD(P)H quinone oxidoreductase 1 (NQO1) and SOD[39]. Besides, Nrf2 contributes to the anti-inflammatory process via multiple different mechanisms, especially negatively regulating NF-ϰB signaling pathway[40]. Several experimental models of CKD have shown an upregulation of Keap1 expression and a decrease in Nrf2 nuclear translocation, resulting in the suppression of antioxidant enzymes[39,41], which were consistent with our findings. Indeed, recent researches have demonstrated that the activation of Nrf2 contributes to the improvement of renal fibrosis[42-44]. Icariin has been demonstrated to alleviate extracellular matrix accumulation and oxidative stress by activating Nrf2 in experimental diabetic kidney disease[45]. Moreover, another study has revealed that icariin enhances mitophagy to suppress the activation of NLRP3 inflammasome through the Keap1-Nrf2/HO-1 axis in diabetic nephropathy rats[17]. Additionally, further studies have suggested that icariin may modulate acute inflammation via the Nrf2/HO-1 and NF-ϰB signaling pathways[46]. These findings collectively support the potential of icariin as a potent activator of Nrf2/HO-1. In our study, further investigations uncovered that icariin treatment restored Nrf2/HO-1 levels both *in vivo* and *in vitro* , whereas inhibition of Nrf2 by ML385 counteracted the protective effects of icariin in TGF-β1-treated HK-2 cells, suggesting that the potential renoprotective effect of icariin was mediated by Nrf2 signaling pathway.

In conclusion, our results demonstrate that icariin protects against renal tubulointerstitial fibrosis and inflammation at least partly through Nrf2-mediated attenuation of mitochondrial oxidative damage, strongly suggesting that icariin may be a promising therapeutic strategy to mitigate TIF and preserve renal function.

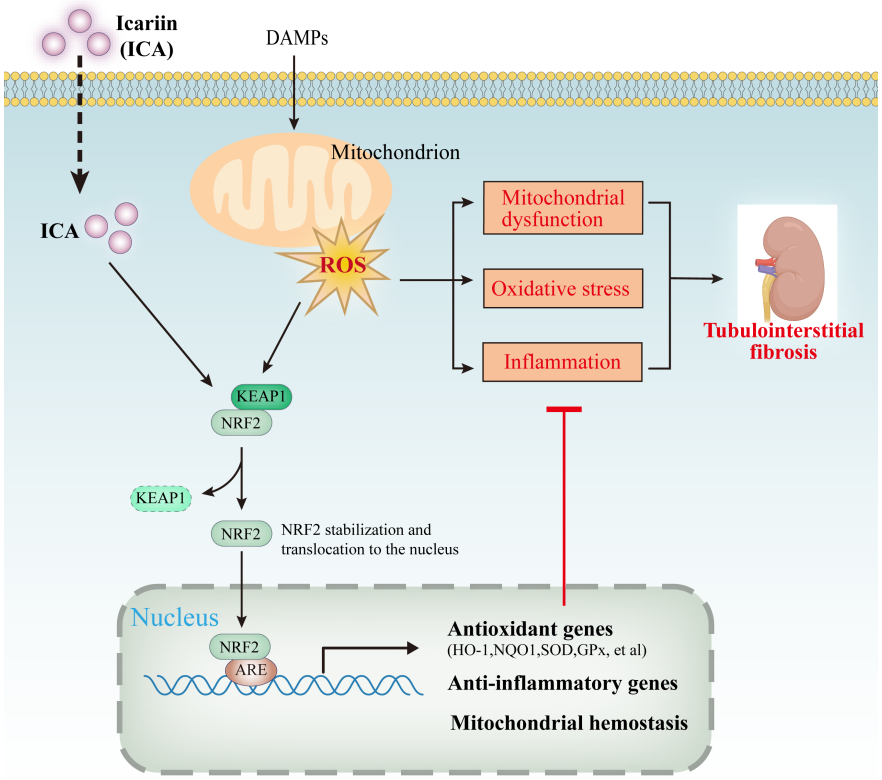



**Figure 7 Schematic diagram of the protective mechanism of icariin in renal tubulointerstitial fibrosis of CKD.** Icariin may inhibit the process of Keap1-dependent Nrf2 degradation, allowing Nrf2 protein to accumulate and translocate to the nucleus. Once in the nucleus, Nrf2 binds to ARE sequence, activates downstream cytoprotective genes, and eventually mitigates tubulointerstitial fibrosis. DAMPs, damage-associated molecular patterns; ARE, antioxidant response element; NQO1, NAD(P)H quinone oxidoreductase-1; HO-1, heme oxygenase-1; GPx, glutathione peroxidase; SOD, superoxide dismutase.

## Acknowledgements

This work was supported by Youth Science Fund Incubation Program of Shandong First Medical University (NO: 202201-075).

## Conflict of interest statement

The authors declare no conflict of interest.

## Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## References

1. Kovesdy CP. Epidemiology of chronic kidney disease: an update 2022.*Kidney Int Suppl (2011)* . 2022;12(1):7-11. doi:10.1016/j.kisu.2021.11.003

2. Humphreys BD. Mechanisms of Renal Fibrosis. *Annu Rev Physiol.*2018;80:309-326. doi:10.1146/annurev-physiol-022516-034227

3. Wang W, Ma B lei, Xu C geng, Zhou X jun. Dihydroquercetin protects against renal fibrosis by activating the Nrf2 pathway.*Phytomedicine.* 2020;69:153185. doi:10.1016/j.phymed.2020.153185

4. Hewitson TD, Holt SG, Smith ER. Progression of Tubulointerstitial Fibrosis and the Chronic Kidney Disease Phenotype - Role of Risk Factors and Epigenetics. *Front Pharmacol.* 2017;8:520. doi:10.3389/fphar.2017.00520

5. Webster AC, Nagler EV, Morton RL, Masson P. Chronic Kidney Disease.*Lancet.* 2017;389(10075):1238-1252. doi:10.1016/S0140-6736(16)32064-5

6. Marchi S, Guilbaud E, Tait SWG, Yamazaki T, Galluzzi L. Mitochondrial control of inflammation. *Nat Rev Immunol.* 2023;23(3):159-173. doi:10.1038/s41577-022-00760-x

7. Monzel AS, Enríquez JA, Picard M. Multifaceted mitochondria: moving mitochondrial science beyond function and dysfunction. *Nat Metab.*2023;5(4):546-562. doi:10.1038/s42255-023-00783-1

8. Emma F, Montini G, Parikh SM, Salviati L. Mitochondrial dysfunction in inherited renal disease and acute kidney injury. *Nat Rev Nephrol.* 2016;12(5):267-280. doi:10.1038/nrneph.2015.214

9. Liu BC, Tang TT, Lv LL, Lan HY. Renal tubule injury: a driving force toward chronic kidney disease. *Kidney Int.* 2018;93(3):568-579. doi:10.1016/j.kint.2017.09.033

10. Ye L, Yu Y, Zhao Y. Icariin-induced miR-875-5p attenuates epithelial-mesenchymal transition by targeting hedgehog signaling in liver fibrosis. *J Gastroenterol Hepatol.* 2020;35(3):482-491. doi:10.1111/jgh.14875

11. Algandaby MM, Breikaa RM, Eid BG, Neamatallah TA, Abdel-Naim AB, Ashour OM. Icariin protects against thioacetamide-induced liver fibrosis in rats: Implication of anti-angiogenic and anti-autophagic properties.*Pharmacol Rep.* 2017;69(4):616-624. doi:10.1016/j.pharep.2017.02.016

12. Qi MY, He YH, Cheng Y, et al. Icariin ameliorates streptozocin-induced diabetic nephropathy through suppressing the TLR4/NF-κB signal pathway. *Food Funct.* 2021;12(3):1241-1251. doi:10.1039/d0fo02335c

13. Wu B, Feng J, Yu L, et al. Icariin protects cardiomyocytes against ischaemia/reperfusion injury by attenuating sirtuin 1-dependent mitochondrial oxidative damage. *Brit J Pharmacology.*2018;175(21):4137-4153. doi:10.1111/bph.14457

14. Yu LM, Dong X, Xu YL, et al. Icariin attenuates excessive alcohol consumption-induced susceptibility to atrial fibrillation through SIRT3 signaling. *Biochim Biophys Acta Mol Basis Dis.*2022;1868(10):166483. doi:10.1016/j.bbadis.2022.166483

15. Qiao C, Ye W, Li S, Wang H, Ding X. Icariin modulates mitochondrial function and apoptosis in high glucose-induced glomerular podocytes through G protein-coupled estrogen receptors. *Mol Cell Endocrinol.* 2018;473:146-155. doi:10.1016/j.mce.2018.01.014

16. Chevalier RL, Forbes MS, Thornhill BA. Ureteral obstruction as a model of renal interstitial fibrosis and obstructive nephropathy.*Kidney Int.* 2009;75(11):1145-1152. doi:10.1038/ki.2009.86

17. Ding X, Zhao H, Qiao C. Icariin protects podocytes from NLRP3 activation by Sesn2-induced mitophagy through the Keap1-Nrf2/HO-1 axis in diabetic nephropathy. *Phytomedicine.* 2022;99:154005. doi:10.1016/j.phymed.2022.154005

18. Yu LM, Dong X, Li N, et al. Polydatin attenuates chronic alcohol consumption-induced cardiomyopathy through a SIRT6-dependent mechanism.*Food Funct.* 2022;13(13):7302-7319. doi:10.1039/d2fo00966h

19. Chung KW, Dhillon P, Huang S, et al. Mitochondrial Damage and Activation of the STING Pathway Lead to Renal Inflammation and Fibrosis.*Cell Metab.* 2019;30(4):784-799.e5. doi:10.1016/j.cmet.2019.08.003

20. Li X, Zhang W, Cao Q, et al. Mitochondrial dysfunction in fibrotic diseases. *Cell Death Discov.* 2020;6(1):1-14. doi:10.1038/s41420-020-00316-9

21. Zheng L, Wu S, Jin H, et al. Molecular mechanisms and therapeutic potential of icariin in the treatment of Alzheimer's disease.*Phytomedicine.* 2023;116:154890. doi:10.1016/j.phymed.2023.154890

22. Zeng Y, Xiong Y, Yang T, et al. Icariin and its metabolites as potential protective phytochemicals against cardiovascular disease: From effects to molecular mechanisms. *Biomed Pharmacother.*2022;147:112642. doi:10.1016/j.biopha.2022.112642

23. Liu Y, Yang H, Xiong J, et al. Icariin as an emerging candidate drug for anticancer treatment: Current status and perspective. *Biomed Pharmacother.* 2023;157:113991. doi:10.1016/j.biopha.2022.113991

24. Du W, Tang Z, Yang F, Liu X, Dong J. Icariin attenuates bleomycin-induced pulmonary fibrosis by targeting Hippo/YAP pathway.*Biomed Pharmacother.* 2021;143:112152. doi:10.1016/j.biopha.2021.112152

25. Zhang L, Wang S, Li Y, Wang Y, Dong C, Xu H. Cardioprotective effect of icariin against myocardial fibrosis and its molecular mechanism in diabetic cardiomyopathy based on network pharmacology: Role of ICA in DCM. *Phytomedicine.* 2021;91:153607. doi:10.1016/j.phymed.2021.153607

26. Chen H, Chen CM, Guan SS, Chiang CK, Wu CT, Liu SH. The antifibrotic and anti-inflammatory effects of icariin on the kidney in a unilateral ureteral obstruction mouse model. *Phytomedicine.* 2019;59:152917. doi:10.1016/j.phymed.2019.152917

27. Qi R, Yang C. Renal tubular epithelial cells: the neglected mediator of tubulointerstitial fibrosis after injury. *Cell Death Dis.*2018;9(11):1126. doi:10.1038/s41419-018-1157-x

28. Jha JC, Banal C, Chow BSM, Cooper ME, Jandeleit-Dahm K. Diabetes and Kidney Disease: Role of Oxidative Stress. *Antioxid Redox Signal.*2016;25(12):657-684. doi:10.1089/ars.2016.6664

29. Galvan DL, Green NH, Danesh FR. The hallmarks of mitochondrial dysfunction in chronic kidney disease. *Kidney Int.*2017;92(5):1051-1057. doi:10.1016/j.kint.2017.05.034

30. Wang FY, Jia J, Song HH, Jia CM, Chen CB, Ma J. Icariin protects vascular endothelial cells from oxidative stress through inhibiting endoplasmic reticulum stress. *J Integr Med.* 2019;17(3):205-212. doi:10.1016/j.joim.2019.01.011

31. Song YH, Cai H, Zhao ZM, et al. Icariin attenuated oxidative stress induced-cardiac apoptosis by mitochondria protection and ERK activation.*Biomed Pharmacother.* 2016;83:1089-1094. doi:10.1016/j.biopha.2016.08.016

32. Zhang C, Cao Z, Lei H, et al. Discovery of a novel small molecule with efficacy in protecting against inflammation in vitro and in vivo by enhancing macrophages activation. *Biomed Pharmacother.*2023;165:115273. doi:10.1016/j.biopha.2023.115273

33. Xiong D, Deng Y, Huang B, et al. Icariin attenuates cerebral ischemia-reperfusion injury through inhibition of inflammatory response mediated by NF-ϰB, PPARα and PPARγ in rats. *Int Immunopharmacol.*2016;30:157-162. doi:10.1016/j.intimp.2015.11.035

34. Su B, Ye H, You X, Ni H, Chen X, Li L. Icariin alleviates murine lupus nephritis via inhibiting NF-ϰB activation pathway and NLRP3 inflammasome. *Life Sci.* 2018;208:26-32. doi:10.1016/j.lfs.2018.07.009

35. Riley JS, Tait SW. Mitochondrial DNA in inflammation and immunity.*EMBO Rep* . 2020;21(4):e49799. doi:10.15252/embr.201949799

36. Zhu Z, Liang W, Chen Z, et al. Mitoquinone Protects Podocytes from Angiotensin II-Induced Mitochondrial Dysfunction and Injury via the Keap1-Nrf2 Signaling Pathway. *Oxid Med Cell Longev.*2021;2021:1394486. doi:10.1155/2021/1394486

37. Xiao L, Xu X, Zhang F, et al. The mitochondria-targeted antioxidant MitoQ ameliorated tubular injury mediated by mitophagy in diabetic kidney disease via Nrf2/PINK1. *Redox Biol.* 2017;11:297-311. doi:10.1016/j.redox.2016.12.022

38. Itoh K, Mimura J, Yamamoto M. Discovery of the negative regulator of Nrf2, Keap1: a historical overview. *Antioxid Redox Signal.*2010;13(11):1665-1678. doi:10.1089/ars.2010.3222

39. Aminzadeh MA, Nicholas SB, Norris KC, Vaziri ND. Role of impaired Nrf2 activation in the pathogenesis of oxidative stress and inflammation in chronic tubulo-interstitial nephropathy. *Nephrol Dial Transplant.* 2013;28(8):2038-2045. doi:10.1093/ndt/gft022

40. Ahmed SMU, Luo L, Namani A, Wang XJ, Tang X. Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochim Biophys Acta Mol Basis Dis.* 2017;1863(2):585-597. doi:10.1016/j.bbadis.2016.11.005

41. Kim HJ, Vaziri ND. Contribution of impaired Nrf2-Keap1 pathway to oxidative stress and inflammation in chronic renal failure. *Am J Physiol Renal Physiol.* 2010;298(3):F662-671. doi:10.1152/ajprenal.00421.2009

42. Lu Y, Sun Y, Liu Z, et al. Activation of NRF2 ameliorates oxidative stress and cystogenesis in autosomal dominant polycystic kidney disease.*Sci Transl Med.* 2020;12(554):eaba3613. doi:10.1126/scitranslmed.aba3613

43. Guerrero-Hue M, Rayego-Mateos S, Vázquez-Carballo C, et al. Protective Role of Nrf2 in Renal Disease. *Antioxidants (Basel).*2020;10(1):39. doi:10.3390/antiox10010039

44. Nezu M, Suzuki N, Yamamoto M. Targeting the KEAP1-NRF2 System to Prevent Kidney Disease Progression. *Am J Nephrol.*2017;45(6):473-483. doi:10.1159/000475890

45. Wang K, Zheng X, Pan Z, et al. Icariin Prevents Extracellular Matrix Accumulation and Ameliorates Experimental Diabetic Kidney Disease by Inhibiting Oxidative Stress via GPER Mediated p62-Dependent Keap1 Degradation and Nrf2 Activation. *Front Cell Dev Biol.* 2020;8:559. doi:10.3389/fcell.2020.00559

46. El-Shitany NA, Eid BG. Icariin modulates carrageenan-induced acute inflammation through HO-1/Nrf2 and NF-kB signaling pathways.*Biomed Pharmacother.* 2019;120:109567. doi:10.1016/j.biopha.2019.109567