INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20F–123/2020/24–6–1665–1671
DOI: 10.17957/IJAB/15.1609
http://www.fspublishers.org

Full Length Article

Selaginella uncinata Aqueous Extract Ameliorates Renal Fibrosis in Rats *via* Antioxidant Pathway

Min Gao[1,2†], Zhi-Wei Sun[4†], Kai Ni[2], Jian Wang[3], Jian Xu[3], Yan-Ping Yang[1], Hua Xiao[1] and Hong-Ling Yuan[1,3*]
[1]*Department of Nephrology, the First Affiliated Hospital of Kunming Medical University, Kunming 650032, P.R. China*
[2]*National Folk Medicine Research Center, Yunnan Traditional Chinese Medicine Institute, Kunming 650223, P.R. China*
[3]*Department of Nephrology, the First People's Hospital of Yunnan Province, Kunming 650032, P.R. China*
[4]*Department of Hepatobiliary Surgery, the First People's Hospital of Yunnan Province, Kunming 650032, P.R. China*
[*]For correspondence: holiyuan@126.com
[†]Contributed equally to this work and are co-first authors
Received 09 July 2020; Accepted 11 August 2020; Published 10 October 2020

Abstract

Selaginella uncinata (Desv.) Spring, known as Cuiyuncao, is a traditional Chinese prescription against some inflammation related diseases. To examine the effect of Cuiyuncao on renal fibrosis in a rat model and the underlying mechanism, a renal fibrosis rat model was established via unilateral ureteral obstruction (UUO). Flow cytometry was used to isolate primary rat kidney fibroblasts and analyze oxidative levels. The mRNA and protein expression of TGF-β1, Col-I, α-SMA was investigated through quantitative PCR (qPCR), western blotting and immunohistochemistry. Antioxidant response element (ARE) motif was examined by using ARE reporter assay and chromatin immunoprecipitation. In the results, it was found that both oxidative stress and the levels of transforming growth factor beta-1 (TGF-β1), collagen-1 (Col-I) and alpha-smooth muscle actin (α-SMA) were higher in the kidneys of UUO rats than in controls. Cuiyuncao aqueous extract ameliorates renal fibrosis in rats of UUO. Furthermore, it reduces both the oxidative stress levels and the expressions of TGF-β1, Col-I and α-SMA, but in different extent in UUO rats. Upon oxidative stress, the antioxidant transcription factor NRF2 enhances the expression of numerous genes regulated by ARE. There are a few potential AREs in the upstream of Col-I and α-SMA respectively, but not TGF-β1. Among these potential AREs one of each of Col-I and α-SMA AREs were confirmed by functional assays. In conclusion, this study indicates that Cuiyuncao aqueous extract has a significant curative effect on renal fibrosis *via* antioxidant pathway. © 2020 Friends Science Publishers

Keywords: *Selaginella uncinate*; Renal fibrosis; Unilateral ureteral obstruction; Oxidative stress; NRF2; Antioxidant response element

Introduction

Selaginella uncinata (Desv.) Spring, known as Cuiyuncao, is a perennial herb which exists in abundance in South China. It is widely used in traditional Chinese medicine (TCM) as a remedy against a wide range of diseases, such as jaundice, dysentery and edema. Phytochemical studies reveal various chemical constituents, including flavonoids (Zheng *et al.* 2011; Zou *et al.* 2014, 2016), chromone glycosides (Ma *et al.* 2003) and steroidal saponins in Cuiyuncao (Zheng *et al.* 2013). These chemicals have broad biological activities. For instance, uncinoside A and B, the two newly discovered chromone glycosides, exhibit antiviral properties and is used to suppress respiratory syncytial virus and parainfluenza type 3 virus (Ma *et al.* 2003). Two new steroidal saponins show a strong neuroprotective effect against anoxia in PC12 cell, a number of flavonoids also exhibit antioxidation activity (Zheng *et al.* 2013), which indicates Cuiyuncao's potential effects on oxidative stress-related diseases.

The global prevalence of chronic kidney disease (CKD) was estimated to be 10–16% (CDC 2007; Jha *et al.* 2013). Renal fibrosis, a common form of CKD, is initially characterized by aberrant angiogenesis and capillary obliteration. The condition is also accompanied by resident cell stimulation *via* pro-inflammatory cytokines. Consequently, tubular epithelial cells and fibroblasts generate and fill the interstitial space with several extracellular matrix (ECM) constituents (Eddy 2000; Liu 2006). COL-I and α-SMA are the two major components of ECM and excessive ECM eventually causes chronic renal failure. The activated fibroblasts mainly contribute to ECM deposition in kidney. It is noteworthy that the blockage of ureters results in acute renal injury, a condition associated with injuries to the ureteral cells, interstitial inflammation and finally fibrosis (Eddy 2000; Liu 2006). In the process of

wound healing, fibroblasts are stimulated by many different signals that are associated with injuries, such as TGF-β1 as well as inflammatory micro environmental factors, which includes the infiltration of immune cells, hyperglycemia, and hypoxia (Strutz and Zeisberg 2006; Kim *et al.* 2018).

Thus, it is crucial to clear the activated fibroblasts to prevent or delay renal fibrosis progression. In this regard, many clinical trials have examined the efficacy of numerous drugs. Currently, the standard treatment to ensure delay of the fibrogenic process involves the use of Corticosteroids and cyclosporine A, both of which are immunosuppressive agents. Unfortunately, when these drugs are used for a long time, they could cause a serious kidney injury. Other regimens like the angiotensin-converting enzyme/antagonists of angiotensin II receptor inhibitors (Sehirli *et al.* 2020) evoke severe side effects (Breyer and Susztak 2016). In the recent past, researchers have been successful in the development of new anti-fibrotic approaches, which can specifically target fibroblasts, and these include antibodies against platelet-derived growth factors (PDGFs), TGF-β1 receptor inhibitors and Ras inhibitor cocktail (Zeisberg and Neilson 2010). Moreover, TCM is also effective in both treatment and prevention of CKD as revealed by clinical and experimental studies (Zhong *et al.* 2015; Lv *et al.* 2018; Alharbi *et al.* 2019; Zhang *et al.* 2019; Tawfik *et al.* 2020).

Oxidative stress has been implicated in renal fibroblasts activation during the progression of kidney diseases (Xu *et al.* 2013; Kang *et al.* 2015). In this study, whether Cuiyuncao aqueous extract could down-regulate cell signaling factors in renal fibrosis and consequently delay the progression of renal fibrosis in rats of unilateral ureteral obstruction (UUO) were investigated. In the molecular level, how Cuiyuncao regulates renal fibrosis closely related genes TGF-β1, Col-I and α-SMA were investigated. Further, it was revealed that the Cuiyuncao's effect on oxidative stress reduction and the mechanism by which the renal fibrosis genes are regulated.

Materials and Methods

Source of *S. uncinata*

Plants of *S. uncinate* (Desv.) Spring (Cuiyuncao) was obtained from Yunnan Institute of Chinese Traditional Medicine and identified according to its morphological characteristics. 1 kg of dry Cuiyuncao herb was ground to powder then suspended in 4 L of water. Next, the suspension was boiled for 2 h then filtered it through nylon mesh. A total amount of 1000 mL extracts were obtained. So, one millimeter of aqueous extracts represents 1.0 g of dry herb.

Model building

This experiment was admitted to The First Affiliated Hospital of Kunming Medical University between February and November 2019. Male SD rats (obtained from Kunming Medical University) weighing 190–220 g were housed at 25 ± 2°C with a 12 h light/dark cycle and given free access to food and water. The left ureter was tied at two points to attain unilateral ureteral obstruction (UUO). This procedure was avoided in sham operation (Yu *et al.* 2017). 81 SPF SD rats were randomly assigned to sham-operation group, UUO saline group and UUO Cuiyuncao group. Rats in each group were randomly divided into 3-day, 7-day and 14-day groups (n = 9 for each).

Cuiyuncao group received 10 mL/kg·day of Cuiyuncao extract for certain days by oral gavage. Animals of other groups received saline at the dose of 10 mL/kg·day. The animals were sacrificed at 3, 7, and 14 days after surgery to remove the left kidneys. Subsequently, the extracted parts were sagittal sliced twice into three parts. One of the parts was preserved at -80°C for mRNA and protein isolation, one part was for histopathological and immunohistochemical examinations and the rest part was for fibroblasts isolation.

Flow cytometric assay

Renal cortexes were cut into pieces and then digested for 30 min with 0.1% collagenase and 3 U/mL of dispose at 37°C. Next, the digest was filtered with a 70 mm nylon strainer (Biosciences, CA, USA), then suspended in DMEM following centrifugation (5 min, 1700 g). CellROX Deep Red kit (Life Technologies, CA, USA) was used to analyze oxidative levels. Canto II cytometer (Biosciences) was utilized to acquire flow cytometric data, which were then analyzed with FlowJo X (TreeStar, OR, USA).

Masson's trichrome staining

Kidney tissues were fixed and dehydrated following a standard workflow (Yu *et al.* 2017). After embedding the tissues in paraffin, they were cut into 4 μm thin sections, then stained with hematoxylin–eosin and Masson's tri-chrome following the standard protocol (Williams *et al.* 1997). The Masson's trichrome staining positive area was quantified using ImageJ.

Immunohistochemical staining

Paraffin sections were deparaffinized, rehydrated, and autoclaved for 10 min in 100 m*M* citrate buffer (pH 6.0). After treatment with 3% H_2O_2 and 10% bovine serum sections were incubated with an antibody against TGF-β1 (ab92486, Abcam), COL-I (ab6308, Abcam) or α-SMA (ab5694, Abcam) for 12 h at 4°C. The bound antibodies were examined using Polink-2 HRP plus rabbit DAB detection system (GoldenBridge, WA, USA). Image Pro Plus was employed to assess the average optical density (AOD) of immunohistochemical (IHC) staining intensities of proteins tested.

qPCR assay

Trizol reagent (Life Technologies) was utilized in the isolation of total RNA from the kidney fibroblasts. The RNA (2 μg per sample) was reverse transcribed to cDNA using the M-MLV Reverse Transcriptase (Promega, WI, USA). The RT-PCR assay was conducted using the standard SYBR Green reagent (Thermo Scientific, M.A., USA) with GAPDH serving as internal control. Quantification of mRNAs was done using 7900HT real-time PCR detection system (BioRad, CA, USA). Each sample was divided, then analyzed separately, and three samples were analyzed for every condition (Table 1).

Western blot analysis

Cells or tissues were collected, lysed and quantified using Bradford method (Ramachandran *et al.* 2012). 50ug protein was boiled and separated by 10% SDS PAGE followed by transferring to Immobilon-P membranes. The membranes were blocked in 10% non-fat milk for 1 h and then incubated with a primary antibody overnight at 4°C and sequentially with HRP conjugated anti-IgG antibody (1:5000) for 1 h. The proteins were visualized using the ECL Plus (Thermo Scientific, M.A., USA). Anti-TGF-β1, anti-COL-I, anti-α-SMA and anti-GAPDH antibodies (ab8245) were purchased from Abcam. Relative intensities were normalized by the ratio between the GAPDH using ImageJ (version 1.47, NIH, MD, USA).

MTT assay

NRK-52E (Rat kidney epithelial cell line) was from the American Type Culture Collection (ATCC, VA, USA) and cultured in DMEM with 10% FBS. Cells were transfected with Lipofectamine 2000 (Life Technologies) and stabilized for 12 h for the downstream experiment. NRK-52E cells were treated with H_2O_2 at 40 μM for 24 h for oxidative stress or sterile Cuiyuncao aqueous extract at 1/20 of the original extract for antioxidant treatment (Clerk *et al.* 2007). The working dilution of Cuiyuncao was determined by MTT assay.

Chromatin immunoprecipitation assay

The upstream sequence of a given gene was retrieved from UCSC genome browser (Tyner *et al.* 2017). The sequence was scanned with a webtool for the antioxidant response element (ARE) motif (Tyner *et al.* 2017). The predicted AREs were validated by NRF2 ChIP-qPCR. The validated AREs were inserted into pT81 luciferase plasmid to construct a reporter (Mulcahy *et al.* 1997). Then a certain reporter was co-transfected with a CMV driven Renilla construct as an internal reference. Luciferase activity was determined using the Dual-Glo luciferase assay (Promega Corporation) in a FlexStation III (Molecular Devices). The NRF2 chromatin immunoprecipitation was carried out in NRK-52E cells following a standard protocol (Blecher-Gonen *et al.* 2013). The NRF2 (ab62352) antibody and IgG (ab190475) as a negative control were from Abcam.

Statistics

Data analysis was conducted using SPSS (Window version 15.0.0) software package. Data are expressed as means ± standard deviation (S.D.). Comparisons between two groups were analyzed by Student's *t*-test was employed to assess the difference between two treatments, whereas one-way ANOVA followed by Tukey test (post-hoc) was used to compare more than two treatments. A P-value less than 0.05 indicated statistical significance.

Results

Cuiyuncao treatment ameliorates renal fibrosis and reduces the oxidative stress in UUO kidneys

Hematoxylin Eosin staining indicates kidney of sham rats were normal, no infiltration of inflammatory cells. Three days post-surgery, the kidneys of UUO presented infiltration of inflammatory cells; Seven days post-surgery vacuolation of renal tubular epithelial cells appeared (Fig. 1A). In the kidney from UUO rats at day 7 post surgery tubulointerstitial infiltration of inflammatory cells increased. At day 14, the lesions of kidney with UUO were further deteriorated. Tubular dystrophy, renal tubular atrophy, disorder of arrangement, and diffuse tubulointerstitial infiltration of inflammatory cells occurred (Fig. 1A). Cuiyuncao treatment largely slowed the development of tubulointerstitial lesions, ameliorated the tubulointerstitial infiltration of inflammatory cells and tubular dilation (Fig. 1A and C).

Masson's trichrome staining revealed that kidneys with sham operation were normal. Interstitial fibrosis presented kidney with UUO after 3 days of surgery and were expanding with the passage of time (Fig. 1B). Masson's trichrome staining revealed tubular epithelial cell shedding, tubular dilation, intensive interstitial fibrosis. Interstitial fibrosis in kidney treated with Cuiyuncao significantly ameliorated at day 14 after surgery (Fig. 1B and C).

The freshly isolated renal cells were subjected to oxidative stress analysis by flow cytometry with CellROX reagent. The sham rats have a stable oxidative stress level throughout the experiments. Whereas the oxidative stress levels in the UUO animals are increasing remarkably with the passage of time. Cuiyuncao treatment of UUO animals can significantly reduce the oxidative stress levels compared to UUO rats of the corresponding day (Fig. 1D).

The effect of cuiyuncao treatment on the expression of TGF-β1, Col-I and α-SMA was evaluated by immunohistochemistry (IHC) in UUO kidneys

As the pro-inflammatory protein and pro-fibrotic protein are both important to tubular interstitial fibrosis formation.

Table 1: qPCR Primers used in this study

Gene name	Product size (bp)	Genbank number	Forward primer	Reverse primer
TGF-β1	128	NM_021578.2	cctggaaagggctcaacac	tgccgtacacagcagttctt
Col-I	68	NM_053304.1	tcctggcaagaacggagat	caggaggtccacgctcac
α-SMA	62	NM_001170325.1	ccggattctggcttctgata	ggcagctctcgacgaagt
GAPDH	87	AF106860.2	atgactctacccacggcaag	ggaagatggtgatgggtttc

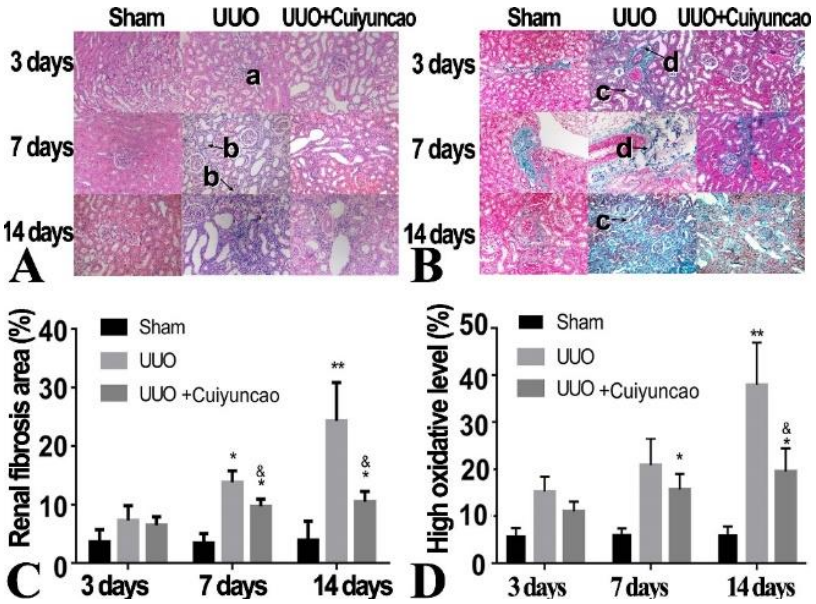

Fig. 1: Cuiyuncao treatment ameliorates renal fibrosis and reduces the oxidative stress in UUO kidneys. (**A**) Representative hematoxylin–eosin (HE) stained kidney sections from sham, UUO, and UUO (Magnification, X 200). a) lymphocte and monocyte cell infiltration; b) cytoplasmic vacuoloation. (**B**) Representative Masson's trichrome stained kidney sections from sham, UUO, and UUO (Magnification, X 200). Increased fibrosis in both glomerulus and tubulointerstitial area, and a prominent inflammatory cell infiltration were seen in tubulointerstitium of UUO rats. c) lumen surface fell and tubular dilation; d) fibrotic hyperplasia of basement membrane and renal interstitium. (**C**) Quantitative analysis of the fibrotic area (the mean ± S.D. of 10 separate observations). (**D**) The oxidative stress level of freshly prepared renal cells was evaluated by DeepRed CellROX. (the mean ± S.D. of renal cells from 9 rats of the same group). Compared with sham group, $^{*}P < 0.05$ and $^{**}P < 0.01$, while compared with UUO group, $^{\&}P < 0.05$. Otherwise, not significant

Transforming growth factor beta 1 (TGF-β1) is one of the key injuries signaling molecules that regulate migration and infiltration of lymphocytes, which lead to inflammation and kidney fibrosis. Cuiyuncao could decrease the TGF-β1 in the day 3 after UUO. Although with a limited statistical significance, Cuiyuncao treatment decreased the expression of TGF-β1 in the day 7 and day 14 after UUO (Fig. 2A and B). Arrows point to the hyperintense sites. There are sporadic TGF-β1 hyperintense sites even in sham samples (Fig. 2A).

The expression and accumulation of ECM components including COL-I and α-SMA are the major events in formation of fibrotic kidney (Lee *et al.* 2013). Unlike TGF-β1, both COL-I and α-SMA have a low expression level with an even distribution across the tissues from kidneys of sham rats. Due to UUO both COL-I and α-SMA are overexpressed with the progression of fibrosis. Cuiyuncao treatment significantly reduced both COL-I and α-SMA (Fig. 2C, D, E and F). Arrows point to the hyperintense sites and there are no hyperintense sites in sham samples (Fig. 2C and E) for both COL-I and α-SMA.

Quantitative RT-PCR and western blot of COL-1 and α-SMA

To avoid the biased interpretation of immunohistochemical data due to the selection of slices and views it is critical to confirm the results from immunohistochemistry staining by quantitative RT-PCR and western blot. The tissues from the same kidneys as used in immunohistochemistry were subjected to RT-qPCR and western blot analysis. The qPCR of Col-I and α-SMA agreed with the immunohistochemically results described in Fig. 3A, B. The western blots of COL-I and α-SMA exhibit the same tendency as the immunohistochemistry data with statistical significance (Fig. 3C, D and E).

Similarly, the TGF-β1 qPCR has the tendency that UUO increased the TGF-β1 expression and Cuiyuncao decreased the induced upregulation of TGF-β1 by UUO. However, the TGF-β1 qPCR data were not statistically significant. Moreover, TGF-β1 western blots in the kidneys from different groups have not the same tendency as in qPCR (data not shown). As TGF-β1 is a soluble protein it could be lost from the tissues during collection and wash.

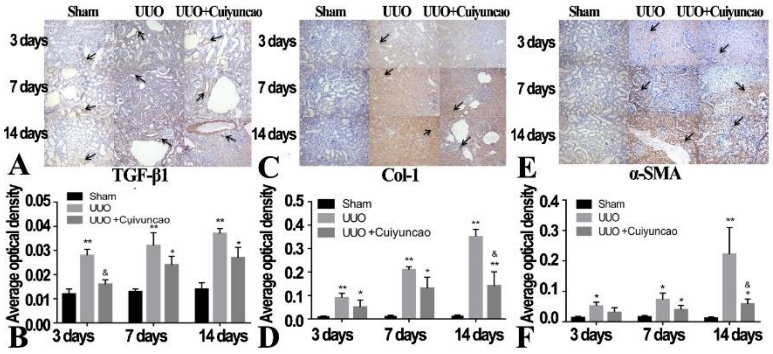

Fig. 2: The effect of Cuiyuncao treatment on the expression of TGF-β1, Col-I and α-SMA in UUO kidneys. (**A**) Representative of immunostaining of TGF-β1, (**B**) Quantitative measurement of TGF-β1, (**C**) Col-I and (**E**) α-SMA in renal sections (×200) from sham, UUO, and Cuiyuncao treated UUO rats at day 3, 7 and 14 post surgery. (**D**) Col-I and (**F**) α-SMA IHC signal. The arrows point sites where the target proteins are highly abundant. Data represent mean ± S.D. for at least 10 independent slices. Compared with sham group, **P* < 0.05 and ** *P* < 0.01; while compared with UUO group, $^{\&}$ *P* < 0.05. Otherwise, not significant

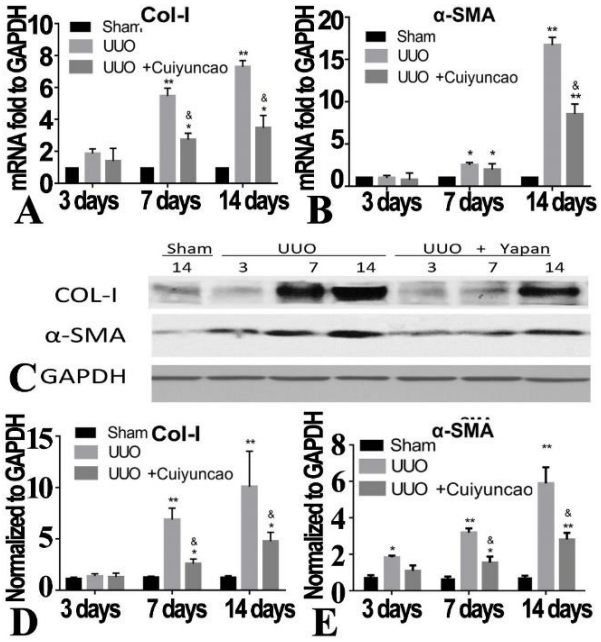

Fig. 3: RT-qPCR and western blot of COL-1 and α-SMA. (**A**) Col-I and (**B**) α-SMA qPCR in kidneys from sham, UUO, and Cuiyuncao treated UUO rats at day 3, 7 and 14 post surgeries. (**C**) Representative western blot of Col-I, α-SMA and GAPDH as control. (**D**) Quantitative analysis of Col-I and (**E**) α-SMA western blot. Data are expressed as means ± S.D. Compared with sham group, **P* < 0.05 and ** *P* < 0.01, while compared with UUO group, $^{\&}$ *P* < 0.05. Otherwise, not significant

Cuiyuncao reduces the expressions of Col-I and α-SMA by downregulating NRF2/ARE pathway

Oxidative stress could trigger the expressions of numerous genes regulated by a cis-acting element called antioxidant response element (ARE). Once oxidative stressed the transcription factor NRF2 will disassociate with KEAP1 to get into the nucleus (Lee *et al.* 2013). Then NRF2 binds to the ARE to enhance the gene expression (Fig. 4D). As the results indicated that Col-I and α-SMA expressions might be enhanced by oxidative stress, we then compared their expression changes between H_2O_2 treatment and Cuiyuncao aqueous extract treatment. Compared to NRK-52E cells in normal condition, Col-I and α-SMA expressions are higher in H_2O_2 treated cells, whereas cells treated with Cuiyuncao were lower in their expressions (Fig. 4A). Cuiyuncao might stabilize the NRF2/KEAP1 complex and therefore reduce the expressions of NRF2-driven genes (Fig. 4D).

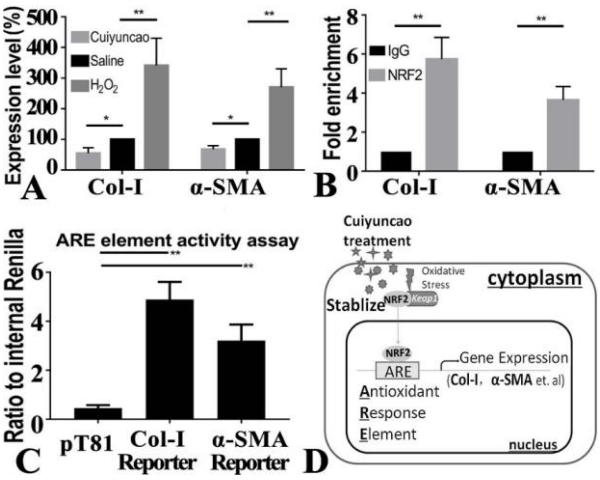

Fig. 4: Cuiyuncao reduces the expressions of Col-I and α-SMA by downregulating NRF2/ARE pathway. (**A**) The Col-I and α-SMA expression comparison between saline, H_2O_2 and Cuiyuncao treated NRK-52E rat kidney cells. (**B**) NRF2 Chromatin immunoprecipitation followed by qPCR with IgG as negative control. (**C**) Luciferase reporter assay of antioxidant response element of α-SMA and Col-I. (**D**) The schematic mechanism by which Col-I and α-SMA are regulated by Cuiyuncao. Data are presented as means ± S.D. *P* < 0.05 and ** *P* < 0.01

In order to figure out if NRF2 binds to the antioxidant response elements of Col-I and α-SMA, we found all the potential AREs according to ARE consensus. The NRF2 ChIP was carried out and IgG ChIP without target as a negative control. The DNA recovered from ChIP was subjected to qPCR. One ARE of each of Col-I and α-SMA was validated (Fig. 4B). To confirm the two AREs validated by NRF2 ChIP, the AREs were cloned into pT81 luciferase plasmid whose luciferase activity is minimal due to the lack of a promoter of full function. Both Col-I ARE and α-SMA ARE can increase the expression of luciferase in response to oxidative stress when treated by H_2O_2 (Fig. 4C). The AREs of Col-I and α-SMA are CAATGGTCATGCTTA and TCAGTGTGACTTTAA respectively.

Discussion

The prevalence of chronic kidney disease (CKD) is on the rise globally as factors that contribute to the condition continue to increase (Lameire *et al.* 2005). Interstitial fibrosis is considered as the main route leading to CKD. Despite the increasing availability of crucial information regarding the mechanisms underlying CKD, current strategies for treating CKD are largely ineffective. Given this, there is a need to further our understanding of CKD, with special focus on the ways in which progression of renal fibrosis could be arrested. Herein, we revealed that Cuiyuncao aqueous extract exhibits significant effect on renal fibrosis.

The UUO rat acted as our experimental model of renal fibrosis and typical features were observed in UUO rats. Based on our results, both aberrant oxidative stress and renal fibrosis were triggered via obstructive nephropathy. It has been long known that excessive oxidative stress in cells and tissues causes severe damage and results in fibrosis in many organs. Although many studies suggest that the excessive oxidative stress associates with renal fibrosis, we provide the direct proof that the cells in kidneys undergoing fibrosis have much higher oxidative stress levels than in normal ones. The Cuiyuncao extract could reduce the oxidative stress in the kidneys undergoing fibrosis and at the same time alleviate the fibrosis. There is a strong correlation between oxidative stress reduction and fibrosis progress delay. However, the question if and how the oxidative stress causes renal fibrosis is still largely opens.

In response to obstructed renal injury, many factors, such as mechanical stretch from urine accumulation, angiotensin II, and inflammatory factors, together contribute to pathological development of kidney. Although inflammation plays a critical role in the fibrogenesis the final step of renal fibrosis is the overproduction and accumulation of ECM components in fibroblasts, such as COL-I and α-SMA. Among the potential therapeutic regimens inactivation of fibroblasts is one of the most promising strategies to treat renal fibrosis. Most of the previous studies on the mechanism of renal fibrosis focused dominantly on anti-inflammation. Our results demonstrated that Cuiyuncao treatment could ameliorate the renal fibrosis by decreasing the levels of the ECM components α-SMA and COL-I with little effect on TGF-β1.

Taken together, we showed that the traditional Chinese herbal medicine Cuiyuncao ameliorates the tubulointerstitial infiltration of inflammatory cells and interstitial fibrosis in kidney with UUO. Besides TGF-β1and ECM pathway, other signaling molecules were also involved in renal fibrosis, such as MAPK and Wnt pathway (Edeling *et al.* 2016). The more detailed mechanism of anti-fibrosis of Cuiyuncao need further investigation. Cuiyuncao aqueous extract contains many substances with broad bioactivities. It is worth for further research to identifying the active ingredient that exerts the real efficacy in the future work.

Conclusion

Traditional Chinese medicine Cuiyuncao significantly ameliorates renal fibrosis in rats. Cuiyuncao reverses both the elevated oxidative stress level and upregulation of key fibrosis factors Col-I and α-SMA in fibrotic kidneys, but not TGF-β1. Cuiyuncao antagonizes renal fibrosis *via* antioxidant pathway.

Acknowledgement

This study was supported by National Natural Science Foundation of China (No 81460649, No 81360662), the Medical Leading Talent Project of Yunnan Province (No L-201606) and the Joint Project of Science and Technology, Department of Yunnan and Traditional Chinese Medicine (No 2017FF116(-047)).

Author Contributions

Min Gao and Zhi-Wei Sun designed the study and prepared the manuscript, Kai Ni, Jian Wang and Jian Xu collected the data and interpreted the data, Yan-Ping Yang and Hua Xiao, Hong-Ling Yuan collected the funds.

References

Alharbi N, M Elobeid, P Virk (2019). Protective effect of quercetin treatment against cadmium-induced oxidative stress in a male rat model. *Pak J Zool* 51:2287–2296

Blecher-Gonen R, Z Barnett-Itzhaki, D Jaitin, D Amann-Zalcenstein, D Lara-Astiaso, I Amit (2013). High-throughput chromatin immunoprecipitation for genome-wide mapping of *in vivo* protein-DNA interactions and epigenomic states. *Nat Protoc* 8:539–554

Breyer MD, K Susztak (2016). The next generation of therapeutics for chronic kidney disease. *Nat Rev Drug Discov* 15:568–588

CDC CFDC (2007). Prevalence of chronic kidney disease and associated risk factors–United States, 1999–2004. *MMWR Morb Mortal Wkly Rep* 56:161–165

Clerk A, TJ Kemp, G Zoumpoulidou, PH Sugden (2007). Cardiac myocyte gene expression profiling during H_2O_2-induced apoptosis. *Physiol Genomics* 29:118–127

Eddy AA (2000). Molecular basis of renal fibrosis. *Pediatr Nephrol* 15:290–301

Edeling M, G Ragi, S Huang, H Pavenstadt, K Susztak (2016). Developmental signalling pathways in renal fibrosis: The roles of Notch, Wnt and Hedgehog. *Nat Rev Nephrol* 12:426–439

Jha V, G Garcia-Garcia, K Iseki, Z Li, S Naicker, B Plattner, R Saran, AY Wang, CW Yang (2013). Chronic kidney disease: Global dimension and perspectives. *Lancet* 382:260–272

Kang HM, SH Ahn, P Choi, YA Ko, SH Han, F Chinga, AS Park, J Tao, K Sharma, J Pullman, EP Bottinger, IJ Goldberg, K Susztak (2015). Defective fatty acid oxidation in renal tubular epithelial cells has a key role in kidney fibrosis development. *Nat Med* 21:37–46

Kim KK, D Sheppard, HA Chapman (2018). TGF-beta1 signaling and tissue fibrosis. *Cold Spr Harb Perspect Biol* 10; Article a022293

Lameire N, K Jager, WV Biesen, DD Bacquer, R Vanholder (2005). Chronic kidney disease: A European perspective. *Kidney Intl Suppl* 68:30–38

Lee J, I Hwang, JH Lee, HW Lee, LS Jeong, H Ha (2013). The selective A3AR antagonist LJ-1888 ameliorates UUO-induced tubulointerstitial fibrosis. *Amer J Pathol* 183:1488–1497

Liu Y (2006). Renal fibrosis: New insights into the pathogenesis and therapeutics. *Kidney Intl* 69:213–217

Lv W, GW Booz, F Fan, Y Wang, RJ Roman (2018). Oxidative stress and renal fibrosis: Recent insights for the development of novel therapeutic strategies. *Front Physiol* 9; Article 105

Ma LY, SC Ma, F Wei, RC Lin, PP But, SH Lee, SF Lee (2003). Uncinoside A and B, two new antiviral chromone glycosides from *Selaginella uncinata*. *Chem Pharm Bull* 51:1264–1267

Mulcahy RT, MA Wartman, HH Bailey, JJ Gipp (1997). Constitutive and beta-naphthoflavone-induced expression of the human gamma-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *J Biol Chem* 272:7445–7454

Ramachandran P, A Pellicoro, MA Vernon, L Boulter, RL Aucott, A Ali, SN Hartland, VK Snowdon, A Cappon, TT Gordon-Walker, MJ Williams, DR Dunbar, JR Manning, NV Rooijen, JA Fallowfield, SJ Forbes, JP Iredale (2012). Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci USA* 109:3186–3195

Sehirli AO, S Sayiner, A Velioglu-Ogunc, N Serakinci, E Eksioglu-Demiralp, B Yegen, F Ercan, G Sener (2020). The influence of N-acetylcysteine alone and in combination with angiotensin converting enzyme inhibitor and angiotensin receptor antagonist on systemic and tissue levels in rats with experimentally-induced chronic renal failure. *Pak J Zool* 52:1263–1274

Strutz F, M Zeisberg (2006). Renal fibroblasts and myofibroblasts in chronic kidney disease. *J Amer Soc Nephrol* 17:2992–2998

Tawfik SS, AA Elkady, ET Mohamed (2020) Protective effect of tamarind against renal injury induced by gamma rays. *Pak J Zool* 52:759–765

Tyner C, GP Barber, J Casper, H Clawson, M Diekhans, C Eisenhart, CM Fischer, D Gibson, JN Gonzalez, L Guruvadoo, M Haeussler, S Heitner, AS Hinrichs, D Karolchik, BT Lee, CM Lee, P Nejad, BJ Raney, KR Rosenbloom, ML Speir, C Villarreal, J Vivian, AS Zweig, D Haussler, RM Kuhn, WJ Kent (2017). The UCSC genome browser database: 2017 update. *Nucl Acids Res* 45:626–634

Williams JH, BL Mepham, DH Wright (1997). Tissue preparation for immunocytochemistry. *J Clin Pathol* 50:422–428

Xu B, YB Zhang, ZZ Li, MW Yang, S Wang, DP Jiang (2013). Hydrogen-rich saline ameliorates renal injury induced by unilateral ureteral obstruction in rats. *Intl Immunopharmacol* 17:447–452

Yu B, W Cai, HH Zhang, YS Zhong, J Fang, WY Zhang, L Mo, LC Wang, CH Yu (2017). *Selaginella uncinata* flavonoids ameliorated ovalbumin-induced airway inflammation in a rat model of asthma. *J Ethnopharmacol* 195:71–80

Zeisberg M, EG Neilson (2010). Mechanisms of tubulointerstitial fibrosis. *J Amer Soc Nephrol* 21:1819–1834

Zhang X, CK Xia, SS Li (2019). Efficacy of cortex mori-liuwei dihuang nanoparticle pills on renal interstitial fibrosis *via* TGF-beta/smad signaling. *Nanosci Nanotechnol Lett* 11:1661–1665

Zheng J, Y Zheng, H Zhi, Y Dai, N Wang, L Wu, M Fan, Y Fang, S Zhao, K Zhang (2013). Two new steroidal saponins from *Selaginella uncinata* (Desv.) Spring and their protective effect against anoxia. *Fitoterapia* 88:25–30

Zheng JX, Y Zheng, H Zhi, Y Dai, NL Wang, YX Fang, ZY Du, K Zhang, MM Li, LY Wu, M Fan (2011). New 3', 8"-linked biflavonoids from *Selaginella uncinata* displaying protective effect against anoxia. *Molecules* 16:6206–6214

Zhong Y, MC Menon, Y Deng, Y Chen, JC He (2015). Recent advances in traditional Chinese medicine for kidney disease. *Amer J Kidney Dis* 66:513–522

Zou H, ML Yi, KP Xu, XF Sheng, GS Tan (2016). Two new flavonoids from *Selaginella uncinata*. *J Asian Nat Prod Res* 18:248–252

Zou H, KP Xu, FS Li, ZX Zou, R Liu, RH Liu, J Li, LH Tan, GS Tan (2014). Unciflavones A-F, six novel flavonoids from *Selaginella uncinata* (Desv.) Spring. *Fitoterapia* 99:328–333