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Attenuation of Tubular Injury and Renal Fibrosis by TI-HU-YIN *via* Reduction in Transforming Growth Factor-β1 Expression in Unilateral Ureteral Obstruction Mice

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Abstract

TI-HU-YIN (JCKD), a compound composed of many Chinese herbs, is hypothesized to attenuate renal tubular injury and interstitial fibrosis. Moreover, its renoprotective effects were assessed in animal and *in vitro* studies. First, male C57BL/6 mice were under sham operation or unilateral ureteral obstruction (UUO) surgery, and then treated with phosphate buffer solution (PBS), aliskirin and valsartan (A+V), and JCKD for 14 days. At 7 and 14 days, mice were sacrificed and the kidney tissues were assessed for histopathological changes and transforming growth factor (TGF)-β1 expression. As compared to sham group, UUO-PBS group had more serious tubular dilatation and injury, α-smooth muscle actin-positive areas, F4/80-positive macrophages, and interstitial fibrosis. Impressively, these pathologic changes were significantly attenuated in UUO mice both treated with JCKD and A+V as compared to UUO-PBS group. At 14 days, TGF-β1 expression was significantly suppressed in kidney tissues of UUO-JCKD group as well as in UUO-A+V group. Second, TGF-β1 production was increased in macrophage J774 cells and NRK-52E proximal tubular cells stimulated by angiotensin (Ang)-II at 10 nM for 24 h and at 1 nM for 48 h, respectively. JCKD (≥ 400 μg/ml) inhibited the TGF-β1 production at baseline and stimulated by Ang II in both cell lines. Our study showed that JCKD reduced renal injury, macrophage infiltration and interstitial fibrosis possibly through suppressing the TGF-β1 expression in UUO mice. Accordingly, JCKD is potential to retard the progression of chronic kidney disease. Further studies are needed to validate its renoprotective effects in the inhibition of TGF-β1 expression and the amelioration of renal fibrosis.

Key Words: angiotensin II, Chinese herbs, kidney injury, renal fibrosis, transforming growth factor-β1, unilateral ureteral obstruction

Introduction

Renal fibrosis is the common pathway of progressive kidney disease. Identification of factors involved in renal fibrosis is important to prevent and treat kidney disease. Transforming growth factor (TGF)-β1 is produced by infiltrated macrophages, myofibroblasts and tubular epithelial cells in unilateral ureteral

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obstruction (UUO) mouse model (4, 16, 17, 19). TGF-β1 promoted the tubular epithelial cells becoming fibroblast through epithelial-mesenchymal transition (EMT) with reduction of E-cadherin and increase of α-smooth muscle actin (α-SMA) expression. Myofibroblast activation increased extracellular matrix (ECM) accumulation in renal interstitium, leading to disruption of tissue architecture and loss of renal function (4, 15, 23).

Cumulative evidence has shown that angiotensin (Ang) II could stimulate TGF-β1 expression (10, 28). In cultured vascular smooth muscle cells, Ang II not only stimulated TGF-β1 transcription but also promoted its conversion to the biologically active form (10). In kidneys, Ang II promoted TGF-β1 expression and upregulated TGF-β1 receptor. UUO mouse model is one of the comprehensively study models to link between renin-angiotensin-aldosterone system (RAAS) and TGF-β1 (4, 16, 17, 19, 28). Ang II increased by 60- to 100-fold in plasma concentration during UUO, and contributed to upregulation of profibrotic factors, induction of EMT and accumulation of ECM. Besides, Ang II together with renin or aldosterone also activated TGF-β1 axis in UUO mouse model through direct and indirect mechanisms (5, 22, 24, 26).

Each RAAS blockade has significantly, but not completely, blocked renal fibrosis (6, 9, 30). In contrast to conventional RAAS blockades, aliskiren blocks the renin system by directly inhibiting plasma renin activity and preventing the formation of Ang II (9, 30). Thus, appropriate combination of RAAS inhibitors may have a better effect on renal fibrosis theoretically (6, 9, 30). Previous studies showed that some traditional Chinese medicines have therapeutic benefits in kidney disease. *Corni Fructus* (Cornaceae), a crude herb, had hypoglycemic, antineoplastic and antimicrobial effects, and improved kidney function (32). Treatment with Iridoid glycosides of *Paederia scandens* (IGPS), an active component isolated from *P. scandens* (*LOUR) MERRILL* (*Rubiaceae*), significantly attenuated kidney injury (7). *Dioscorea alata* (DA) extract caused a decrease in α-SMA and MMP-2 levels, and an increase in E-cadherin expression in kidney tissues (14). A Ginger extraction exhibited protective effects against renal complications in streptozotocin-induced diabetic rats (3). Moreover, Shugan-Huayu powder, a traditional Chinese medicine, could prevent hepatic fibrosis in rat model (21).

JCKD (TI-HU-YIN; Healthy-Kidneys Enterprise Co. Ltd., Taiwan), a compound composed of many Chinese herbs, was used in an uncontrolled study, showing that estimated glomerular filtration rate (GFR) was improved from 18 to 48 ml/min/1.73 m^2 in 60 chronic kidney disease (CKD) patients following 6 months of JCKD treatment. Accordingly, in the present study, we aimed to validate whether JCKD could attenuate renal fibrosis in UUO mouse model, and down-regulate the TGF-β1 expression in UUO kidney tissues. We further explored whether TGF-β1 could be down-regulated by JCKD in macrophages and proximal tubular epithelial cells *in vitro*.

Materials and Methods

Ethical Approval

All procedures were approved by the ethics committee of Institutional Animal Care and Use Committee of National Yang-Ming University, Taipei, Taiwan.

Study Protocols

Male C57BL/6 mice, aged 8 to 10 weeks old and weighted 20-25 g, were purchased from BioLASCO Taiwan. Mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body wt) before UUO surgery. Left kidney and ureter were dissected, then completely ligated at the proximal ureter and placed back inside the body carefully. UUO mice were kept warm under a lamp and recovered in the cages. For sham-operated group, mice underwent the same surgery but ureter was not ligated. Mice were sacrificed at 7 and 14 days after ureteral ligation, and the obstructed kidney tissues were preserved in 4% paraformaldehyde or in liquid nitrogen for further examination. Mice were randomly divided into four treatment groups as follows: [1] sham; [2] UUO + phosphate buffer solution (PBS); [3] UUO + aliskiren and valsartan (A+V); [4] UUO + JCKD. Mice in the latter two groups were fed by oral gavage with aliskiren (6 mg/kg) combined with valsartan (10 mg/kg) or JCKD (9.375 g/kg) per day, respectively. Four study groups containing 5-8 mice were performed for 14 days.

Histology, Tubular Injury and Interstitial Fibrosis Score

Kidneys were embedded in paraffin wax as described previously (27). The sections were then stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS) reagent (Sigma-Aldrich, MO, USA), and Masson's trichrome (Sigma-Aldrich, MO, USA) stain. Tubular injury was defined as renal tubular dilation, brush border loss and tubular epithelial cell necrosis or loss. Convert the tubular dilation score which was determined by the number of points overlying dilated tubular spaces to a percentage (30). The tubular injury score was graded in each PAS-stained section from 0 to 4 (0, no change; 1, changes affecting < 25%; 2, changes affecting 25 to < 50%; 3, changes affecting 50 to < 75%; 4, changes affecting 75-100% of the sections) (2). The interstitial fibrosis score was graded in each Masson's trichrome-stained section from 0 to 4 (0, no staining;

1, < 25% staining; 2, 25 to <50% staining; 3, 50 to < 75% staining; 4, 75 to 100% staining) (18). Twenty randomly selected cortical fields per mouse were assessed at a magnification of ×400.

Immunohistochemistry

Immunohistochemical staining was performed as described previously (11). Sections were incubated with primary antibodies for α-SMA and TGF-β1 (Abcam, USA) or F4/80 (eBioscience, San Diego, USA), respectively. Afterwards, sections were incubated with the Envision avidin–biotin-free horseradish peroxidase (HRP)-labeled polymer (Dako Cytomation, Glostrup, Denmark). α-SMA–positive area and macrophages by counting F4/80–positive cells were assessed in 20 randomly chosen cortical fields at a magnification of ×400 for each section.

Ang II−Stimulated J774 and NRK-52E Cell Lines

J774 mouse macrophage cell line is a kind gift from Dr. Nien-Jung Chen, Institute of Microbiology and Immunology, National Yang-Ming University, Taiwan. NRK-52E rat proximal tubular epithelial cell line was purchased from American Type Culture Collection (ATCC). The 2×10^6 cells were seeded into 15 cm cell culture dish containing 20 ml medium.

For Ang II stimulation, J774 or NRK-52E cells were seeded in 24 wells culture dish initially. After overnight culture, the cells were washed by 0.5 ml Dulbecco's phosphate buffered saline (DPBS) once and replaced with 0.5 ml serum free DMEM at day 1. Then the cells were washed once by DPBS and replaced with JCKD containing serum free DMEM. After 1 h culture, the cells were stimulated with 1 or 10 nM Ang II at day 2. After 24 or 48 h culture, the supernatant was collected and stored at −20°C for further measurement.

JCKD (Healthy-Kidney Enterprise Co. Ltd., Taiwan) is a compound containing *Poria cocos* (Schw.) *Wolf*., *Glycyrrhiza uralensis* Fisch., *Crassostrea gigas*, *Dioscorea opposita* Thunb, *Lablab vulgaris Savi*, *Imperata apillaries* Beauvois var. *Koenigii* Durant et Schinz, *Lilium lancifolium* Thunb, *Citrus medica* var. *sarcodactylis* (Noot.) Swingle, *Rubus idaeus L*., *Gallus gallus domesticus Brisson*, *Crataegus pinnatifida* Bunge, *Folium Mori*, *Zaocys dhumnades* Cantor, *Euryale ferox* Salisb, *Zingiber zerumbet* Smith, and *Ziziphus jujuba* Mill. var. *inermis* (Bge.) Rehd.

TGF-β1 Enzyme-Linked Immunosorbent Assay (ELISA)

Protein concentrations of TGF-β1 were determined by the ELISA (R&D System; Minneapolis, IN, USA) for kidney tissues and cell culture supernatants, respectively.

Cell Proliferation Assay

For measurement of cell proliferation, thiazolyl blue tetrazolium bromide (MTT) solution was added to the cultured cells. After incubation for 1 h, the supernatant was discarded after centrifugation and the cells were lysed with DMSO. MTT value was determined at OD 540 nm.

Statistical Analysis

Data were presented by means ± standard errors of means (SEM). Statistical analysis was performed using unpaired Student's *t*-test between two-group comparisons. For comparison of three or more groups, we used one-way analysis of variance (ANOVA), followed by Duncan's multiple-comparison *post-hoc* test. Statistics were analysed by the computer software Statistical Package of Social Science (version 16.0; SPSS, Chicago, IL, USA). A P-value < 0.05 was considered statistically significant.

Results

Previous reports showed that A+V treatment reduced UUO-induced renal fibrosis. Hence, UUO mice fed with PBS served as negative control and with A+V as positive control to identify the therapeutic effect of JCKD in renal injury and fibrosis of UUO mice. Grossly, the UUO-PBS kidneys showed profound pelvis dilatation and renal parenchyma reduction compared to sham-operated kidney on day 7 and day 14 after UUO (Fig. 1). Treatment with A+V or JCKD restored renal parenchyma loss compared to PBS group after UUO.

JCKD Attenuated Renal Injury and Fibrosis in UUO Mouse

The obstructed kidneys from mice treated with PBS showed more severe tubular dilatation (H&E stain), tubular injury (PAS stain), and interstitial fibrosis (Masson's trichrome stain) than those with sham operation group on day 7 and day 14 after UUO. Administration of A+V or JCKD significantly attenuated the tubular dilation (Figs. 2 and 3A), tubular injury (Figs. 2 and 3B), and interstitial fibrosis (Figs. 2 and 3C) as compared with those treated with PBS for 2 weeks.

JCKD Reduced Macrophage Infiltration in UUO Kidneys

Immunohistochemical staining showed that the obstructed kidneys from mice treated with PBS had more α-SMA expression in myofibroblasts and

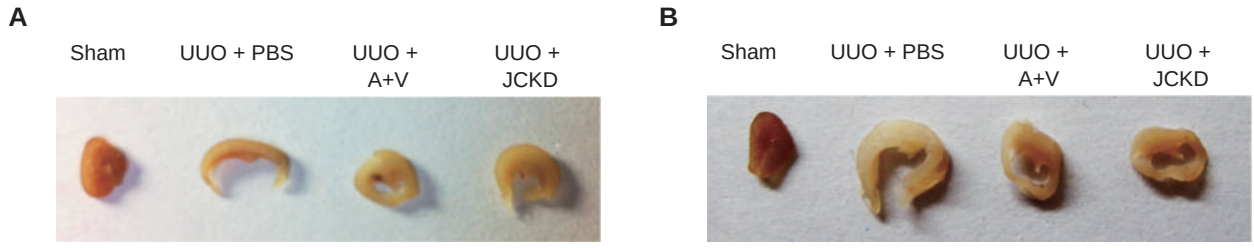

Fig. 1. The morphological change of kidneys from mice receiving sham-operation and unilateral ureteral obstruction (UUO) treated with PBS, A+V, and JCKD for 7 days (A) and 14 days (B), respectively. The kidneys were cross-sectioned for histological analysis. A+V, aliskiren and valsartan; PBS, phosphate buffer solution.

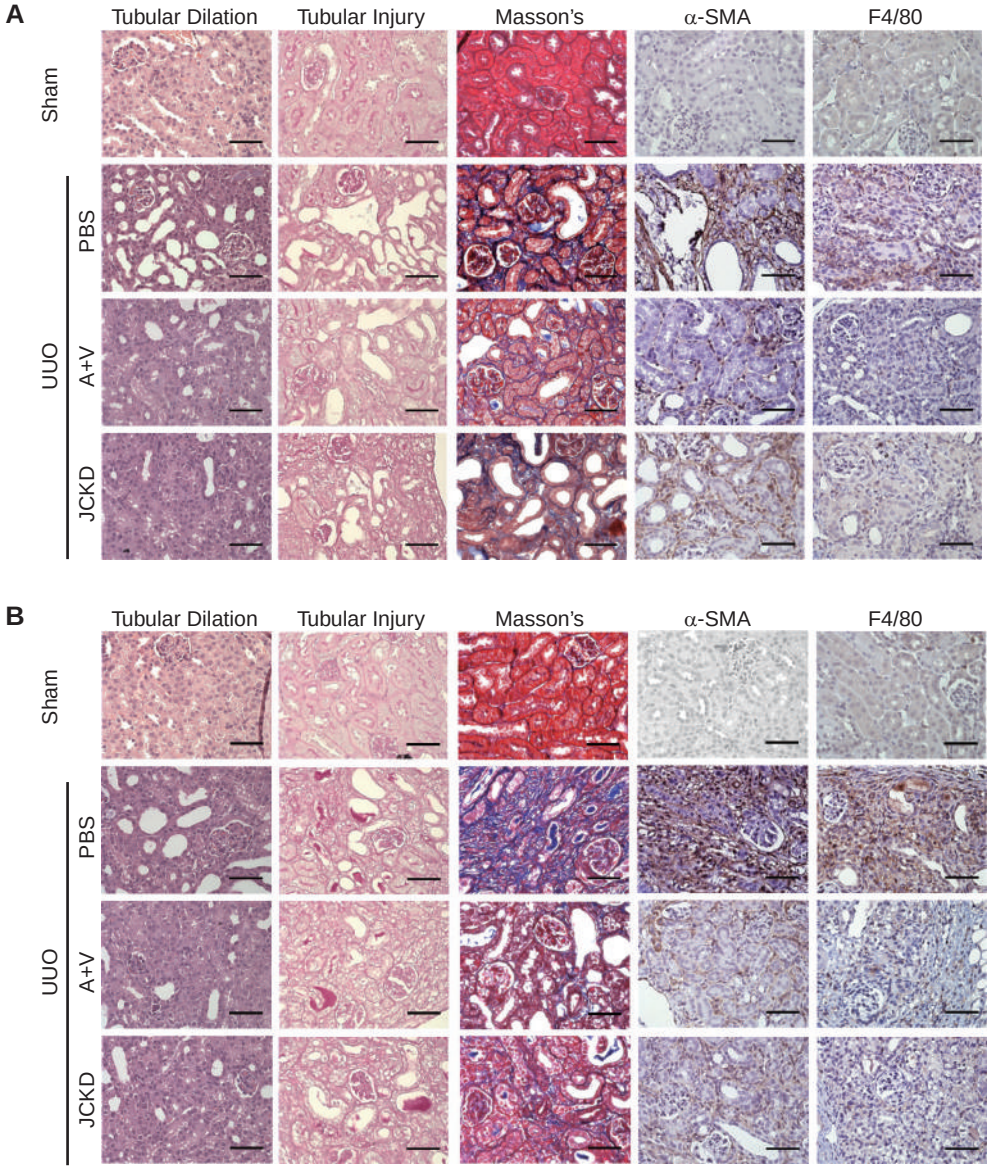

Fig. 2. Histopathology and immunohistochemistry in kidney tissues of mice receiving sham-operation and UUO. PBS, A+V and JCKD were administered *via* oral gavage to UUO mice for 7 days (A) and 14 days (B). The kidney tissue sections were stained by H&E stain for tubular dilatation stain, PAS stain for tubular injury, Masson's trichrom-stain for interstitial fibrosis, as well as immunohistochemical stain for α-SMA-positive areas and F4/80-positive macrophages, respectively. Scale bar: 50 mm. A+V, aliskiren and valsartan; PBS, phosphate buffer solution; SMA, smooth muscle actin; UUO, unilateral ureteral obstruction.

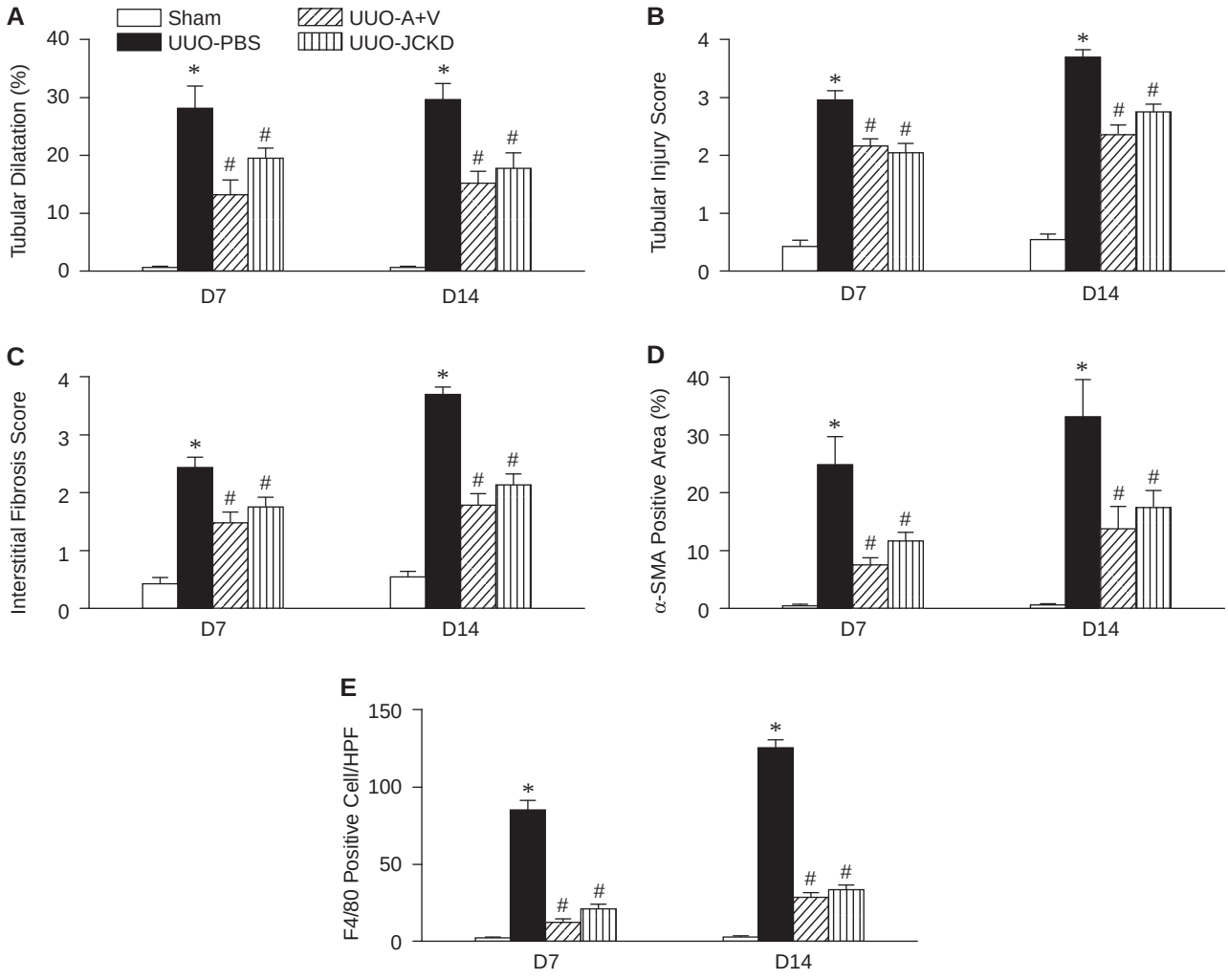

Fig. 3. Quantitative analyses of tubular dilatation (A) and injury (B), interstitial fibrosis (C), α-SMA-positive area (D), and F4/80-positive cell in kidney tissues of mice receiving sham-operation and UUO. PBS, A+V and JCKD were administered *via* oral gavage to UUO mice for 7 days or 14 days. The number of mice was 5 to 8 in each group. Data were presented by means ± standard errors of the means (SEM). $^{*}P < 0.05$ compared to sham group; $^{\#}P < 0.05$ compared to UUO-PBS group. A+V, aliskiren and valsartan; PBS, phosphate buffer solution; SMA, smooth muscle actin; UUO, unilateral ureteral obstruction.

F4/80−positive macrophages than those with sham operation group on day 7 and day 14 after UUO. Administration of A+V or JCKD significantly reduced α-SMA−positive area (Figs. 2 and 3D) and F4/80−positive macrophages (Figs. 2 and 3E) as compared with those treated with PBS for 2 weeks.

JCKD Down-Regulated TGF-β1 Expression in UUO Kidney Tissues

Previous studies showed TGF-β1 plays a central role in modulating fibrosis. Hence, we measured TGF-β1 protein level in obstructed kidney tissues with different treatment. On day 7 and 14, TGF-β1 levels markedly increased in UUO-PBS group as compared to sham operation group (Fig. 4). On day 7 after UUO, TGF-β1 levels significantly reduced in UUO-A+V group as compared to UUO-PBS group (Fig. 4A). On day 14, TGF-β1 levels in UUO mice treated with A+V and JCKD were significantly lower than that in UUO-PBS group (Fig. 4B). The findings were consistent with the decreased expression of TGF-β1 in tubular epithelial cells and interstitial monocytes by IHC staining in UUO mice treated with A+V and JCKD for 14 days (Fig. 5A).

JCKD Inhibited TGF-β1 Production in Macrophages and Tubular Cells

During RAAS system activation, Ang II stimulated tubular epithelial cells and macrophages through AT1 receptor (AT1R) to activated NF-κB and lead to TGF-β1 production. Accordingly, we tested whether JCKD could inhibit TGF-β1 expression through inter-

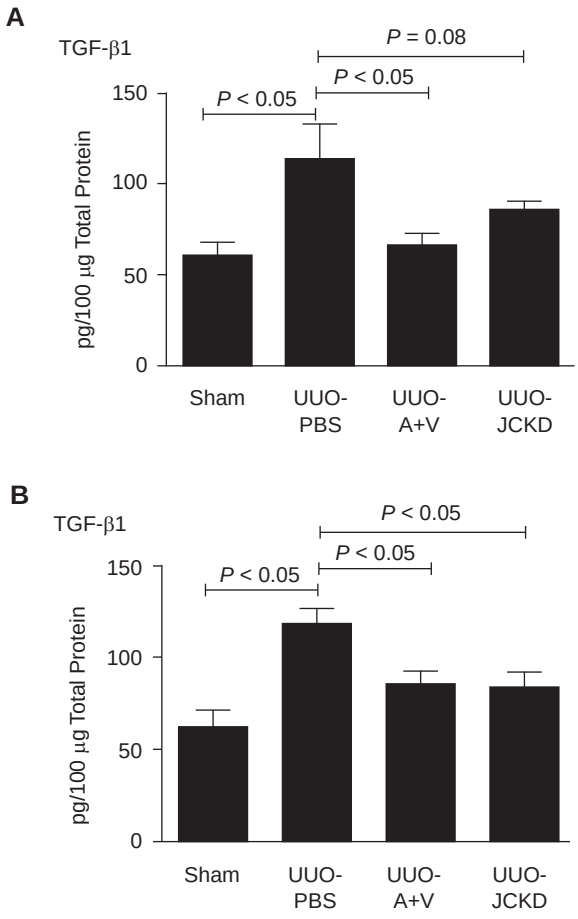

Fig. 4. TGF-β1 expression in kidney tissues of sham-operated and UUO mice receiving PBS, A+V and JCKD. Kidney tissues were homogenized in each group (n = 5-8) and TGF-β1 protein level was determined by ELISA at 7 days (A) and 14 days (B). A+V, aliskiren and valsartan; PBS, phosphate buffer solution; UUO, unilateral ureteral obstruction.

fering Ang II-AT1R axis. First, J774 cells were stimulated with Ang II at concentrations from 1 nM to 10 μM for 24 h. The results showed that there was no difference in cell proliferation, and Ang II at 10 nM increased TGF-β1 levels compared with control group (Fig. 6). To identify the inhibitory effect of JCKD on TGF-β1 production, J774 cells were treated with 0 to 1000 μg/ml of JCKD alone (Fig. 7A), or pretreated with JCKD for 1 h and then treated with Ang II at 10 nM for 24 h (Fig. 7B). Our data showed that JCKD (≥400 μg/ml) markedly reduced TGF-β1 expression at baseline (Fig. 7C) and stimulated by Ang II for 24 h (Fig. 7D).

Second, NRK-52E cells were stimulated with Ang II from 1 nM to 10 μM for 24 h or 48 h. Following 24 h of Ang II stimulation, there was no significance in cell proliferation and TGF-β1 expression (Fig. 8). Following 48 h, Ang II at 1 nM concentration signifi-

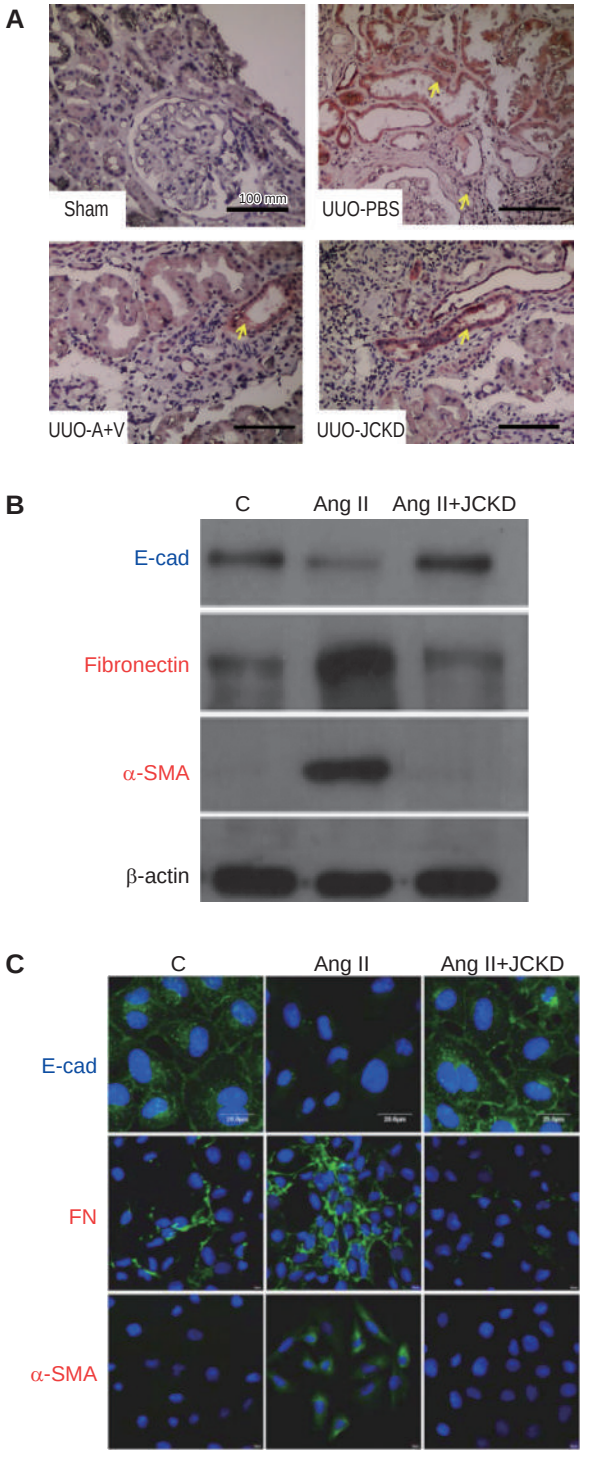

Fig. 5. TGF-β1 expression by immunohistochemical staining in kidney tissues. UUO mice were treated with PBS, A+V and JCKD for 14 days (A). The TGF-β1 expression in tubular epithelial and interstitial monocytes was indicated by yellow arrows. The expressions of E-cadherin (E-cad), fibronectin (FN) and α-smooth muscle actin (α-SMA) in NRK-52E cells upon angiotensin II (Ang II) stimulation or pretreated with JCKD (400 μg/ml) for 1 h and then treated with Ang II at 1 nM for 48 h by Western blotting (B) and immunoflorescent confocal microscopy (C), respectively.

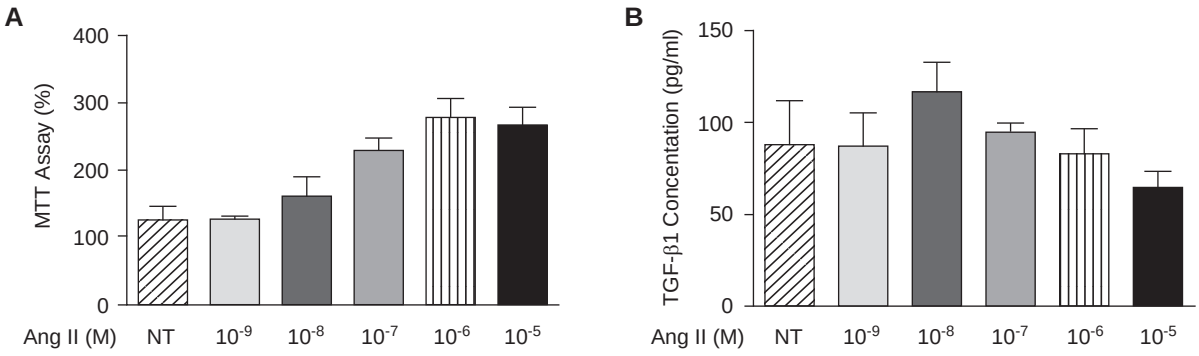

Fig. 6. TGF-β1 synthesis in response to Angiotensin II (Ang II) stimulation in J774 cells. The J774 cells were starved in serum free medium for 16 h and stimulated with different concentrations of Ang II for 24 h. Cell viability of Ang II-stimulated J774 cells was detected by MTT assay (A). The supernatants were collected for determination of TGF-β1 protein levels by ELISA (A). Results are representative of three independent experiments.

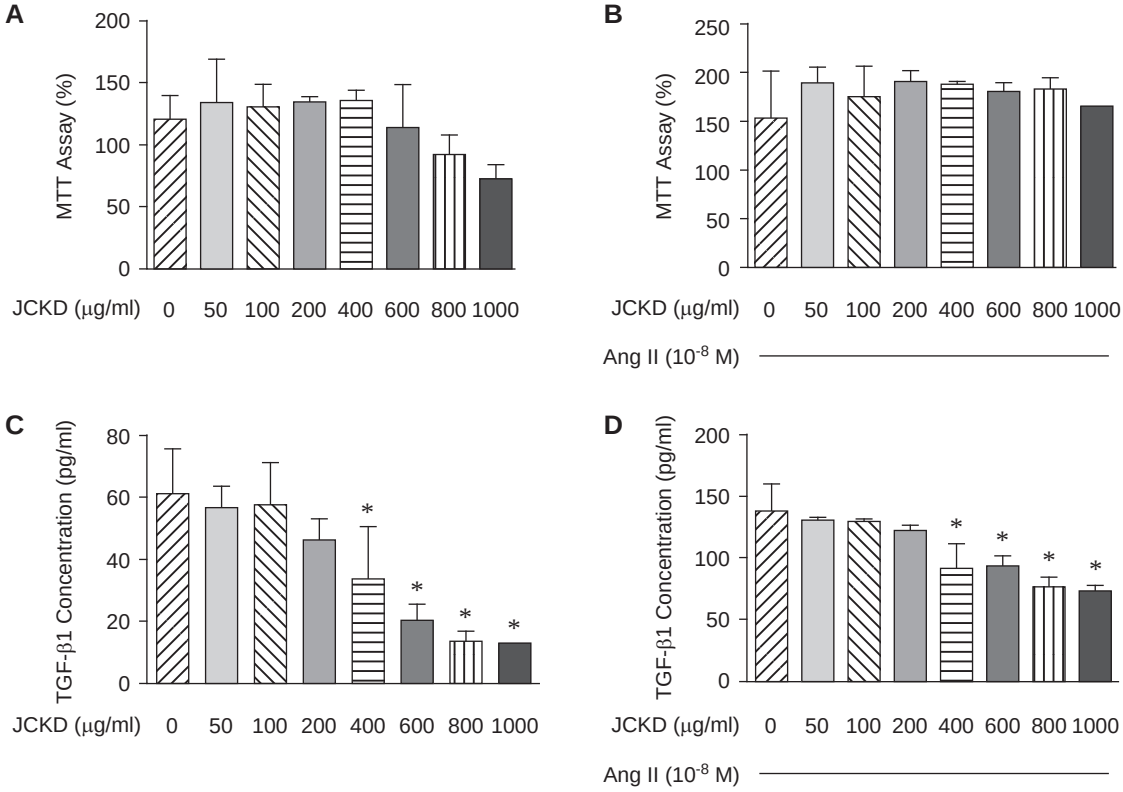

Fig. 7. JCKD inhibited TGF-β1 production in J774 cells. The J774 cells were starved in serum free medium for 16 h and co-cultured with JCKD alone (A & C) or JCKD plus 10 nM of Ang II for 24 h (B & D). Cell viability of stimulated J774 cells was detected by MTT assay. The supernatant was collected and TGF-β1 protein level was determined by ELISA. Results are representative of three independent experiments. *$P < 0.05$ compared to JCKD at 0 mg/ml.

cantly increased TGF-β1 expression but cell proliferation showed no significant difference (Fig. 8). NRK-52E cells were treated with JCKD (0 μg/ml to 1000 μg/ml) alone (Fig. 9A), or pretreated with JCKD for 1 h and then treated with Ang II at 1 nM for 48 h (Fig. 9B). Our data showed that JCKD (≥ 400 μg/ml) markedly reduced TGF-β1 expression at baseline (Fig. 9C) or stimulated with Ang II (Fig. 9D).

Third, following Ang II (1 nM) stimulation for 48 h, the E-cadherin expression was down-regulated and the expressions of fibronectin (FN) and α-SMA were up-regulated in NRK-52E cells by Western blotting (Fig. 5B). The EMT transformation was converted in NRK-52E cells pretreated with JCKD (400 μg/ml) for 1 h and then treated with Ang II for 48 h. These findings were further validated by immuno-

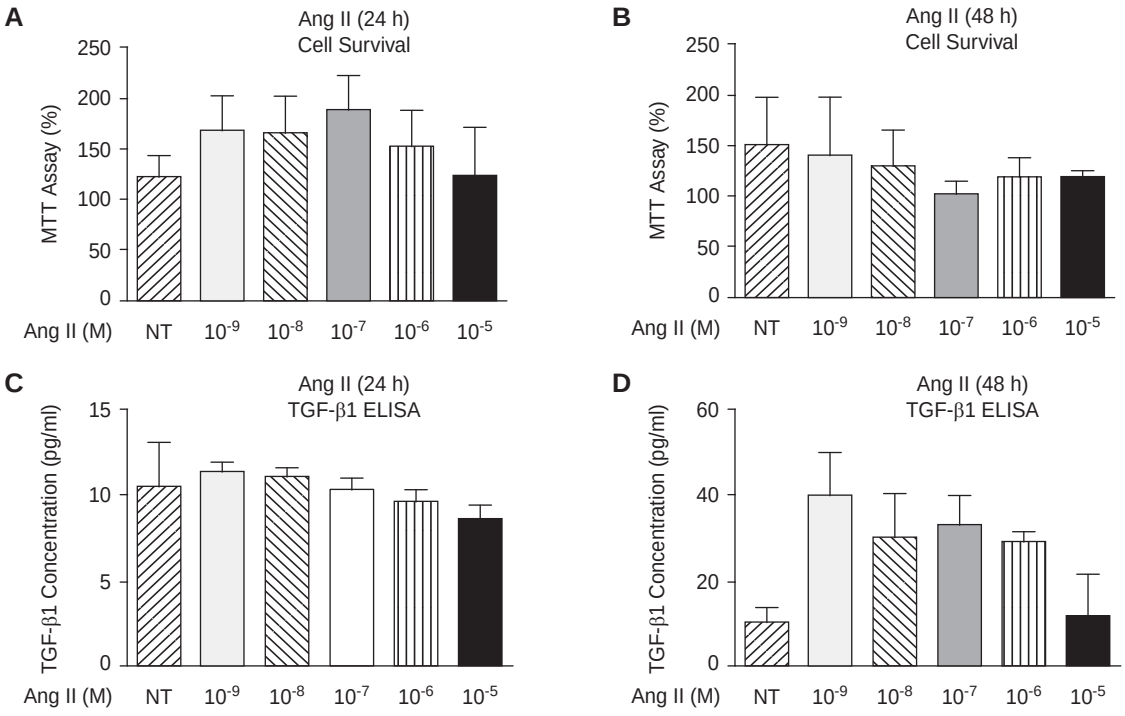

Fig. 8. TGF-β1 synthesis in response to Angiotensin II (Ang II) stimulation in NRK-52E cells. The NRK-52E cells were starved in serum free medium for 16 h and stimulated with different concentrations of Ang II for 24 or 48 h. Cell viability of Ang II-stimulated NRK-52E cells was detected by MTT assay at 24 h (A) or 48 h (B). The supernatants were collected for determination of TGF-β1 protein levels by ELISA at 24 h (C) or 48 h (D). Results are representative of three independent experiments.

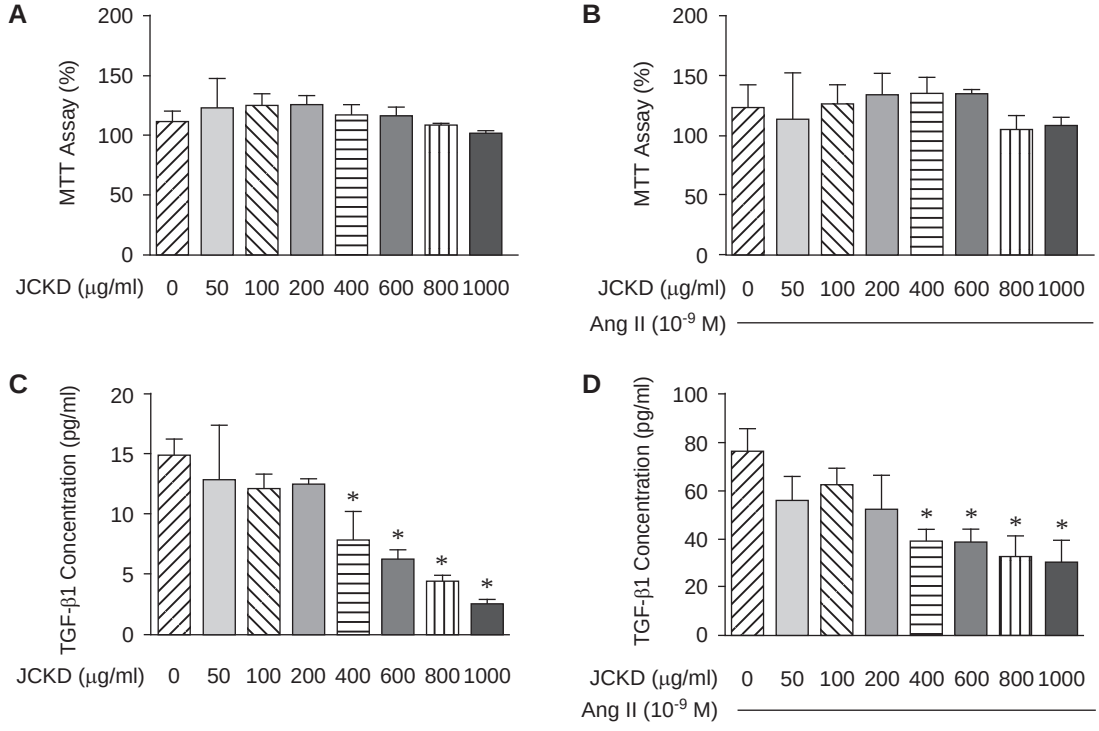

Fig. 9. JCKD inhibited TGF-β1 production in NRK-52E cell lines. The NRK-52E cells were starved in serum free medium for 16 h and co-cultured with JCKD alone (A & C) or JCKD plus 1 nM of Ang II for 48 h (B & D). Cell viability of stimulated NRK-52E cells was detected by MTT assay. The supernatant was collected and TGF-β1 protein level was determined by ELISA. Results are three independent experiments. $*P < 0.05$ compared to JCKD at 0 mg/ml.

florescent confocal microscopy (Fig. 5C) showing consistency.

Discussion

CKD is a global public health issue and is highly prevalent in Taiwan (29, 31). The causes of CKD include diabetes, hypertension, aging, cardiovascular disease, drug abuse and genetic disorder (13). Activation of RAAS plays a pivotal role in renal fibrosis and CKD progression. During RAAS activation, Ang II is produced from Ang I by angiotensin-converting enzyme (28, 33). Ang II binds to AT1 receptor (AT1R) and activates downstream signaling through MAP kinase such as ERK, p38 and JNK, to up-regulate the TGF-β1 expression (28). Wu *et al.* (30) demonstrated that block of RAAS through combined treatment with A+V attenuated renal tubular injury and interstitial fibrosis in mouse UUO kidneys. In the present study, we for the first time found that administration of JCKD also ameliorated tubular injury, in terms of tubular dilatation, tubular cell vacuolization and detachment, and brush border lost, as well as diminished the interstitial widened space and fibrosis score in UUO mice at 7 and 14 days. Macrophage infiltration is the inflammatory hallmark for renal injury. Both A+V and JCKD treatment could equally reduced myofibroblast formation and F4/80−positive macrophage infiltration in renal cortex for 2 weeks.

RAAS is one of the most complex physiological systems (10, 28). It regulates body's blood pressure and fluid balance, which influence systemic vascular resistance and blood volume. The kidneys and RAAS are almost responsible for long-term blood pressure adjustments. Ang II is a potent vasoactive peptide to increase systemic blood pressure directly. It also maintains the intra-glomerular pressure and keeps normal glomerular perfusion and filtration in the kidneys. However, unremitting kidney injury also induces the RAAS activation. The progression of kidney disease to end-stage renal failure is driven in part by Ang II. Relationship between Ang II and TGF-β1 has been well known in the UUO mouse model (4, 16, 17, 19, 27). Ang II in plasma increased to 60 to 100-fold during UUO, and then upregulated the TGF-β1 expression (10). TGF-β1 in turn promoted renal fibrosis through activation of fibroblasts and ECM deposition in interstitium (5, 22, 24, 28). Intriguingly, in our study, TGF-β1 expression was significantly attenuated in kidney tissues of UUO mice treated with JCKD for 14 days as compared to those treated with PBS.

The blockade of RAAS through an ACE inhibitor, Ang II receptor blocker (ARB), or direct renin inhibitor (DRI), is proven to be effective in the treatment of hypertension, heart failure, as well as diabetic and non-diabetic kidney disease (10, 24). In contrast to conventional RAAS blockades, aliskiren directly inhibits plasma renin activity and prevents the formation of both Ang I and Ang II as shown by basic and clinical studies (9, 30). Recent clinical evidence showed that the addition of aliskiren to valsartan in patients with hypertension more effectively reduced blood pressure than either agent alone (6). Accordingly, our study also showed that administration of A+V to mice with UUO significantly reduced the TGF-β1 expression in kidney tissues on day 7 and day 14, respectively.

During RAAS activation, Ang II stimulated tubular epithelial cells and macrophages through AT1R to activate NF-κB and subsequent expression of TGF-β1 (26). Infiltrated macrophages and tubular epithelial cells were the major sources of TGF-β1 production in kidney injury animal model (28). Due to the central role of Ang II in TGF-β1 expression, we further assessed whether JCKD could inhibit TGF-β1 expression in macrophage and tubular epithelial cell lines *in vitro*. Our data showed that JCKD (≥ 400 μg/ml) markedly reduced the basal TGF-β1 expression and further inhibited the TGF-β1 expression stimulated with Ang II in J774 and NRK-52E cells. Further identifying the compounds of JCKD in HPLC showed no any RAAS blockade (data not published). Therefore, we hypothesized that the mechanisms of JCKD in suppressing the basal and Ang II-stimulated TGF-β1 expression might not be through interference with Ang II-AT1R interaction.

Approximately 50-70% of all protein-coding genes might be controlled by microRNAs (miRNAs). MiRNAs are approximately 19-25 nuceotides long, and noncoding RNA molecules that post-transcriptionally regulate gene expression *via* RNA interference by binding either to the 3'-UTR or 5'-UTR or the coding sequence of protein-encoding mRNAs (8, 12, 20). Recent studies showed that miRNAs influenced the TGF-β1 pathways at multiple levels (1). MiR-141 and miR-200a directly inhibited TGF-β2 in rat NRK-52E cells and their down-regulation might be responsible for the development and progression of TGF-β-dependent EMT and fibrosis (25). Therefore, further studies are needed to validate the possible role of JCKD in the interaction between TGF-β1 signaling and miRNAs.

Acknowledgments

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