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# Tanshinone IIA attenuates renal fibrosis and inflammation via altering expression of TGF-β/Smad and NF-κB signaling pathway in 5/6 nephrectomized rats

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ABSTRACT

*Purpose:* In traditional Chinese medicine, Tanshinone IIA is used to treat chronic kidney disease (CKD). However, its biological activity and mechanism of action in renal fibrosis and inflammation are not fully identified. The current study was conducted to determine the effects of Tanshinone IIA treatment on CKD by assessing potential modulation of the TGF-β/Smad and NF-κB signaling pathway.

*Methods:* CKD was produced in rats by 5/6 nephrectomy. They were then divided into the following groups: control (sham operation); CKD (5/6 nephrectomy); 5/6 nephrectomy + Tanshinone IIA (10 mg/kg in average, once a day for 16 weeks). Serum and urine samples were obtained from animals in each group, and serum creatinine (Scr), blood urea nitrogen (BUN) levels and 24 h urinary protein excretion were measured. Tissue samples from the kidney were used for morphometric studies (Masson's trichrome). The expression of fibronectin protein and collagen types I, III, IV, and TGF-β, TNF-α, CXCL-1, MCP-1, RANTES mRNA were evaluated using immunohistochemistry and RT-PCR analysis; the TGF-β/Smad and NF-κB signaling pathway was detected by immunohistochemistry and Western blot analysis.

*Results:* The following effects were observed in CKD rats treated with Tanshinone IIA: (1) marked improvements in Scr, and 24 h urine protein excretion; (2) significant reductions in protein and mRNA levels of fibronectin, collagen III, and collagen IV and TNF-α, MCP-1, and CXCL-1; (3) significantly inhibited the TGF-β/Smad and NF-κB signaling activation.

*Conclusions:* These results suggest that Tanshinone IIA suppresses renal fibrosis and inflammation via altering expression of TGF-β/Smad and NF-κB pathway in the remnant kidney, thus supporting the potential of Tanshinone IIA as a new therapeutic agent for slowing the progression of CKD.



## 1. Introduction

Chronic kidney disease (CKD) is a major cause of morbidity, recurrent hospitalization and accelerated death, affecting 10–11% of the population in both Europe and the United States [1]. In a substantial proportion of such patients, deteriorating kidney function leads to the development of end-stage kidney disease (ESKD), requiring dialysis or transplantation to preserve life. Studies conducted almost 20 years ago highlighted the importance of blood pressure control and blockade of the renin–angiotensin system in attenuating the progression of CKD towards its end stage [2]. Unfortunately, while substantial progress has been made in our understanding of renal pathophysiology, there has been little in the way of new therapies since that time. Thus, additional intervention, including treatment with novel therapeutic compounds from natural products, may be effective for the treatment of CKD.

Tanshinone IIA, the rhizome of Salvia miltiorrhiza (Danshen), is the most abundant diterpene quinone isolated from Danshen, which has been used in treating CKD for more than 2000 years in China. The two active hydrophilic components of Danshen are danshensu and magnesium tanshinoate B, whereas cryptotanshinone and Tanshinone IIA are the two lipophilic components [3]. Tanshinone IIA has been shown to attenuate oxidative stress injury and decrease endoplasmic reticulum stress-mediated apoptosis in rat kidneys during hypothermic preservation [4]. It has been reported that Tanshinone IIA induces vasodilation and reduces blood pressure via endothelial nitric oxide synthase stimulation in renovascular hypertension model hamsters, and that Tanshinone IIA has renoprotective effects on the progression of diabetic nephropathy [5,6]. Recently, it has been reported that Tanshinone IIA

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attenuates the structural manifestations of renal disease progression and ameliorates the effects of renal dysfunction in CKD rats [7], but the mechanism of its desirable effects on renal fibrosis remains unclear.

Matrix deposition and inflammatory cell infiltration within the glomerulus and interstitium accompanied with a loss of functioning nephrons are common pathologic features of progressive kidney diseases [8,9]. Transforming growth factor-β1 (TGF-β1) has been recognized as a powerful multifunctional cytokine that plays roles in cell proliferation, differentiation, migration, immunomodulation, and extracellular matrix (ECM) turnover in the kidney [8]. Increasing evidence suggests that dysregulation of TGF-β1 may be a pathogenic mechanism in the progression of CKD [10]. TGF-β1 exerts its biological and pathological activities via Smad-dependent and -independent signaling pathways. Of them, the Smad-dependent mechanism has been well studied and considered to be a major pathway in many pathophysiological processes associated with TGF-β1. The binding of TGF-β1 to its receptor II (TβRII) activates the TGF-β receptor type I (TβRI) kinase. Then TβRI phosphorylates Smad2 and Smad3. Subsequently, phosphorylated Smad2 and Smad3 bind to Smad4, the common Smad, and form the Smad complex. This complex then translocates into the nucleus to regulate the target gene transcription, including Smad7. Smad7 is an inhibitory Smad that negatively regulates Smad2 and Smad3 activation and functions. It is now well accepted TGF-β/Smad signaling is a major pathway for renal fibrosis [10]. In the context of renal fibrosis, Smad2 and Smad3 are strongly activated in experimental and human kidney diseases, including diabetic nephropathy, obstructive kidney diseases, 5/6 nephrectomy, and hypertensive nephropathy [11–14].

The NF-κB family comprises five members, including relA (p65), relB, c-Rel, p105/50, and p100/52, which associate as homo- or heterodimers and are regulated by diverse transduction cascades [15]. The subcellular location of NF-κB is controlled by a family of inhibitory proteins, IκB proteins (IκBs), such as IκBα and IκBβ, which bind to NF-κB and mask its nuclear localization signal, thereby preventing nuclear uptake. IκBs are phosphorylated by the IKK complex, which leads to ubiquitination and subsequent degradation by the 26S proteasome [16,17]. Finally, the free NF-κB complexes translocate into the nucleus for target gene transcription activation, and the IκBs are degraded [18]. NF-κB is a ubiquitous and well-characterized transcription factor with a pivotal role in control of the inflammation, among other functions. Thus, NF-κB controls the expression of genes encoding proinflammatory cytokines (TNF-α,), chemokines (MCP-1, CXCL-1, RANTES), and immune receptors, all of which play critical roles in controlling most inflammatory processes [19]. Over the last decade, in vitro studies, animal models and human studies have provided evidence that upregulation of the canonical NF-κB isoform (in tubular epithelial cells, podocytes, mesangial cells, macrophages) has a pathogenic role in mediating chronic inflammation in CKD [20]. NF-κB indirectly regulates fibroblast accumulation through paracrine effects of infiltrating inflammatory models, as shown in animal models of ureteric obstruction using inhibitors of angiotensin or TNF receptor mice [21–23]. Many of the models in which NF-κB inhibition showed an anti-inflammatory effect, were also associated with improvements in renal fibrosis. For example, inhibition of NF-κB activation in the unilateral ureteral occlusion (UUO) model reduced interstitial collagen IV deposition and α-SMA accumulation [22–24]. Moreover, chronic treatment with pyrrolidine dithiocarbamate (PDTC, an inhibitor of NFκB) for more than 90 days also attenuated renal fibrosis in the remnant kidney model [25]. More direct evidence using molecular methods to suppress NF-κB activation have also shown the same results [26–30]. Inhibition of NF-κB activation by Smad7-gene therapy also reduced injury in rats with remnant kidney and crescentic glomerulonephritis [31,32].

This study evaluated the influence of Tanshinone IIA on the renal function, proteinuria, and the expression of fibronectin, TGF-β1, collagen I collagen III, collagen IV and macrophage infiltration, TNF-α, CXCL-1, MCP-1, and RANTES in order to further explore the related mechanisms of Tanshinone IIA influencing TGF-β/Smad and NF-κB pathway in the remnant kidney of rats with CKD induced by 5/6 nephrectomy.

## 2. Materials and methods

### 2.1. Tanshinone IIA

Tanshinone IIA was purchased from the Chinese Institute for Drug and Biological Product Control (Guangzhou, China). Tanshinone IIA was solved in 0.9% saline for oral administration.

### 2.2. Animals and experimental design

Male Sprague–Dawley rats (obtained from Experimental Animal Center of Southern Medical University, China, with the certification number of SCXK (Yue) 2006-0015) weighing 180–220 g were housed in a room at a constant temperature with a 12 h light–dark cycle and were given free access to food and water. These rats were kept according to the guidelines of Care and Use of Laboratory Animals formulated by Ministry of Science and Technology of China and all experimental procedures concerned were approved by the ethics committee of Southern Medical University. All rats were subjected to either sham surgery or 5/6 nephrectomy (NX) performed under isoflurane general anesthesia using the full sterile technique. Using a retroperitoneal approach, two poles of the left kidney were removed and hemostatic absorbable collagen sponges were used to stop bleeding. One week later the right kidney was removed [33]. In the sham-operated rats, a sham operation was performed. Then, at 1 week after the operation, the 5/6 nephrectomy group was randomly divided into two groups: CKD group (5/6 NX, $n = 10$): CKD rats who received no treatment and Tanshinone IIA group (5/6 NX + Tanshinone IIA, $n = 10$): CKD rats that were orally administrated a dose of 10 mg/kg of Tanshinone IIA daily. At the end of the 16 weeks of intervention, two CKD rats, and one Tanshinone IIA treatment rat died, but no rat died in control group.

### 2.3. Blood and urine examination

The 24 h urine samples were collected by using metabolic cages. Immediately before euthanasia, blood samples were drawn from the retro-orbital sinus for analysis of renal function parameters. Scr and BUN were measured using a commercial kit (Roche Diagnostics, Roche, Basel, Switzerland) and 24 h urinary protein excretion was measured with another commercial kit (Tonein-TPII, Ot-suka, Tokushima, Japan) according to the instructions of the manufacturers.

### 2.4. Renal semiquantitative morphometry

Remnant or control kidneys were removed, fixed in 10% buffered formalin, embedded in paraffin, and sliced to 3 mm-thick sections. Sections were stained by Masson's trichrome. Renal pathology and morphological analyses were performed by an experienced pathologist blinded to the source of the tissue.

The extent of glomerular sclerosis was assessed as described previously [34]. At least 30 glomeruli from each kidney were graded according to the following criteria: 0, no sclerosis; 1, less than 25% cross-sectional sclerosis; 2, 25–50% sclerosis; 3, 50–75% sclerosis; and 4, over 75% sclerosis. The mean score per glomerulus in each kidney was determined as the sclerosis index. Similarly, sections from the cortex of each kidney were graded for the severity of interstitial fibrosis: 0, no evidence of interstitial fibrosis; 1, less than 10% involvement; 2, 10–25% involvement; 3, 25–50%involvement; 4, 50–75% involvement; and 5, more than 75% involvement. The score for each section was recorded as the mean of 20 random high-power fields per section.