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**Original Paper**

# Tempol, a Superoxide Dismutase-Mimetic Drug, Ameliorates Progression of Renal Disease in CKD Mice

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**Key Words**
Chronic kidney disease • Fibrosis • Inflammation • Reactive oxygen species

**Abstarct**
***Background:*** Oxidative stress has been implicated in the pathogenesis of chronic kidney disease (CKD) and antioxidants may ameliorate disease progression. We investigate the beneficial effect of Tempol, a superoxide dismutase-mimetic drug, on progression of disease in a mouse model of CKD. ***Methods:*** CKD was surgically induced in c57BL/6 mice by 5/6 nephrectomy. Mice were randomly divided into 3 groups: sham group, 5/6 nephrectomized group (Nx) and Nx+Tempol (2 mmol/l in drinking water). Mice were sacrificed at the end of 12 weeks. Renal function, structure as well as expression of key molecules involved in the pathogenesis of inflammation, fibrosis and progression in mice were measured. ***Results:*** Reduced body weight and impaired renal function (elevation on serum creatinine, blood urea nitrogen, urine albumin, segmental sclerosis and tubulointerstitial damage) was demonstrated in Nx mice but was significantly improved by Tempol administration. Nx animals exhibited significantly elevated proinflammatory and profibrotic factors, activation of NF-κB, increased expression of NADPH oxidase related subunits (p47phox, p67phox, gp91phox), and elevated activation of TGF-β/Smad3, EGFR, MAPK signaling pathway. Tempol inhibited NF-κB mediated inflammation, TGF-β/Smad3-induced renal fibrosis as well as EGFR and MAPK signaling pathway activation. ***Conclusions:*** Tempol administration attenuated renal injury in CKD mice through NF-κB, TGF-β/Smad3, redox-senstive EGFR activation and c-Raf/MEK/ERK pathways.



## Introduction

Chronic kidney disease (CKD) is a slow, progressive and irreversible loss of renal function. The pathogenesis of CKD in most cases involves a complex interaction of inflammatory and

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hemodynamic processes, which leads to end stage renal disease (ESRD) characterized by glomerulosclerosis, tubulointerstitial fibrosis and the complete loss of renal function [1]. Oxidative stress is an important feature of CKD and a major mediator of its complications [2]. Reactive oxygen species (ROS) play an important role in normal cellular physiology. However, in pathologic conditions, a surplus of ROS in tissue results in oxidative stress with detrimental consequences such as inflammation and fibrosis. Upregulation of NADPH oxidase, a major source of ROS, has been shown in CKD rats [3]. ROS activates the redox-sensitive NF-κB, which is the general transcription factor for pro-inflammatory cytokines and adhesion molecules that drive the inflammation in CKD [4]. Several lines of evidence point to a central role for inflammation in CKD [5]. The levels of the inflammatory markers TNF-α and IL-6 and the white blood cell count are elevated in CKD [6], although it is not certain whether these changes represent a contribution to, or are a result of, CKD progression.

Transactivation of epidermal growth factor receptor (EGFR) was involved in the progression of CKD [7, 8]. EGFR was widely expressed in the mammalian kidney including glomerular mesangial cells, proximal tubular and medullary interstitial cells [9, 10]. Genetic or pharmacologic blockade of EGFR pathway attenuates renal injury [11], suggesting that EGFR signaling acts as a pivotal role in renal development and pathology. EGFR activates downstream mitogen activated protein kinase (MAPK) pathways. The involvement of MAPK pathway has been implicated in several models of kidney disease [12]. Thus, ROS may stimulate MAPK pathways and induce renal injury. For instance, angiotensin II stimulates the production of the superoxide ion through NADPH oxidases and subsequently activates MAP kinases, that leads to renal injury [13].

ROS are important for the pathogenesis of CKD and antioxidants may slow or prevent disease progression [14]. Oxidative stress in CKD is caused by a combination of excessive ROS production and antioxidant depletion. Increased antioxidant defenses may ameliorate progression of renal injury. Nitroxides exert redox metabolic actions and Tempol is the most extensively studied nitroxide [15]. It is a cell membrane permeable amphilite that dismutates superoxide catalytically and limits the formation of toxic hydroxyl radicals. Tempol is effective in detoxifying ROS in cell and animal studies [16]. Administration of Tempol has multiple effects on the kidney. Available data on the effect of administration of Tempol on progression of CKD are limited. In this study, we investigate the beneficial effect of Tempol on progression of disease in CKD mice.

## Materials and Methods

*Reagents and antibodies*

Tempol (4 - hydroxyl - 2,2,6,6 - tetramethyl - piperidine - 1 - oxyl) was purchased from Sigma-Aldrich. Antibodies against phospho-IκB, phospho-EGFR, total EGFR, phospho-c-Raf, phospho-MEK1/2, phospho-ERK1/2 and phospho-Smad3 were purchased from Cell Signaling Technology. Antibodies against fibronectin (FN), NF-κB P65, CTGF were purchased from Santa Cruz Biotechnology.

*Animals and experimental protocol*

Animal study was conducted according to the guidelines for the care and use of animals established by Fudan University. C57BL/6 male mice, weighing 22-25 g, were used. CKD in mice was induced by two-stage 5/6 nephrectomy [17]. Mice were randomly segregated into three groups: (1) sham-operated mice and treated with tap water as vehicle (Sham+V); (2) Nx mice were induced by 5/6 nephrectomy and treated with tap water (Nx+V) and (3) Nx mice were treated with Tempol (2 mmol/l of drinking water) (Nx+Tempol). After 12 weeks the mice were sacrificed by exsanguination and kidney was used for histologic, immunohistologic and molecular studies. Section of kidney tissue was affixed in 4% buffered paraformaldehyde for histological examination and the remainder of kidney was snap-frozen in liquid nitrogen for subsequent protein and mRNA analysis.

*Biochemical measurement*

Serum creatinine, blood urea nitrogen (BUN) and urinary albumin excretion and creatinine levels were measured by ELISA kits (Exocell) while serum 8-OHdG was determined using mouse 8-OHdG ELISA

kit (BMAssay). Renal protein levels of Malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) were detected using commercial kits (Jiancheng Bioengineering Research Institute and Cayman Chemical Company, respectively).

*Histologic examination and Immunohistochemistry*

Kidney samples were sectioned at 3 μm slices and stained with periodic acid-Schiff reagent (PAS) and Masson's trichrome. Glomerular damage was measured according to published methods [18]. The severity of tubulointerstitial damage was graded according to interstitial collagen deposition using Masson's trichrome staining [19]. For immunohistochemistry, paraffin was removed from the sections. The 3 μm slices were stained with mouse anti-CTGF (1:200), anti-FN (1:200), and anti-NF-κB p65 antibody, respectively and counterstained with hematoxyal. The average number of CTGF, FN and NF-κB p65-positive cells in glomerular section was quantified by counting the cells in 20 glomeruli. All histological and immunohistochemistry evaluations were performed without prior knowledge of the experimental group being studied.

*Western blot*

Mouse renal protein (30 mg) were denatured in boiling water for 15 min, separated by SDS-PAGE gel and transferred onto nitrocellulose membranes. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline, followed by incubation for 2 h with rabbit anti-phospho-EGFR, phospho-IκB, total EGFR, phospho-c-Raf, phospho-MEK1/2, phospho-ERK1/2, or phospho-Smad3 antibody at a dilution of 1:1000, respectively. After washing with Tris-buffered saline and incubated with horseradish peroxidase conjugated secondary antibody (1:2000), the blots were visualized with Enhanced ECL Kits.

*Real-time PCR*

Gene expression of mouse p47phox, p67phox, gp91phox, TNF-α, IL-1β, TGF-β, collagen IV and GAPDH were analyzed using real-time PCR. Briefly, kidney tissues were homogenized and total RNA was isolated by Invitrogen RNA isolation kit. cDNA synthesis was carried out using Fermentas cDNA synthesis kit. PCR amplication of cDNA was performed with ABI Biosystems 7500 Detection System. Comparative $2^{-\Delta\Delta CT}$ method was used to calculate the relative amounts of target gene. The primers of these target genes were listed in Table 1.

*Electrophoresis mobility shift assay (EMSA)*

EMSA methods were used to detect the binding activities of NF-κB in nuclear extracts. NF-κB oligonucleotide's sequence was 5'-AGTTGAGGGGACTTTCCCAGGC-3' end-labeled with [γ-$^{32}$P]-ATP (Beyotime Institute of Biotechnology). Nucleus extracts (20 μg) were treated with poly(dI-dC) and the [γ-$^{32}$P] labeled DNA probe in buffer for 20 min at 37°C. Then the binding reactions were separated by electrophoresis on 4% gel at 200 V for 1 hour. The protein-DNA complex was then visualized by autoradiography and the relative intensity was measured by a scanning densitometer [20].

*Statistical analysis*

Results were presented as means ± SEM. One-way ANOVA for repeated measures was used to compare mean values between two groups. $p < 0.05$ was considered to be statistically significant.

**Table1.** Sequence of primer pairs for real time PCR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase

| Name | Forward primer | Reverse primer |
|---|---|---|
| P47phox | 5'-CACCGGCTATTTCCCATCCA-3' | 5'-TGTCCTTTGTGCCATCCGT-3' |
| P67phox | 5'-CTGTGGTGTGAGCATACGGT-3' | 5'-TGTCCTTTGTGCCATCCGT-3' |
| gp91phox | 5'-GAGGTTGGTTCGGTTTTGGC-3' | 5'-CTCCGTCCAGTCTCCAACAA-3' |
| TNF-α | 5'-AAGTTCCCAAATGGCCTCCC-3' | 5'-CTTGGTGGTTTGCTACGACG-3' |
| IL-1β | 5'-GCTACCTGTGTCTTTCCCGT-3' | 5'-CGTCACACACCAGCAGGTTA-3' |
| TGF-β | 5'-AGCTTTGCAGGGTGGGTATC-3' | 5'-CCTTCGGGTGAGACCACAAA-3' |
| Collagen IV | 5'-CACCGTTCGTTGAATGCCAG-3' | 5'-AAACCTGGCACCTGCTGATT-3' |
| GAPDH | 5'-TCAGCCGCATCTTCTTTTG-3' | 5'-AAATCCGTTGACTCCGACC3' |

## Results

*Body weight and impaired renal function in Nx mice is improved in Nx+Tempol mice*

The physiological parameters in mice are shown in Fig. 1. Body weight was significantly decreased in Nx+V mice relative to Sham+V mice (24.8±0.3 g versus 30.2±0.4 g). Final body weight was significantly improved in Nx+Tempol mice (28.1±0.2 g) relative to Nx+V mice although still lower than Sham+V mice (Fig. 1A). Serum creatinine and BUN levels were

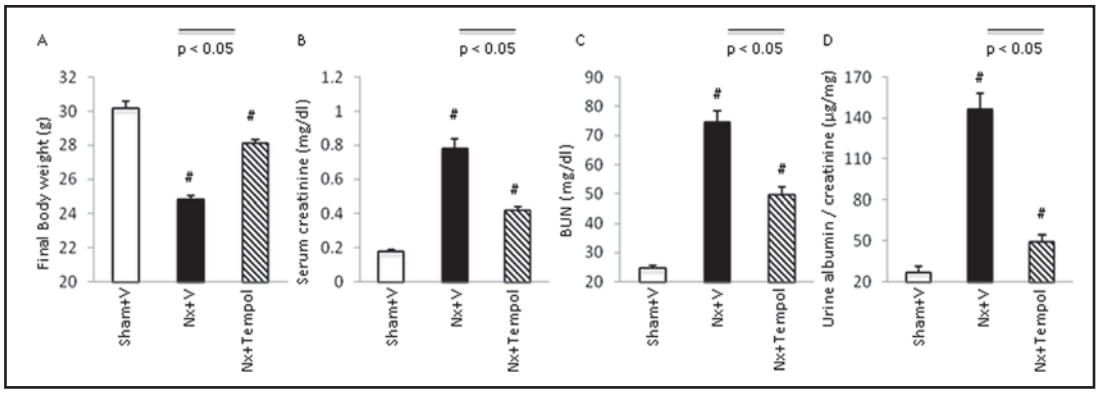


**Fig. 1.** Physiological parameters in mice at the end of 12-week study. Three groups of mice were included, Sham+V, Nx+V and Nx+Tempol. Mice were fed *ad libitum*. A: body weight; B: serum creatinine; C: BUN; and D: urine albumin/creatinine ratio. Results are expressed as means±SEM (n=8). Nx+V and Nx+Tempol mice were compared with Sham+V mice. In addition, Nx+V mice were also compared to Nx+Tempol mice. # $p < 0.05$.

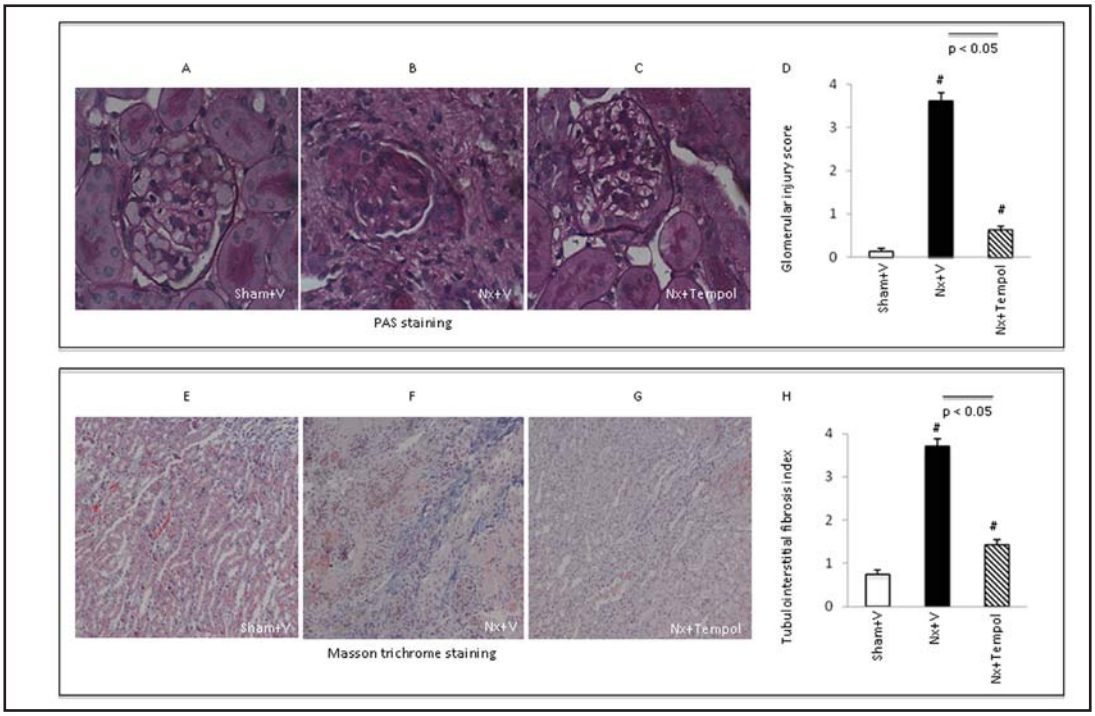


**Fig. 2.** Kidney histopathology. Representative photomicrographs of PAS-stained kidney sections (A, B & C, Magnification, × 400) and Masson's trichrome-stained kidney sections (E, F & G, Magnification, × 200). A & E: Sham+V group; B &F: Nx+V group; C & G: Nx+Tempol group. Glomerular injury score (D) and tubulointerstitial fibrosis index (H) were evaluated as described in the methods. Values are expressed as means±SEM (n=3). Nx+V and Nx+Tempol mice were compared with Sham+V mice. Nx+V mice were also compared to Nx+Tempol mice. # $p < 0.05$.

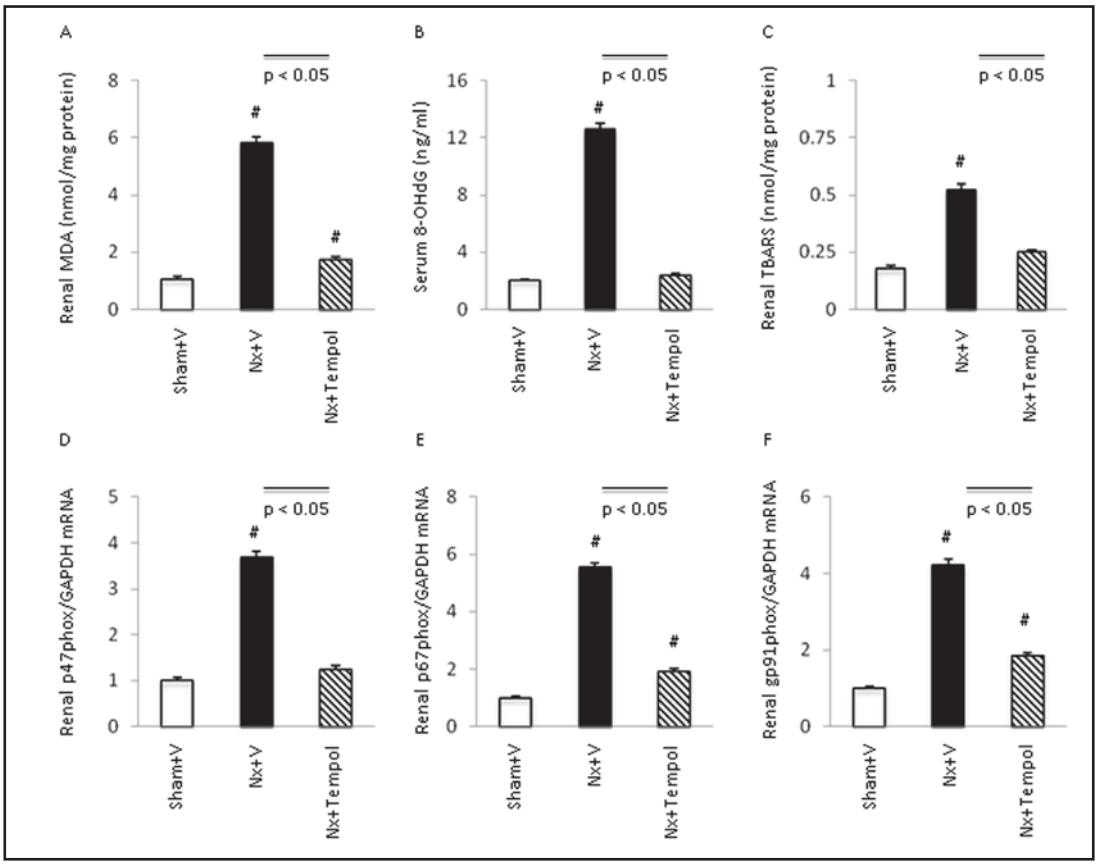


**Fig. 3.** Reactive oxygen species production in mice. Renal MDA (A), serum 8-OHdG (B) and renal TBARS (C) were measured. Renal mRNA level of p47phox (D), p67phox (E) and gp91phox (F) were detected by real-time PCR and normalized to the expression of GAPDH. Values are expressed as means±SEM (n=3). Nx+V and Nx+Tempol mice were compared with Sham+V mice. Nx+V mice were also compared to Nx+Tempol mice. $^{\#}p < 0.05$.

elevated in Nx+V mice (0.8±0.1 mg/dl and 74.7±3.8 mg/dl, respectively) than Sham+V mice (0.2±0.0 mg/dl and 24.8±0.8 mg/dl, respectively). Serum creatinine and BUN levels were reduced in Nx+Tempol mice (0.4±0.0 mg/dl and 49.6±2.7 mg/dl, respectively) than Nx+V mice (Fig. 1B and 1C). Similarly, urine albumin/creatinine ratio was increased in Nx+V mice relative to Sham+V mice (146.6±11.3 μg/mg versus 27.3±4.2 μg/mg) and was improved in Nx+Tempol mice (49.1±5.1 μg/mg) (Fig. 1D).

*Nx-induced glomerular injury and tubulointerstitial fibrosis is attenuated in Nx+Tempol mice*

Renal histopathology of Nx mice was examined. Baseline glomerular injury score for Sham+V mice were shown (0.14±0.06) (Fig. 2A and 2D). Nx+V mice exhibited severe glomerular injury characterized by cell proliferation, increased extracellular matrix and glomerulosclerosis (3.62±0.20) (Fig. 2B and 2D). Furthermore, glomerular injury score was significantly reduced in Nx+Tempol mice (0.63±0.09) relative to Nx+V mice (Fig. 2C and 2D). Tubulointerstitial fibrosis score in Sham+V mice was 0.74 ± 0.12 (Fig. 2E and 2H). Increased collagen deposition and renal fibrosis was evident in Nx+V mice (3.71±0.18) (Fig. 2F and 2H). Treatment with Tempol significantly decreased renal interstitial collagen accumulation. Tubulointerstitial fibrosis index was markedly reduced in Nx+Tempol mice (1.42±0.1) relative to Nx+V mice (Fig. 2G and 2H).

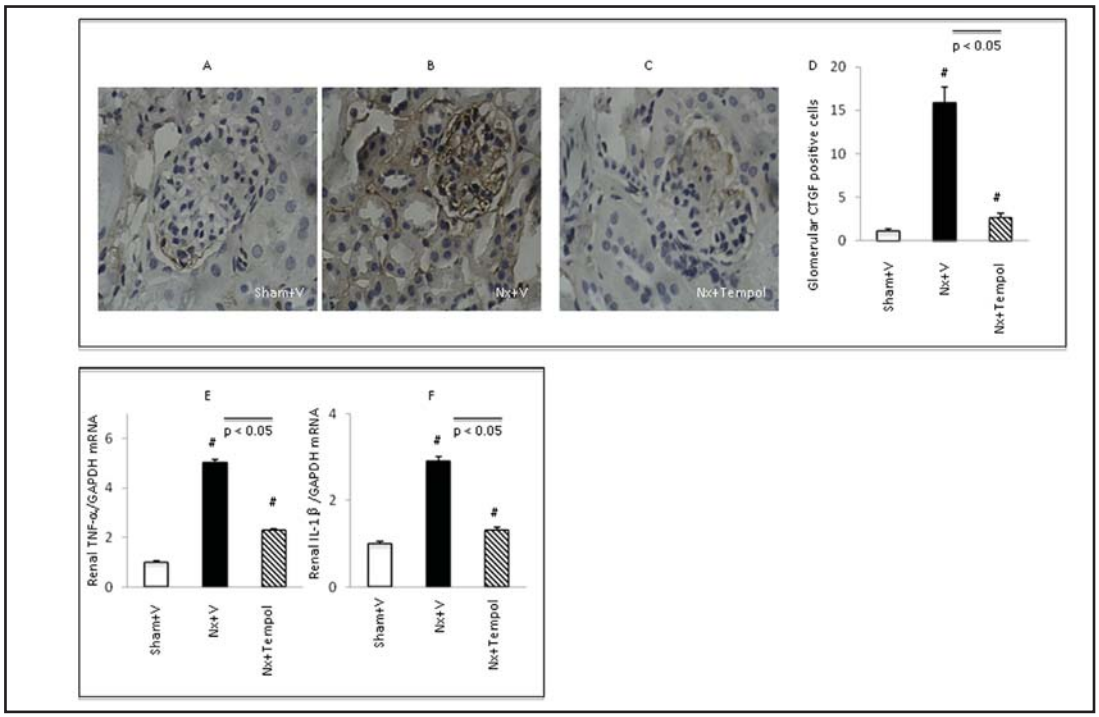


**Fig. 4.** Expression of inflammatory cytokines in the kidney. Renal CTGF protein expression was detected by Immunohistochemistry (A-D). A: Sham+V group; B: Nx+V group; C: Nx+Tempol group; D: Semi-quantitative analysis of CTGF positive cells. Renal mRNA expression of TNF-α (E) and IL-1β (F) were detected by real-time PCR and normalized to the expression of GAPDH. Values are expressed as means±SEM (n=3). Nx+V and Nx+Tempol mice were compared with Sham+V mice. Nx+V mice were also compared to Nx+Tempol mice. $^{\#}p < 0.05$.

*Nx-induced oxidative stress is suppressed in Nx+Tempol mice*

Oxidative stress was evaluated by determination of renal MDA and TBARS levels, serum 8-OHdG levels as well as renal mRNA levels of NADPH oxidase subunits p47phox, p67phox and gp91phox. Renal MDA and TBARS protein levels were significantly increased in Nx+V mice (5.8±0.2 nmol/mg protein and 0.5±0.01 nmol/mg protein, respectively) than Sham+V mice (1.1±0.1 nmol/mg and 0.2±0.01 nmol/mg, respectively) (Fig. 3A and 3C). Serum 8-OHdG levels were also increased in Nx+V mice relative to Sham+V mice (12.6±0.4 ng/ml versus 2.0±0.1 ng/ml) (Fig. 3B). Moreover, renal mRNA levels of p47phox, p67phox and gp91phox were significantly increased in Nx+V mice relative to Sham+V mice (Fig. 3D, 3E and 3F). Treatment with Tempol significantly ameliorated oxidative stress in Nx mice. Serum 8-OHdG levels, renal TBARS protein levels and renal p47phox were normalized in Nx+Tempol mice whereas renal MDA levels and renal p67phox mRNA and gp91phox mRNA levels were significantly reduced in Nx+Tempol mice relative to Nx+V mice although still higher than Sham+V mice.

*Tempol attenuates renal inflammation and activation of NF-κB in Nx mice*

Renal immunohistochemistry with CTGF antibody is illustrated in Fig. 4A–C. Compared with Sham+V mice, glomerular cells that were positive for CTGF were significantly increased in Nx+V mice (15.9±1.1 versus 1.1±0.2). Treatment with Tempol significantly reduced the number of CTGF positive cells in Nx+Tempol mice (2.7±0.5) than Nx+V (Fig. 4D). Renal TNF-α and IL-1β mRNA level was significantly elevated in Nx+V mice (5.0 fold and 2.9 fold, respectively) than Sham+V mice and treatment with Tempol markedly reduced renal TNF-α and IL-1β mRNA levels relative to Nx+V mice (Fig. 4E and 4F). Renal NF-κB level

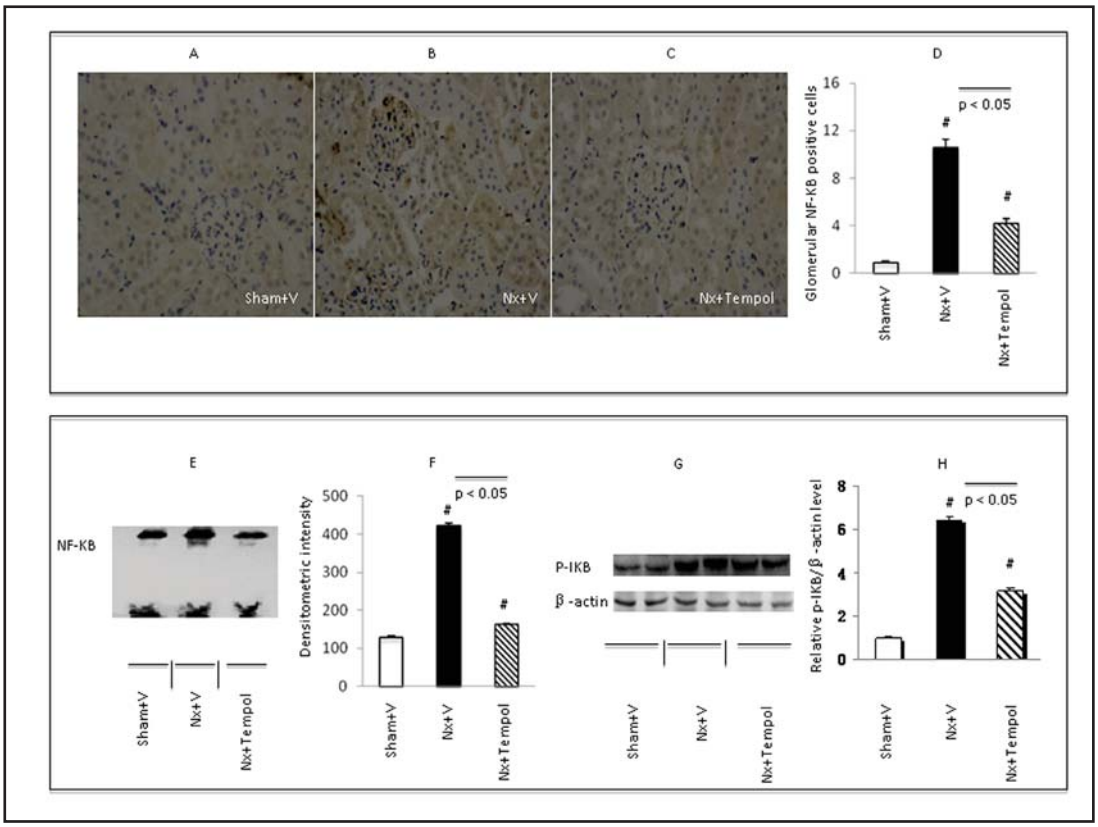


**Fig. 5.** NF-κB expression in mice. Expression of NF-κB p65 subunit was detected by Immunohistochemistry (A-D). A: Sham+V group; B: Nx+V group; C: Nx+Tempol group; D: Semi-quantitative analysis of p65 subunit positive cells in renal section. NF-κB was also determined by EMSA assay (E & F). Renal p-IκB was determined by Western blot after normalization to the expression of β-actin (G & H). Values are expressed as means±SEM (n=3). Nx+V and Nx+Tempol mice were compared with Sham+V mice. Nx+V mice were also compared to Nx+Tempol mice. $^{\#}p < 0.05$.

in Nx mice was quantified using immunohistochemistry, EMSA and western blot (Fig. 5). Compared with Sham+V mice (0.9±0.1), glomerular cells that were positive for NF-κB p65 subunit were markedly increased in Nx+V mice (10.6±0.7) (Fig. 5D). Treatment with Tempol significantly reduced the number of NF-κB positive cells (4.2±0.4) relative to Nx+V mice (Fig. 5D). Similarly, renal NF-κB–DNA binding activity was significantly elevated in Nx+V mice compared with Sham+V mice group (422.3±7.6 unit versus 128.2±3.9 unit). NF-κB–DNA binding activity was significantly inhibited in Nx+Tempol mice (163.3±3.1 unit) relative to Nx+V mice (Fig. 5F). Similarly, Compared with Sham+V mice, the IκB phosphorylation was increased significantly in Nx+V mice, and the increment of P-IκB was inhibited by Tempol (Fig. 5G, H).

*Tempol attenuates renal fibrogenic pathways in Nx mice*

Renal immunohistochemistry with FN antibody is illustrated in Fig. 6A–C. Compared with Sham+V mice, glomerular cells that were positive for FN were significantly increased in Nx+V group mice (8.2±0.9 and 0.9±0.2, respectively). Treatment with Tempol significantly reduced the number of FN positive cells in Nx+Tempol mice (3.9±0.3) relative to Nx+V mice (Fig. 6D). Renal TGF-β and collagen IV mRNA levels were significantly increased in Nx+V mice (9.3-fold and 4.0-fold, respectively) relative to Sham+V mice whereas renal TGF-β and collagen IV mRNA levels were notably decreased in Nx+Tempol mice compared to Nx+V mice (Fig. 6E and 6F). We also investigated the significance of TGF-β/Smad3 pathway in NADPH

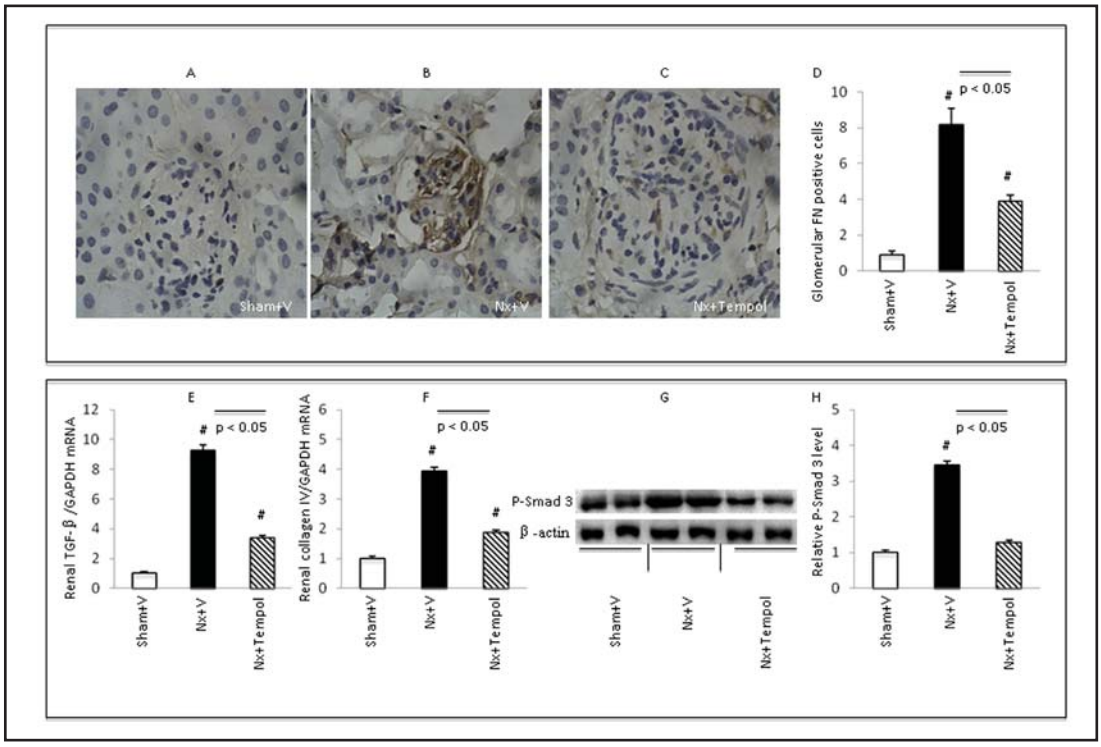


**Fig. 6.** Expression of fibrotic genes in the kidney. Renal FN protein was detected by Immunohistochemistry (A-D). A: Sham+V group; B: Nx+V group; C: Nx+Tempol group; D: Semi-quantitative analysis of FN positive cells. Renal mRNA expression of TGF-β (E) and collagen IV (F) were detected by real-time PCR and normalized to the expression of GAPDH. Renal p-Smad3 was determined by Western blot after normalization to the expression of β-actin (G & H). Values are expressed as means±SEM (n=3). Nx+V and Nx+Tempol mice were compared with Sham+V mice. Nx+V mice were also compared to Nx+Tempol mice. $^{\#}p < 0.05$.

oxidase-mediated renal injury. Renal P-Smad3 protein level was significantly increased in Nx+V mice (3.4-fold) relative to Sham+V mice. Importantly, renal P-Smad3 protein level was normalized in Nx+Tempol mice (Fig. 6G and 6H).

*Tempol attenuates Nx-induced overexpression of EGFR signaling*

We quantified the levels of EGFR and ERK1/2 phosphorylation in mice. Increased phosphorylation of renal EGFR (2.1-fold) was detected in the Nx+V mice relative to Sham+V group, and tempol treatment markedly inhibited this response (Fig. 7A). Phosphorylation of renal c-Raf/MEK/ERK1/2 were significantly elevated (1.89-fold, 3.19-fold and 4.07-fold, respectively) in Nx+V mice compared with Sham+V group (Fig. 7B-D). Treatment with Tempol significantly reduced the levels of renal p-c-Raf, p-MEK1/2 and p-ERK1/2 protein in Nx+Tempol mice relative to Nx+V mice.

## Discussion

Oxidative stress played a key role in the progression of renal disease. ROS accelerate the development of renal injury by promoting inflammation and intracellular signaling pathways [2, 21]. NADPH oxidase is a major source of ROS in kidney disease. Renal MDA and TBARS levels, serum 8-OHdG levels as well as renal mRNA levels of NADPH oxidase subunits p47phox, p67phox and gp91phox were significantly increased in Nx+V mice (Fig. 3). Our results are in agreement with previous reports in which increased expression of NADPH oxidase is an important cause of oxidative stress in remnant kidney model [22]. Oxidative

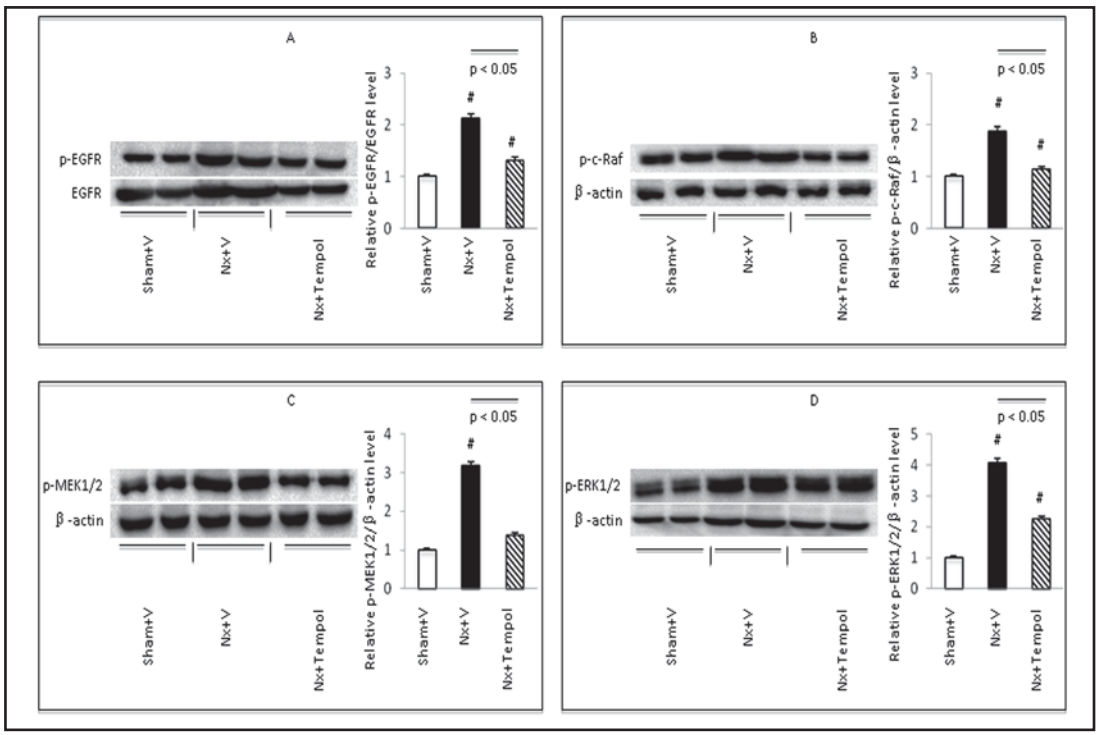


**Fig. 7.** EGFR and MAPK signaling expression in mice. Protein expression of p-EGFR (A), p-c-Raf (B), p-MEK1/2 (C) and p-ERK1/2 (D) were measured by western blot. Values are expressed as means±SEM (n=3). Nx+V and Nx+Tempol mice were compared with Sham+V mice. Nx+V mice were also compared to Nx+Tempol mice. $^{\#}p < 0.05$.

stress in CKD is not only the result of increased generation on ROS but also because of the depletion of antioxidant defenses. Recently, Dornas *et al* showed that antioxidant treatment with tempol could reduce blood pressure and suggested that ROS may play a role in the pathogenesis of increased blood pressure in the hypertension models [23]. In present study, we demonstrated that Tempol, a superoxide dismutase-mimetic drug, attenuated renal injury in CKD mice through NF-κB, TGF-β/Smad3, redox-senstive EGFR activation and c-Raf/MEK/ERK pathways.

At the end of the 12-week study, final body weight and renal function (serum creatinine, BUN and proteinuria) was significantly improved in Nx+Tempol mice relative to Nx+V mice (Fig. 1). As expected, the remnant kidney in the Nx+V mice showed significant glomerulosclerosis and tubulointerstitial damage. Importantly, glomerulosclerosis and the extension of tubular injury were reduced by more than one-half in the Nx+Tempol mice (Fig. 2). The favorable results seen in Nx+Tempol mice are likely related to the antioxidant property of this compound. Indeed, Tempol treatment normalized or significantly reduced markers of oxidative stress in Nx mice (Fig. 3).

Previous studies indicated that Tempol treatment (1 mmol/L in drinking water) was incapable of reducing renal oxidative stress and failed to improve renal function and structure in the remnant kidney model [2, 24]. Ding *et al* also showed that tempol (1 mmol/L in drinking water) treatment reduced immunofluorescence for 3-nitrotyrosine and HIF 1A mRNA, but had little impact on polycystic kidney disease [25]. Interestingly, higher dose of Tempol (3 mmol/L in drinking water) reduced food intake and weight gain in normal rats [24]. In the present study, we selected the optimal dose of Tempol as 2 mmol/L in drinking water. Of note, administration of Tempol (0.29 mmol/L to 5.8 mmol/L) modulates serum leptin levels and influences food intake and weight gain in mice [26]. However, the mechanism of energy metabolism of Tempol on CKD is beyond the scope of current investigation.

The number of glomerular CTGF-positive cells as well as renal TNF-α and IL-1β mRNA levels was significantly increased in Nx mice but was markedly reduced by the administration of Tempol (Fig. 4). Our findings point to the inhibitory effects of Tempol on activation of pro-inflammatory transcription factors. Oxidative stress activates the redox-sensitive NF-κB, which is the general transcription factor for pro-inflammatory cytokines such as renal TNF-α, IL-1β and CTGF and drives the inflammation in CKD. Increased activation of NF-κB, as evidenced by increased p65 subunit, was demonstrated in CKD rats. Previously, we showed that pyrrolidine dithiocarbamate, an inhibitor of nitric oxide synthase, attenuated the activation of NF-κB and aldosterone/salt-induced CKD progression [20], Fujihara showed that inhibition of NF-κB attenuates renal injury in the CKD rodents [27]. In the present study, we found that glomerular cells staining positively for NF-κB p65 subunit was increased in Nx+V mice and was significantly reduced in Nx+Tempol mice. Treatment with Tempol suppressed the activation of NF-κB in remnant kidney (Fig. 5). We concluded that Tempol at 2 mmol/l prevents translocation and DNA binding of NF-κB and, thereby, suppresses renal production of pro-inflammatory cytokines such as TNF-α, IL-1 and CTGF in CKD. Our results were in accordance with a recent report. Tempol reduces the activation of NF-κB in a mouse model of acute inflammation [28]. The precise role of NF-κB in human renal disease is uncertain. In human renal disease, there is histologic evidence of NF-B activation in diabetic nephropathy, glomerular disease and AKI. NF-κB activates macrophages and glomerular and tubular cells and correlates with parameters of severity of disease such as proteinuria and inflammation [29]. However, these data are largely descriptive and can only been interpreted as supportive for the role of NF-κB in promoting inflammation in CKD because inflammation *per sec* will also promote NF-κB activation.

Oxidative stress promotes pro-fibrotic responses through TGF-β signaling, a key mediator of renal fibrosis in CKD [30]. Inhibition of NADPH oxidase suppressed EMT and matrix protein production in renal tubular epithelial cells and renal fibroblasts [31]. Upregulation of TGF-β and its receptors lead to the onset of renal fibrosis while neutralizing TGF-β1 activity by anti-TGF-β1 antibody ameliorated kidney fibrosis [32, 33]. TGF-β binds to its receptor and activates downstream signaling pathway, such as Smad2 and Smad3, and mediate tubular epithelial transforming to myofibroblast and stimulate extracellular matrix production [34]. Redox pathways may regulate TGF-β/Smad signaling in the development of renal fibrosis [35]. We demonstrated that Tempol suppressed renal TGF-β and collagen IV mRNA level, p-Smad3 level as well as number of glomerular FN cells in Nx mice (Fig. 6). The mechanism of the enhancing effect of ROS on TGF-β-induced Smad phosphorylation is unclear. Activation of TGF-β signaling requires phosphorylation of the type I receptor (ALK5) on serine and threonine residues in the GS domain upon ligand binding, while phosphorylated ALK5 in turn induces phosphorylation of Smad2/3 and thereby initiates downstream signaling events. ALKs phosphorylation is negatively regulated by protein phosphatase (PP1) 1 and PP 2A. There is evidence that PP1 and PP2A are redox-sensitive and ROS is able to activate ALK5 and Smad phosphorylation. In addition to regulating Smad phosphorylation, ROS may also modulate TGF-β via Smad-independent pathways, including MAPK members, c-Jun N-terminal kinase (JNK) and p38 [35]. Both JNK and p38 are redox sensitive. Hence, it is possible that ROS may facilitate TGF-β induced signaling by enhancing JNK and p38 activation and indirectly induce phosphorylation of Smad3.

EGFR activation plays an important role in the progression of renal vascular and glomerular fibrosis. Increased transactivation of EGFR correlates with interstitial fibrosis in human renal allograft biopsies [36] and angiotensin II-induced renal fibrosis [37]. Thus, inhibition of EGFR signaling may attenuate progression of CKD through the suppression of renal proliferation and fibrosis [38]. Liu and co-workers reported that genetic or pharmacologic blockade of EGFR inhibits renal fibrosis [11]. Wassef *et al* demonstrated that EGFR inhibition attenuated early kidney enlargement in experimental diabetes [39]. Our study showed that transactivation of EGFR was significantly increased in Nx+V mice and was suppressed by Tempol (Fig. 7A). The role of extracellular-regulated kinase (ERK)1/2 in the transduction of renal proliferation and fibrosis has been described. MEK/ERK signaling

mediated renal proliferation via EGFR transactivation in mesangial cells while albumin activated ERK1/2 in human renal epithelial cells through transactivation of EGFR [40, 41]. The c-Raf protein is part of the ERK1/2 pathway as a MAP kinase kinase kinase (MAP3K) that functions downstream of the Ras subfamily of membrane associated GTPases. The most important targets of all Raf enzymes are MEK1 and MEK2. Our study demonstrated that c-Raf, MEK1/2 and ERK1/2 signaling cascades were activated in Nx mice and was suppressed by Tempol treatment (Fig. 7).

In conclusion, the present study demonstrated that Tempol administration attenuated oxidative stress, inflammation, fibrosis and deterioration of the remnant kidney function and structure in mice with renal ablation. Further studies are needed to explore the efficacy of Tempol in the progression of CKD in humans, particularly since the results of other antioxidant therapies are more compelling in animal models than in human diseases. If proven effective, Tempol treatment would be an attractive adjunctive therapy for CKD since it is inexpensive and can be orally administrated.

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## Disclosure Statement

The authors declare that there are no conflicts of interest.

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