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# Molecular Mechanisms of Nephro-Protective Action of HE-86 Liquid Extract in Experimental Chronic Renal Failure

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## 1. Introduction

Chronic renal injury can be mediated by angiotensin II (Ang II) through hemodynamic and inflammatory mechanisms and attenuated by individual suppression of these mediators. Hypertension is usually associated with the development of vascular and renal fibrosis [3]. This pathophysiological process is characterized by structural changes in vasculature caused by increased synthesis and rearrangement of extracellular matrix proteins, such as the collagen type I [4]. Several studies support a major role for the renin-angiotensin system in the development of fibrosis [5, 6].

Hypertension injures blood vessels and thereby causes end-organ damage. The mechanisms are complicated and although they have been studied for decades in experimental animal models [7], they are only currently being elucidated. From the efforts of many investigators, we are now in the position of constructing a chain of events from the endothelium to the underlying matrix, to the vascular smooth muscle cells, and beyond to the adventitia, and surrounding tissues. The endothelial layer acts as a signal transduction interface for hemodynamic forces in the regulation of vascular tone and chronic structural remodeling of arteries [8]. Infiltration of the permeabilized endothelium by leukocytes sets the stage for an inflammatory cascade, involving cytokines, chemokines, growth factors, and matrix metalloproteinases. Altered integrin signaling, the production of tenascin, epidermal growth factor signaling, tyrosine phosphorylation, and activation of downstream pathways culminate in vascular smooth muscle cell proliferation [9]. Evidence is accumulating that matrix molecules provide an environment which decreases the rate of programmed cell death [10].

Hypertension is a major risk factor for renal and cardiac damage, however, the mechanisms are incompletely understood. Angiotensin (Ang) II, the key effector of the local and circulating renin-angiotensin system (RAS), plays a central role [11-12]. In addition to its vasoactive and growth-promoting action, Ang II stimulates circulating leukocytes and endothelial cells, thereby promoting inflammation and interstitial extracellular matrix accumulation [13-17]. Many inflammation-mediating genes are activated by the transcription nuclear factor- $\kappa$ B (NF- $\kappa$ B), which resides inactive and bound to the inhibitory protein I- $\kappa$ B in the cytoplasm of T lymphocytes, monocytes, macrophages, endothelial cells, and smooth muscle cells [18-19]. Ang II stimulates NADPH oxidase, which generates reactive oxygen species (ROS) [20]. ROS may act as signal transduction messengers for several important transcription factors, including NF- $\kappa$ B and AP-1 (activator protein-1) [21]. Recently, Ozes et al [22] showed that Akt/protein kinase B (Akt) is essential in tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-induced activation of NF- $\kappa$ B. Takahashi et al, [23] as well as Ushio-Fukai et al, [24] have demonstrated Akt activation by Ang II, which may involve ROS. Akt-induced activation of NF- $\kappa$ B upregulates numerous genes, including interleukin (IL)-1, IL-6, IL-8, interferon- $\gamma$ , TNF- $\alpha$ , intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and the chemokine MCP-1 (monocyte chemoattractant protein-1). Several reports [25-27] indicated that angiotensin converting enzyme (ACE) inhibition decreased NF- $\kappa$ B in renal disease.

We have previously demonstrated that traditional Chinese medicine prescription documented in the ancient Chinese pharmacopoeia or monographs promoted blood circulation, decreased blood stasis, and improved renal function. They decreased urinary protein excretion, balanced lipid metabolism and enhanced the effects of antioxidant in the treatment of patients with early and middle stage chronic renal failure [28-32].

It has been shown broad foreground to postpone progression of chronic renal dysfunction. But it is unclear that effective composition and mechanism of renal protection. Therefore, the study presented here was designed to test the hypothesis that HE-86 liquid extract, which is effective unite refined from above Chinese prescription, would prevent chronic renal failure rats induced by nephrectomized, in association with decreased expression of angiotensin II and AT- II receptors, further to suppress high expression of inflammatory and growth factors. In an attempt to obtain more effective renal protection, research design consisted of a group of Nx rats receiving a HE-86 liquid extract treatment comparing with chronic renal failure rats induced by subtotal nephrectomized without treatment. At same time, in the present study, we also assess the influence of renal mass reduction (RMR) caused by subtotal (5/6) nephrectomy on gene expression for NF- $\kappa$ B, TNF- $\alpha$  and TGF-beta1 and evaluate the correlation between expression of these genes and activity of the intrarenal renin-angiotensin systems. The research result showed HE-86 played a critical role in improving renal disease and was a key mediator in delay process of vascular fibrosis, characterized by reduced lumen diameter and arterial wall thickening attributable to excessive deposition of extracellular matrix (ECM) through by the model study.

## 2. Materials and methods

### 2.1 Experimental design

Thirty-six of the normal kidney mass were removed from adult male Munich-Wistar rats (BiKai, Shanghai, China) weighing 200–210 g to make animal models of CRF. In a first

session, two thirds of the left kidney were removed. One week after the first operation, the right kidney was removed. These procedures were performed under anaesthesia with sodium pentobarbital (The ShuGuang pharmaceutical factory in Shanghai). Two weeks after 5/6-nephrectomy, 24 rats were divided into pairs such that both rats in each pair exhibited almost the same levels of serum creatinine, blood urea nitrogen (BUN) (Table 1). One rat from each pair was assigned to (i) control uraemic group (n=12), the other to (ii) treatment uraemic group (n=12) which received HE-86, extract liquid which is effective composition isolated from Chinese medicine prescription, everyday at a dose of 0.75 g/100 g body weight for 8 weeks. For normal controls, rats underwent a sham operation consisting of laparotomy and manipulation of the renal pedicles but without damage to the kidney(n=12). The treatment group were administered by HE-86 infuse the stomach as pair-fed with the control uraemic rats, and the normal rats were fed ad libitum with standard solid chow (BiKai Animal Lab. Company, Shanghai, China) containing 24.5% protein.

	N	BUN(mmol/L)	Scr( $\mu$ mol/L)
sham	12	7.51 $\pm$ 0.75	19.00 $\pm$ 4.00
control	12	16.17 $\pm$ 0.99*	49.50 $\pm$ 6.53*
treatment	12	16.18 $\pm$ 2.42*	49.23 $\pm$ 9.36*

Table 1. The variation of serum creatinine and blood urea nitrogen before treatment.

Blood pressure was measured before treatment and every two weeks after surgery. The levels of serum creatinine (Scr), Blood urea nitrogen (BUN), 24h urine protein excretion and urine TGF- $\beta$  were determined at 4 or 8 weeks after starting the administration of HE-86, respectively. The remnant kidneys were removed after perfusion at the end of experiment for histopathological and gene expression studies.

## 2.2 Analytical procedures

### Renal Function Assessment and Blood Pressure Measurement

Serum creatinine (Scr) and Blood urea nitrogen (BUN) were measured using a Beckman Cx4 analyser (Fullerton, CA, USA), respectively.

24h Urinary protein concentrations were determined by the Bradford method, adapted to a microtiter plate assay. Coomassie reagent (USB, Cleveland, OH) was added to the diluted urine samples. After 10 minutes, the absorbance at 595-nm wavelength was read on ELX800 microplate reader (Bio-Tek Instruments, VT). The protein concentrations were calculated by reference to bovine serum albumin (Sigma) standards.

Systolic blood pressure was recorded by tail plethysmography using the BP2000 blood pressure analysis system (Visitech Systems, Inc., Apex, NC) in conscious rats at baseline and every 2 weeks throughout the experimental time course.

### 2.3 Immunohistochemical analysis

Immunostaining of NF- $\kappa$ b (Sigma) in renal tissue sections was performed using the streptavidin-biotinylated peroxidase complex (SABC) method. The tissue specimens were divided into thin sections (4- $\mu$ m thick) that were then deparaffinized. The sections were washed three times with distilled water for 5 min. The sections were treated with Protease K (Try box produced by BSD living creature technique company of Wuhan) in distilled water at 37°C for 15 min, and washed three times with PBS for 10 min. Endogenous peroxidase activity was blocked by incubating the sections with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min at room temperature. The sections were washed three times with PBS for 5 min. The sections were incubated with 10% rabbit serum at 37°C for 60 min to reduce the non-specific background staining, and washed three times with PBS for 5 min. Then, the sections were incubated with a monoclonal anti- NF- $\kappa$ b antibody (7  $\mu$ g/ml) dissolved in PBS containing 3% BSA and 0.1% NaN<sub>3</sub> at 4°C overnight, and washed three times with PBS for 10 min; followed by incubation with a biotinylated rabbit antibody against mouse IgG+IgA+IgM (10  $\mu$ g/ml) at 37°C for 40 min. The sections were washed three times with PBS for 5 min, and then incubated with peroxidase-labelled streptavidin at 37°C for 30 min. After washing three times with PBS for 10 min, the reaction was completed by the addition of diaminobenzidine-H<sub>2</sub>O<sub>2</sub> solution for 15 min, and washed three times with distilled water for 5 min, then the slides were counter-stained with methylgreen.

The primary anti- NF- $\kappa$ b antibody (1 : 100) was incubated with NF- $\kappa$ b (10 mg/ml) at 4°C overnight. After centrifuging the mixture at 10,000xg for 30 min, the supernatant was used as negative control for the primary antibody solution followed by the usual SABC method. There was no positive staining in the renal cortex when the primary antibody was pre-incubated with NF- $\kappa$ b.

The immunostaining of NF- $\kappa$ b was quantified using an image analyser IMS (FUDAN university of medical science portrait examination center) by evaluating the positively stained area of the sections under the same light intensity for microscopy. The intensity of colour component for red, green or blue was graded from 0 to 256°. Areas which showed intense brown color were extracted from the microscopic fields (number of fields for each tissue sample, six fields; magnification on the display: x300) under the following conditions; red component ranging from 104 to 158°, green component from 81 to 129°, and blue component from 70 to 123°.

### 3. Real-time quantitative Polymerase Chain Reaction (PCR) for TNF- $\alpha$ , Ang II and AT1R

To investigate the expression of TNF- $\alpha$  mRNA, Ang II and AT1R real-time PCR (BC living creature technique company, Shanghai, China) was performed with the Opticon real-time PCR machine (FX scientific research Inc. Shanghai, China). Briefly, total RNA was extracted from renal tissues. All of the RNA samples were treated with the RNase-free DNase I (GIBCO BRC Inc, Shanghai, China) before the RT-PCR. Real-time quantitative one-step RT-PCR assay was performed to quantify mRNA using real-time PCR machine (FX scientific research Inc. Shanghai, China). The primers used for real-time RT-PCR were as follows: TNF- $\alpha$ : forward 5'-CTCATTCCCGCTCGTGG-3' reverse 3'-CGTTGGTGGTTCGTCCTCC- 5';

AT1R: forward 5'-CTTGTTCCCTTTCCTTATC -3' reverse 3'-ACTCCACCTCACTGTCCA -5'. Ang II : forward 5'- ACCTG CATGA GTGTT GATAGG-3' reverse 3'-ACTTCA ATATC GTCAGT AACTGGAC-5'.

Total RNA of osteoblasts was isolated by using TRIzol reagent (Invitrogen) and reverse transcription was performed follow manufacturer's manual (BioTNT, Shanghai, China). Quantitative real-time PCR, enabling the quantification of relative gene expression, was performed using SYBR green DNA binding fluorescent dye. 10  $\mu$ L of QuantiTect™ SYBR Green PCR Master Mix, 4  $\mu$ L of QuantiTect™ SYBR Green primer assay (osteocalcin, b-actin; all provided by BioTNT), 5  $\mu$ L of RNase free water and 1  $\mu$ L of cDNA (1 ng/ $\mu$ L) were used for one reaction. Quantitative real-time PCR was performed in triplicates with the following cycler program: 95°C 10 min, denaturation step: 95°C 15 s, annealing step: 60°C 15 s, elongation step: 72°C 30 s; dissociation: 95°C 15 s, 60°C 1min, 95°C 15 s, 40 cycles were performed in total. B-actin was taken as an endogenous standard and relative gene expression was determined using the  $\Delta\Delta$ Ct method. Gene expression was compared by setting control cultures to 1 (reference value) as indicated in the relevant figures.

Quantitative analyses of TNF,  $\alpha$ , Ang II and AT1R expression were performed using a quantitative image analysis system (FR-2000, FR Science and technology Inc, Shanghai China). Because the pattern of expression of TNF $\alpha$ , Ang II and AT1R are diffuse in nature, the percentage of positive staining in the renal tissue was quantified under a  $\times 20$  power field of microscope. Briefly, up to 10 random areas of kidney with the early stage (media:intima  $\geq 1$ ) and advanced stage (media:intima  $< 1$ ) were chosen from each tissue section and examined. The examined area was outlined, the positive staining patterns were identified, and the percent positive area in the examined area was then measured. Data were expressed as the percentage of mean  $\pm$  SEM.

#### 4. Characterization of monoclonal anti-TGF- $\beta$ antibody

The reactivity of the produced monoclonal antibodies with Urine TGF- $\beta$  was screened by enzyme-linked immunosorbent assay (ELISA) using kit produced by Section living creature technique limited company of Hangzhou, China (NO,13409007) The sample solution (40  $\mu$ l) was incubated with the monoclonal anti- TGF- $\beta$  antibody (40  $\mu$ l) at room temperature for 1 h in an TGF- $\beta$ -transferrin attached microplate. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20, 0.1 ml of peroxidase-labelled goat F(ab')<sub>2</sub> fragment to mouse IgG(Fc) was added into the microplate, followed by incubation at room temperature for 1 h. After washing with PBS containing 0.05% Tween 20, 0.2 ml of o-phenylenediamine hydrochloride (1 mg/ml) containing 0.0124% H<sub>2</sub>O<sub>2</sub> was added to the microplate, and then incubated at room temperature for 30 min. The reaction was terminated with 1.3 M H<sub>2</sub>SO<sub>4</sub>. The absorption at 492 nm was measured.

##### 4.1 Statistical analysis

Data obtained from this study are expressed as the means  $\pm$  SEM. Statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA). Differences in blood pressure, serum creatinine, blood urea nitrogen, 24h urine protein and Urine TGF- $\beta$  at different time points (weeks 0 to 8) within the groups, and differences of Ang II and

AT1R activation, TNF- $\alpha$  expression and NF- $\kappa$ b accumulation in sham, control and HE86-treated animals were assessed by one-way analysis of variance, followed by t-test. Results were considered statistically significant when the P value was <0.05.

## 5. Result

Renal and systemic parameters obtained at 0 (before treatment), 32 and 64 days after Nx are given in Table 1-5, Figure 1-5. Nx groups exhibited limited growth compared with Sham. In all Nx groups except treatment group, body weights were statistically different from those observed before treatment. Average food intake was similar among groups.

## 6. Effects of HE-86 administration on biochemical parameters in uraemic rats

Table 2-3 shows the summary of renal function and 24h urine protein level. There was significant change in body weight between the control uraemic (control) and HE-86 treated uraemic (treatment) rats, although they were pair-fed. body weight of treatment group was showed more than control uraemic. Even 4 weeks after 5/6-nephrectomy, the levels of serum creatinine and BUN were markedly increased as compared to sham rats. Not only at 4 week but also at 8 week, the uraemic rats treated with HE-86 were manifested significantly decreased levels of serum creatinine, BUN, respectively. Urinary protein excretion was also suppressed obviously at 8 week as comparing with control uraemic rats.

	N	BUN(mmol/L)	Scr( $\mu$ mol/L)
sham	12	6.79 $\pm$ 0.70	26.25 $\pm$ 1.04
control	12	12.09 $\pm$ 3.37	50.56 $\pm$ 15.83
treatment	12	9.81 $\pm$ 2.93	38.83 $\pm$ 12.00#

Table 2. Serum creatinine and blood urea nitrogen after 4 week treatment. #P<0.05, ##P<0.01, when compared against empty vector-treated controls

	N	BUN(mmol/L)	Scr( $\mu$ mol/L)	24h urine protein(mg)
sham	12	9.31 $\pm$ 1.05	18.88 $\pm$ 1.55	22.34 $\pm$ 4.4
control	12	14.85 $\pm$ 2.83	53.38 $\pm$ 12.05	41.47 $\pm$ 8.07
treatment	12	13.62 $\pm$ 2.81	41.00 $\pm$ 10.51##	29.14 $\pm$ 5.68##

Table 3. Serum creatinine, blood urea nitrogen and twenty-four-hour urinary protein excretion after 8 week treatment. #P<0.05, ##P<0.01, when compared against empty vector-treated controls

## 7. Effects of HE-86 administration on mean arterial blood pressure in uraemic rats

After subtotal nephrectomy, hypertension developed in both HE-86 treatment and control uremic rats. Blood pressure was significantly elevated from second to eighth week after nephrectomy compared to sham-operated animals (P < 0.05-0.01), and the rise in blood pressure was equivalent (systolic blood pressure 180 to 200 mmHg) in control group. After using HE-86 liquid extract, hypertension was obviously suppressed in treatment group, showing average systolic blood pressure 140 to 160 mmHg (Table 4).

	Before treatment	After treatment			
		Second week	Forth week	Sixth week	Eighth week
sham	137.31±14.72	139.13±14.06	125.50±7.15	150.56±13.97	129.63±29.16
control	140.50±23.55*	212.46±43.26	199.92±23.55	156.33±20.72	202.44±15.09
treatment	141.77±26.45*	148.50±38.82**	152.46±29.54**	141.00±14.73#	176.00±30.70#

Table 4. Systolic blood pressure. Data represent the means  $\pm$  SEM for groups of twelve rats treated with either HE-86 or empty vector (#P<0.05, ##P<0.01, when compared against empty vector-treated controls; \*P<0.05, \*\*P<0.01, when compared to normal sham-controls).

### 8. Effects of HE-86 administration on urine TGF- $\beta$ 1

High excretion of urine TGF- $\beta$ 1, which express both glomerular and tubulointerstitial injuries. To demonstrate further the anti-inflammatory effect of HE-86 on rat chronic renal failure, we determined the TGF- $\beta$ 1 levels within the urine by ELISA. Results demonstrated that compared with vehicle, He-86 treatment significantly reduced urinary TGF- $\beta$ 1 levels, corrected by decrease level of serum creatinine, throughout the entire disease course (P<0.05), indicating that HE-86 treatment may primarily suppress the local immune and inflammatory response within the diseased kidney. In contrast, overexpression of urine TGF- $\beta$ 1 was found in control uraemic rats as compared with normal rats (Table 5). The experimental result showed the administration of HE-86 significantly inversed high expression of urine TGF- $\beta$  in uraemic rats, manifesting HE-86 to attenuate the development of glomerular sclerosis.

	N	Urine TGF- $\beta$ (ug/L)
sham	12	1.83±0.64
control	12	1.90±0.56*
treatment	12	1.77±0.43#

Table 5. Effect of HE-86 liquid extract on urine TGF- $\beta$  excretion in 5/6 nephrectomy in rats. (#P<0.05, ##P<0.01, when compared against empty vector-treated controls; \*P<0.05, \*\*P<0.01, when compared to normal sham-controls)

### 9. Effects of HE-86 administration on localization of NF- $\kappa$ B in renal tissue

Immunohistochemical analysis was performed to determine the localization of NF- $\kappa$ B in the renal cortex (Fig.1-2). NF- $\kappa$ B, a critical transcriptional factor for controlling inflammatory response, has been shown to play a central role in inflammatory diseases, including kidney diseases [33]. In normal rats, only tubular epithelial cells were weakly stained by the monoclonal anti-NF- $\kappa$ B antibody, while glomeruli were hardly stained. In control uraemic rats, however, proximal tubular epithelial cells, especially of dilated tubules, were intensively stained by the anti-NF- $\kappa$ B antibody. In contrast, in the HE-86-treated uraemic rats activation of the NF- $\kappa$ B in tubular epithelial cells was less prominent as compared with that in the control uraemic rats. The staining of NF- $\kappa$ B as shown in the control uraemic rats found increased NF- $\kappa$ B -positive (intensively stained) area in the renal cortex, whereas HE-86-treated rats showed markedly decreased NF- $\kappa$ B -positive area as compared to the control uraemic rats. These data demonstrate that HE-86 markedly reduces the overexpress of NF- $\kappa$ B on the remnant tubular cells.

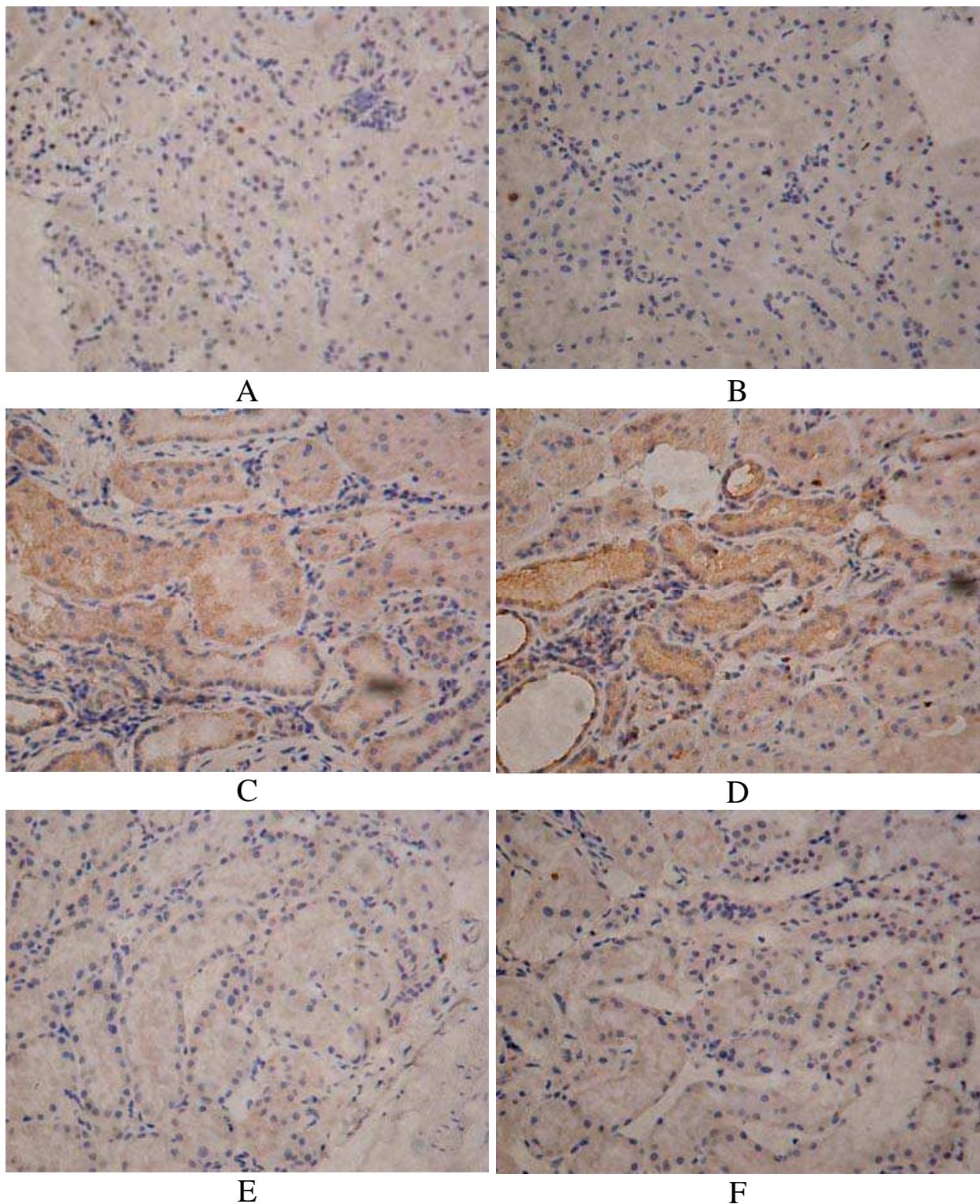


Fig. 1. Immunohistochemistry demonstrates that HE-86 inhibits renal NF- $\kappa$ B accumulation within the kidney. The accumulation of NF- $\kappa$ B in the glomerular and tubulointerstitium is markedly increased in empty vector-treated animals (C, D), compared to normal sham-controls (A,B), which is substantially inhibited in 5/6 nephrectomized rats treated with HE-86 (E, F). Original magnifications,  $\times 100$ .

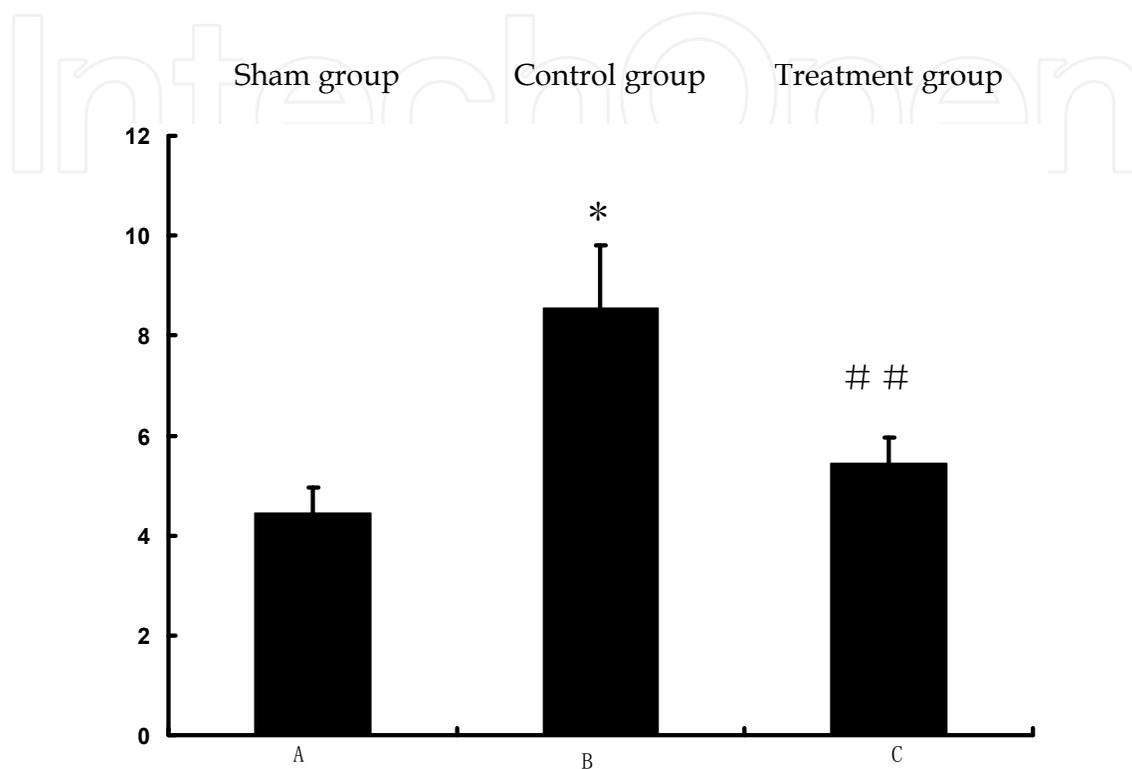


Fig. 2. Semiquantitative analysis of the therapeutic effect of HE-86 on NF- $\kappa$ B localization in the glomerulus and tubulointerstitium using the Quantitative Image System. A: Percentage of glomerular and tubulointerstitial NF- $\kappa$ B deposition in sham group. B: Percentage of NF- $\kappa$ B localization in glomerular and tubulointerstitial without treatment C: Percentage of glomerular and tubulointerstitial NF- $\kappa$ B accumulation in twelve rats treated with HE-86 was decreased significantly. Each bar represents data (mean  $\pm$  SEM) #,  $P < 0.05$  and ##,  $P < 0.001$ , when compared to empty vector-treated controls; \*,  $P < 0.05$  and \*\*,  $P < 0.01$ , when compared to the normal sham-control.

### 10. Effects of HE-86 administration on mRNA levels of TNF- $\alpha$ , Ang II and AT II R in renal tissue

The effects of HE-86 on the gene expression of Ang II (Figure 3), AT1R (Figure 4) and TNF- $\alpha$  (Figure 5) in the renal cortex were examined. We investigated the potential

mechanisms whereby HE-86 suppressed rat tubular interstitial fibrosis and glomerular cirrhosis.  $TNF-\alpha$ , being key proinflammatory cytokines in anti-GBM glomerulonephritis, and a group of chemotactic and adhesion molecules including ICAM-1, MCP-1, was examined. In vehicle-treated chronic renal failure rats, there was a substantial increase in renal mRNA expression of  $TNF-\alpha$ . Treatment with HE-86 significantly reduced upregulation of  $TNF-\alpha$  inflammatory genes examined ( $P < 0.05$ ). Furthermore, HE-86 was capable of attenuating renal cortical mRNAs for Ang II and AT1R as compared with the control uraemic rats when they were administered after the establishment of nephrectomized. However, the renal mRNA levels of Ang II and AT1R were markedly increased in control uraemic rats as compared with normal rats. The variation in the mRNA levels of  $TNF-\alpha$ , Ang II and AT1R in both HE-86-treated and control uraemic rats are related to variation in the extent of CRF.

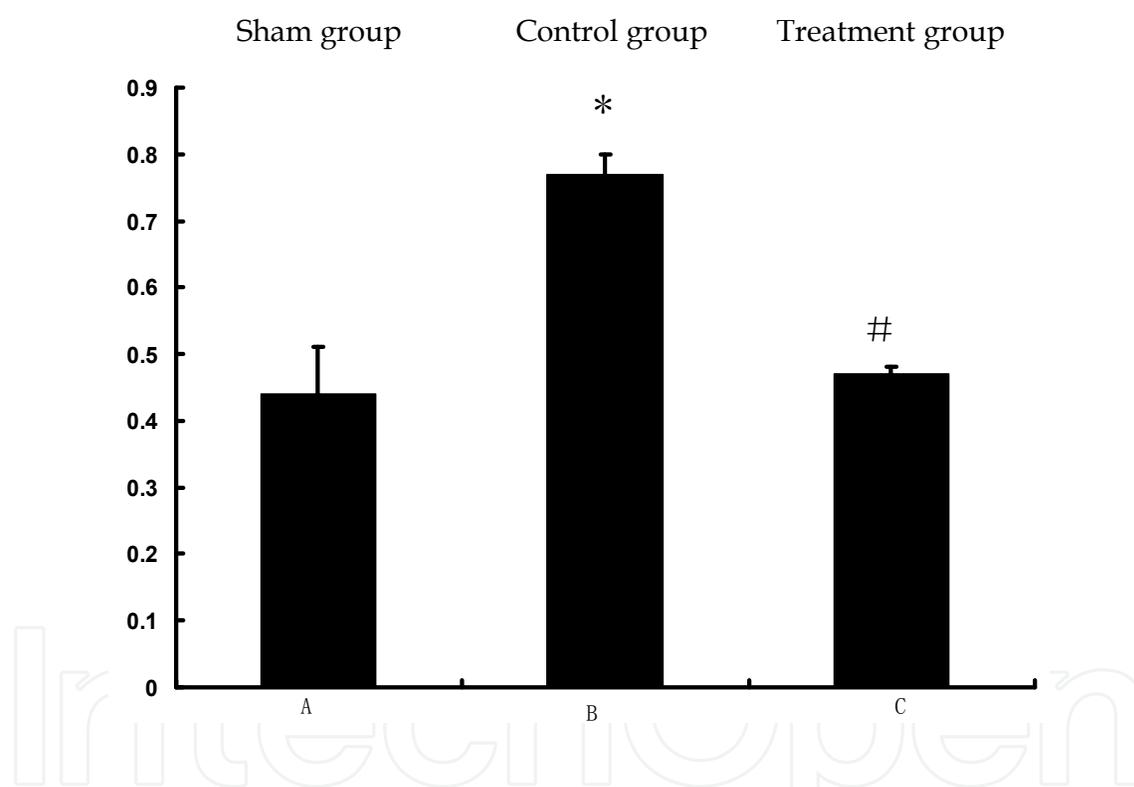
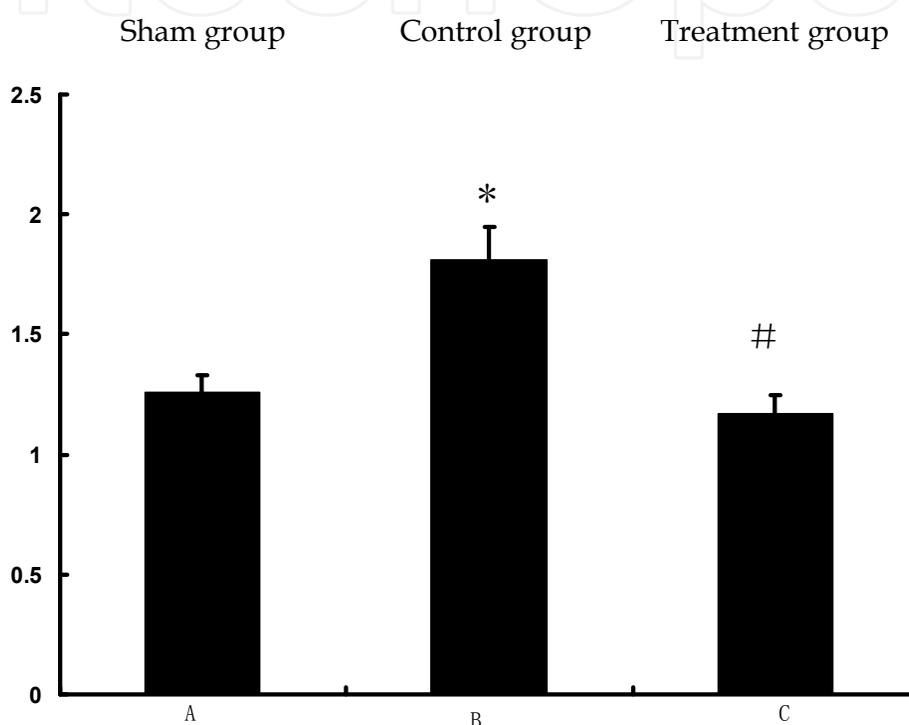


Fig. 3. Real-time PCR reveals the inhibitory effect of HE-86 liquid extract on renal Ang II mRNA expression(A). and Semiquantitative analysis of the therapeutic effect of HE-86 on Ang II mRNA localization in the glomerulus and tubulointerstitium using the FR-2000 Image Analyze System. A: Degree of glomerular and tubulointerstitial Ang II mRNA expression in sham group. B: Numbers of Ang II mRNA expression in glomerular and tubulointerstitial without treatment C: Numbers of glomerular and tubulointerstitial cells with nuclear localization of Ang II mRNA in twelve rats treated with HE-86 was decreased significantly. Each bar represents data (mean  $\pm$  SEM) #,  $P < 0.05$  and ##,  $P < 0.01$ , when compared to empty vector-treated controls; \*,  $P < 0.05$  and \*\*,  $P < 0.01$ , when compared to the normal sham-control.

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Fig. 4. Real-time PCR reveals the inhibitory effect of HE-86 liquid extract on renal AT1RmRNA expression(B). and Semiquantitative analysis of the therapeutic effect of HE-86 on AT1RmRNA localization in the glomerulus and tubulointerstitium using the FR-2000 Image Analyze System. A: Degree of glomerular and tubulointerstitial AT1RmRNA expression in sham group. B: Numbers of AT1RmRNA expression in glomerular and tubulointerstitial without treatment C : Numbers of glomerular and tubulointerstitial cells with nuclear localization of AT1RmRNA in nephrectomized rats treated with HE-86 was decreased significantly. Each bar represents data (mean  $\pm$  SEM) #,  $P < 0.05$  and # #,  $P < 0.01$ , when compared to empty vector-treated controls; \*,  $P < 0.05$  and \*\*,  $P < 0.01$ , when compared to the normal sham-control

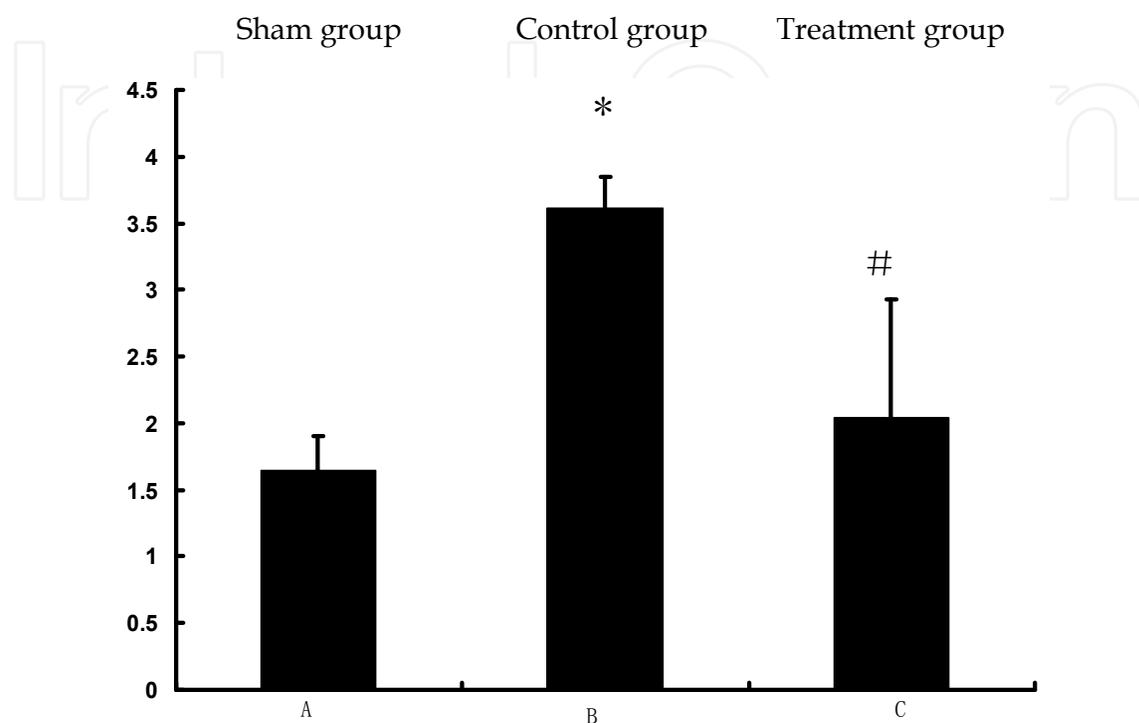


Fig. 5. Real-time PCR reveals the inhibitory effect of HE-86 liquid extract on renal TNF- $\alpha$  mRNA expression (C). and Semiquantitative analysis of the therapeutic effect of HE-86 on TNF- $\alpha$  mRNA within the glomerulus and tubulointerstitium using the FR-2000 Image Analyze System. A: Degree of glomerular and tubulointerstitial TNF- $\alpha$  mRNA expression in sham group. B: Numbers of TNF- $\alpha$  mRNA expression in glomerular and tubulointerstitial without treatment C: Numbers of glomerular and tubulointerstitial cells with nuclear localization of TNF- $\alpha$  mRNA in nephrectomized rats treated with HE-86 was decreased significantly. Each bar represents data (mean  $\pm$  SEM) #,  $P < 0.05$  and # #,  $P < 0.01$ , when compared to empty vector-treated controls; \*,  $P < 0.05$  and \*\*,  $P < 0.01$ , when compared to the normal sham-control.

## 11. Discussion

Renal fibrosis is a final common pathway to end-stage renal disease. Recent studies have shown that hypertensive nephropathy is a major leading cause of end-stage renal disease and the renin-angiotensin system plays a pivotal role in the development of progressive renal injury [34-35]. Clinical trials have shown that blocking the effects of angiotensin II (Ang II) with angiotensin-converting enzyme inhibitors and angiotensin-receptor blockers

can prevent or slow the progression of kidney damage in patients with diabetes and hypertension [34-36].

As expected, 5/6 renal ablation promoted growth retardation, systemic arterial hypertension, impaired renal function, and severe albuminuria. These functional changes were accompanied by severe glomerulosclerosis, as well as expansion and intense macrophage infiltration of the interstitial area. Mounting evidence indicates that these renal structural abnormalities, which are characteristic of the Nx and other models of progressive nephropathies, are a consequence of the concerted action of mechanical stress, caused by glomerular hypertension and hypertrophy [37-38], and inflammatory phenomena, comprising cell infiltration and/or proliferation and extracellular matrix accumulation [38-39]. Moreover, a causal relationship appears to exist between these phenomena, because the distension of the glomerular walls due to intracapillary hypertension may trigger the local release of cytokines, growth factors, and, particularly, Ang II and AT-1 receptors [40-41].

The beneficial effect of RAS suppressors was initially attributed to amelioration of the glomerular hemodynamic dysfunction associated with progressive nephropathies. However, recent observations suggest that the nonhemodynamic effects of RAS suppressors may be equally important, given the strong proinflammatory and profibrotic effects of Ang II [42]. A substantial fraction of this proinflammatory ANG II may originate in the renal parenchyma, rather than in renal vessels or in the systemic circulation [43]. Increased intrarenal production of ANG II was described in various models of renal fibrosis [44-46]. A preliminary report has suggested that, in the 5/6 renal ablation (Nx) model, ANG II is expressed in renal interstitial cells, paralleling the severity of renal injury [47].

Increasing evidence shows that angiotensin II (Ang II) plays a critical role in cardiovascular disease and is a key mediator in the process of vascular fibrosis, characterized by reduced lumen diameter and arterial wall thickening attributable to excessive deposition of extracellular matrix (ECM). Vascular fibrosis is a major complication of hypertension and diabetic mellitus. It has been shown that upregulated tissue rennin-angiotensin system is involved in development of vascular lesions in both human and experimental vascular diseases [48-49]. This observation is confirmed by the finding that infusion of Ang II is able to induce vascular fibrosis in rats [50]. The functional importance of Ang II in vascular fibrosis is further supported by the evidence that blockade of Ang II inhibits vascular fibrosis in diabetic and subtotal nephrectomy rats and NO-deficient mice [51-53].

Both the hemodynamic and proinflammatory effects of Ang II are mediated by AT-1 receptors (AT1R) [54], extensively expressed in renal tissue. In the normal rat kidney, AT1R are predominantly expressed in tubular cells and vessels [55]. Recent data obtained with the Nx model have suggested that AT1R expression is shifted from the glomerular to the tubulointerstitial compartment 4 wk after ablation [56]. However, the renal distribution of AT1R in this model and its temporal evolution have not been established.

Beyond its hemodynamic effects, Ang II is recognized as a cytokine with an active role in cardiovascular remodeling. It is well known that Ang II signals through its Ang II receptor 1 (AT1) receptor to exert most of its biological functions [57]. After binding to the AT1 receptor, Ang II activates multiple downstream intracellular signaling pathways, including tyrosine kinase, mitogen-activated protein kinase (MAPK), p38, and Janus family kinase

[58]. Activation of these pathways leads to numerous heterogeneous downstream events that play essential roles in the biological activities of Ang II, such as cell growth and migration, ECM production, and apoptosis [58].

Renal expression of AT1R in rats appeared mostly in tubular cells, and to a lesser extent, at the interstitial area, whereas weaker expression was seen in vessels and glomeruli. This pattern was completely disrupted after Nx, when dense AT1R expression could be demonstrated in interstitial cells, far exceeding in intensity the expression of AT1R in tubules. The exact meaning of this finding and the cell types involved are uncertain. Several inflammatory cells known to infiltrate the renal interstitium in the Nx model have the potential to express AT1R, such as lymphocytes [59] and macrophages [60]. In addition, AT1R may be expressed by myofibroblasts originating from tubular cell transdifferentiation [61]. This hypothesis is particularly attractive because it helps to explain the progressive shift in AT1R expression, from tubules to the interstitial area, observed in Nx rats, and also because tubular cells already express AT1R under normal conditions. The simultaneous presence at the interstitial area of large amounts of Ang II and of the AT1R may accelerate the progression of the nephropathy by a positive-feedback mechanism. Consistent with this view is the aggravation of the renal structural injury of Nx, which was paralleled by the intensity of the inflammatory infiltration and of the interstitial expression of Ang II.

It is well accepted that NF- $\kappa$ B is a key transcriptional factor to regulate a variety of inflammatory responses [75]. NF- $\kappa$ B is composed of p50 and p65 subunits, among which p65 is a potent transcriptional activator, strongly promoting inflammatory reaction in kidney diseases [76]. NF $\kappa$ B total protein expression, and inflammation, which may have resulted from blockade of the oxidative stress pathway [77-78]. This was accompanied by a substantial attenuation in renal fibrosis, which might have resulted from the modulating actions of vitamins on lipid peroxidation and profibrotic activity involved in renal tissue damage [79-82]. In this study, marked activation of NF- $\kappa$ B was closely correlated with the renal inflammation. In our study, using liquid extract isolated from clinical effective Chinese prescription, we were able to show that overexpression activation of NF- $\kappa$ B was substantially suppressed as compared with control group. These findings are consistent with the improving renal function and correcting high blood pressure.

Tumour necrosis factor- $\alpha$ (TNF- $\alpha$ ) is a potent pro-inflammatory cytokine which is produced by many cell types including monocytes/macrophages, and renal mesangial and epithelial cells. It induces the expression of major histocompatibility complex (MHC) class I and II molecules, endothelial adhesion molecules and procoagulant activity of endothelium. TNF- $\alpha$  stimulates the release of other pro-inflammatory cytokines, chemokines and growth factors, including interleukin-1 $\beta$ (IL-1 $\beta$ ), monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor- $\beta$ (TGF- $\beta$ ) [83-84]. The biological effects of TNF- $\alpha$  are mediated by binding to specific receptors which are widely distributed. TNF- $\alpha$  binds to two types of receptor: TNF receptor type 1 and TNF receptor type 2, which have molecular weights of 55 kDa (p55) and 75 kDa (p75), respectively. Both receptors are necessary and act synergistically for cell proliferation and maturation, cytotoxicity and antiviral activity, but p55 is responsible for activation of NF $\kappa$ B and mediation of apoptosis [85].

TNF- $\alpha$  may contribute to renal damage by inciting an inflammatory response within the kidney via induction of a variety of chemokines and adhesion molecules [86-87]. There is a

mounting evidence to implicate TNF- $\alpha$  in the pathogenesis of glomeruli of rodents with experimental nephritis, and is found in renal biopsies, sera and urine of patients with different types of glomerulonephritis [88-91]; In vitro and in vivo studies document that TNF- $\alpha$  is produced locally within inflamed glomeruli by mesangial and epithelial cells, as well as by infiltrating monocytes/macrophages [89,91]; Systemic administration of TNF- $\alpha$  results in glomerular damage in rabbits [92] and exacerbates the degree of glomerular injury in nephrotoxic nephritis in rats [93]; and blocking endogenous TNF- $\alpha$  in nephrotoxic nephritis in rats ameliorates acute glomerular inflammation [94], and down-regulates glomerular IL-1 $\beta$ mRNA and circulating TNF- $\alpha$  concentrations [95].

Treatment of Nx rats with the HE-86 promoted a significant regression of hypertension, high level of creatinine and blood urea nitrogen, albuminuria, and inflammatory signs such as urine TGF- $\beta$  and renal tissue TNF- $\alpha$ , NF- $\kappa$ B, Ang II and AT1R expression, whereas the parameters of renal structural tissue injury were strongly attenuated, compared with pretreatment levels. The protection achieved with effective unit from clinical prescription treatment was much greater than that obtained with traditional prescription alone. On the basis of the present study, we cannot exclude the hypothesis that the success of HE-86 was due to a particularly effective hemodynamic action, although previous observations from this laboratory [96] indicated that NOF, a new nonsteroidal anti-inflammatory, had no significant effect on glomerular hemodynamics. Because treatment with NOF alone had no effect on blood pressure, it seems unlikely that the hemodynamic effect of NOS was directly intensified by its association with NOF. Therefore, the efficacy of extract HE-86 was likely due to the simultaneous blockade of the hemodynamic and proinflammatory actions of Ang II, AT1R and its derivatives as TNF- $\alpha$ , NF- $\kappa$ B, TGF- $\beta$  and by abrogation of the complex interplay between hypertension and inflammation. The present findings support other scholars' observations of the Nx model, which similarly indicated the superiority of the combination of a RAS suppressor with an anti-inflammatory agent [97-99]. It is noteworthy that HE-86 afforded partial regression of the nephropathy associated with Nx even though it was started 4 week after surgery, when renal injury was already established. This observation suggests that both continued stimulation of Ang II and AT1 receptors and production of inflammatory factors continue to play an important pathogenic role even during the late phases of the process, necessitating vigorous and persistent treatment to prevent further renal deterioration.

Taken together with our previous data and the present results, it is likely that HE-86-induced reduction of renal rennin-angiotensin system is mediated, at least partly, by reducing the overload of inflammatory factors activity on remnant kidney unit. In summary, HE-86 effective composition coming from clinical validly treating patients with chronic renal failure especially for early and middle stage, partially reversed the nephropathy and renal inflammation associated with the Nx model, showing much more effective protection than with traditional Chinese medicine prescription.

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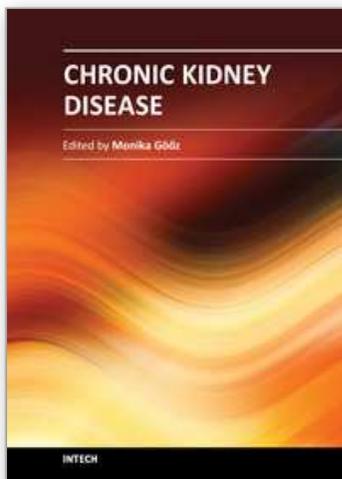
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## **Chronic Kidney Disease**

Edited by Prof. Monika Gööz

ISBN 978-953-51-0171-0

Hard cover, 444 pages

**Publisher** InTech

**Published online** 16, March, 2012

**Published in print edition** March, 2012

Chronic kidney disease is an increasing health and economical problem in our world. Obesity and diabetes mellitus, the two most common cause of CKD, are becoming epidemic in our societies. Education on healthy lifestyle and diet is becoming more and more important for reducing the number of type 2 diabetics and patients with hypertension. Education of our patients is also crucial for successful maintenance therapy. There are, however, certain other factors leading to CKD, for instance the genetic predisposition in the case of polycystic kidney disease or type 1 diabetes, where education alone is not enough.

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Li-qun He, Dong Feixia, Qiang Fu and Jun Li (2012). Molecular Mechanisms of Nephro-Protective Action of HE-86 Liquid Extract in Experimental Chronic Renal Failure, *Chronic Kidney Disease*, Prof. Monika Gööz (Ed.), ISBN: 978-953-51-0171-0, InTech, Available from: <http://www.intechopen.com/books/chronic-kidney-disease/molecular-mechanisms-of-nephro-protective-action-of-he-86-liquid-extract-in-experimental-chronic-ren>

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