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Antihypertensive and renal protective effect of Shunaoxin pill combined with captopril on spontaneous hypertension rats

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ABSTRACT

Introduction: According to previous reports, hypertension has become the most common chronic disease in the world. Captopril, an angiotensin-converting enzyme inhibitor, has been widely used for the therapy of arterial hypertension and cardiovascular diseases therapy. Besides, Shunaoxin pill (SNX) as a traditional Chinese prescription showed antihypertensive effect in our previous research.
Objective: This study means to investigate whether SNX combining with captopril could show antihypertensive and renal protective effects on spontaneous hypertension rats (SHRs).
Methods: SHRs were randomly assigned to four treatment groups, including non-treated group, captopril, SNX, and captopril + SNX-treated groups. Their body weight and systolic blood pressure (SBP) were measured weekly. Histopathological examination was analyzed through Masson staining and hematoxylin and eosin staining. Biochemical analyses, ELISA, and western blot were used to analyze their combining mechanism.
Results: In this experiment, this combinatorial therapy significantly reduced aortic wall thickness, increased the content of NO, NOS and eNOS, decreased the content of bradykinin and endothelin 1(ET-1), and regulated the levels of TG, TC and HDLC back to normal, which suggested they could induce vasodilation and lower blood pressure. Meanwhile, histological examination alleviated that captopril + SNX remarkably inhibited renal injury, including tubular disorder, inflammatory cell infiltration and fibrosis. They down-regulated the serum levels of BUN and Cr, protein expression of IL-1β, NF-κB, Bax, Cyt c, caspase 3, 8 and 9 in kidney tissues and significantly increased the levels of Bcl-2 in kidney tissues compared with monotherapy group.
Conclusion: The combinatorial treatment of SNX and captopril lowered blood pressure through adjusting NO/NOS, ET-1 and dyslipidemia profile. Furthermore, this treatment alleviated the kidney damage via reducing the release of inflammatory factors and the expression of apoptotic markers. Therefore, these results provided a rationale for future clinical use of SNX combined with captopril in antihypertensive and protecting renal functions in hypertension.

Abbreviations: Ang II, angiotensin II; BUN, blood urea nitrogen; cGMP, cyclic guanosine monophosphate; Cr, creatinine; Cyt c, cytochrome c; eNOS, endothelial nitric oxide synthase; ET-1, endothelin 1; HDLC, high-density lipoprotein cholesterol; HE, hematoxylin and eosin; IL, interleukin; LDLC, low-density lipoprotein cholesterol; NF-κB, nuclear transcription factor-κB; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; TC, total cholesterol; TG, triglyceride; TNF-α, tumor necrosis factor-alpha; TNOS, total nitric oxide synthase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; WKY, wistar kyoto rat

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1. Introduction

According to previous reports, hypertension has become the most common chronic disease in the world [1]. Meanwhile, more than a quarter of Chinese adults had high blood pressure, whose number was rising sharply [2]. Hypertension was accompanied by a variety of complications, such as kidney damage, fatty liver and so forth [3]. As an angiotensin converting enzyme inhibitor, captopril was widely used in the treatment of hypertension and cardiovascular diseases. It alleviated hypertension-induced renal damage through the inhibition of inflammation and NF-κB activation [4].

Shunaoxin pill (SNX), a traditional Chinese medicine product, is

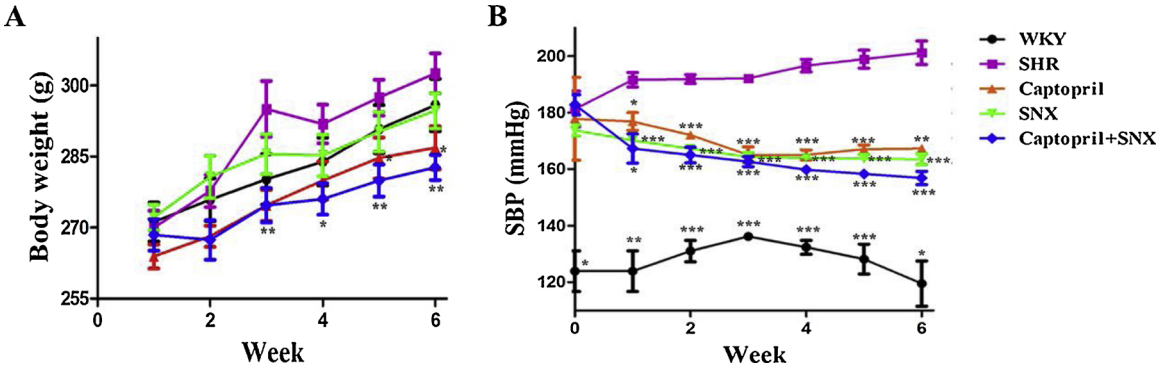

Fig. 1. Effect on body weight and SBP of different groups. (A) Body weight, (B) Systemic blood pressure (SBP). The results were presented as the means ± standard error (n = 6), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. SHR group.

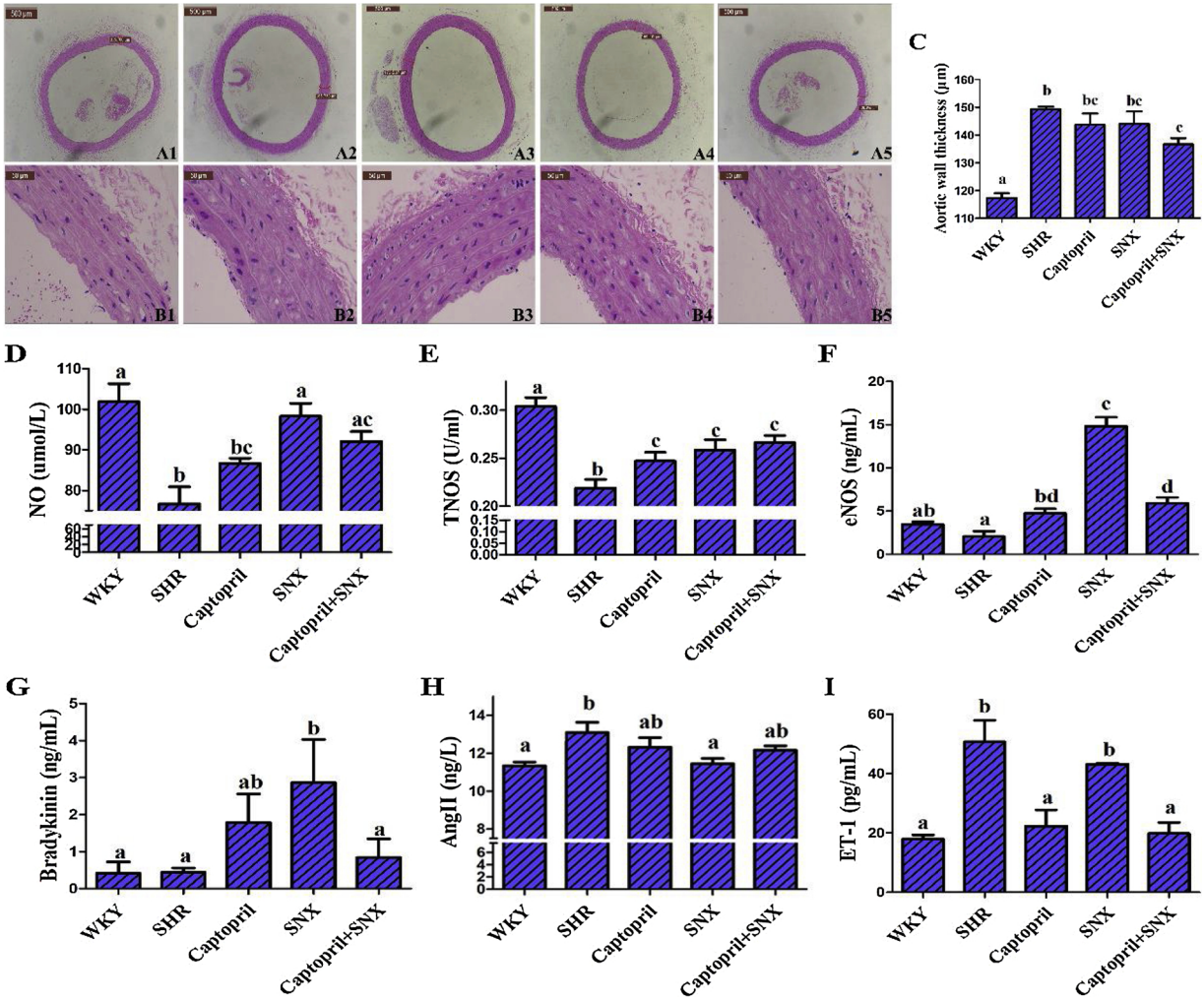

Fig. 2. Effects of drug combination on histopathologic examination and NO, NOS, eNOS, Bradykinin, Ang II and ET-1 in rats. Representive photomicrographs for aorta, magnification: 40 X (A) and 400 X (B). Groups listed as follows, (1) WKY group, (2) SHR group, (3) Captopril group, (4) SNX group and (5) Captopril + SNX group. (C) Analysis of aortic wall thickness. The levels of (D) NO, (E) TNOS, (F) eNOS, (G) Bradykinin, (H) Ang II and (I) ET-1 in serum. The results were presented as the means ± standard error (n = 6). Different letters meant significant differences between two groups. Letters shared in common between or among the groups indicated no significant differences ($p < 0.05$).

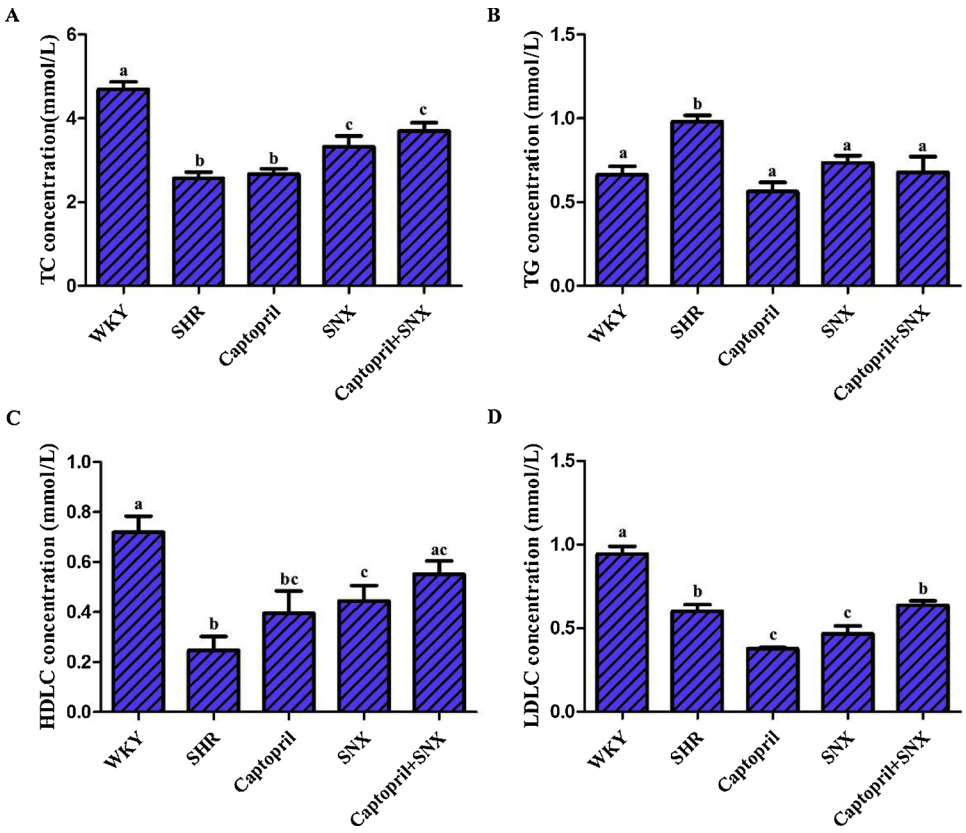

Fig. 3. Effects on lipid metabolism in rats. The levels of (A) TC, (B) TG, (C) HDLC and (D) LDLC in serum. The results were presented as the means ± standard error (n = 6). Different letters meant significant differences between two groups. Letters shared in common between or among the groups indicated no significant differences ($p < 0.05$).

developed from the "Decoction of Xionggui" that it originated from an ancient book of the Ming dynasty named Puji Fang. It was a famous classical recipe in traditional Chinese prescription composed of *Chuanxiong* (*Ligusticum chuanxiong Hort*, *Umbelliferae*) and *Danggui* (*Angelica sinensis radix*, *Umbelliferae*). In our previous studies, the active components in SNX were identified by UPLC-Q-TOF-MS, mainly containing ferulic acid, senkyunolide I, senkyunolide A, Z-ligustilide and levistolide A. SNX has an effect on reducing blood pressure of spontaneously hypertensive rat, which may be related to reduce angiotensin II (Ang II) level and increase nitric oxide synthase level [5]. SNX induced both of endothelium-dependent and endothelium- independent vascular relaxation [6]. In clinic, SNX combined with conventional medical treatment can better reduce the pulse pressure level of hypertensive patients, reduce the variability of patients' blood pressure, and make the blood pressure drop steadily [7]. Furthermore, it demonstrated that both *Chuanxiong* and *Danggui* could reduce kidney injury through the reduction of the inflammatory response [8,9]. The combination of *Chuanxiong* and *Danggui* could also synergistically promote vasorelaxation in isolated rat aortas and spontaneously hypertensive rats (SHR) [10].

In the treatment of hypertension, combination therapy has more advantages than monotherapy, including complementary effects of antihypertensive mechanisms, superposition of efficacy, and ultimate reduction of adverse reactions of the disease [11]. In this study, we tried to explore the combination of SNX and captopril on blood pressure and renal protection in SHR.

2. Materials and methods

2.1. Drugs

SNX were donated by Tianjin Zhongxin Pharmaceutical Group Co., Ltd. (Tianjin, China). Captopril tablets were obtained from Shanghai HengShan Pharmaceutical Co. (Shanghai, China).

2.2. Animals

24 male spontaneously hypertensive rats (SHR) (210 ± 10 g, 12 weeks olds) and 6 normotensive control Wistar Kyoto rats (WKY) (210 ± 10 g, 12 weeks olds) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (License No. SCXK (Jing) 2016-0006, Beijing, China). They were housed under standard conditions with free access to food and water. Before experiments, the rats were allowed a one-week of acclimation in the animal quarters under controlled temperature (23 ± 2 ℃), humidity (55 ± 15 %) and photoperiod (12 h light, 12 h dark). This animal study was approved by the Institutional Animal Care and Use Committee of China, and institutional guidelines for animal welfare and experimental conduct were followed.

2.3. Drug administration and blood pressure measurement

SHRs were randomly assigned to four treatment groups. Group I (SHR): gavage of 0.9 % saline solution every day. Group II (Captopril): gavage of captopril (18.75 mg/kg body weight) in 0.9 % saline solution every day. Group III (SNX): gavage of SNX (1008 mg/kg body weight) in 0.9 % saline solution every day. Group IV (Captopril + SNX): gavage of captopril (18.75 mg/kg body weight) and SNX (1008 mg/kg body weight) in 0.9 % saline solution every day. WKY group were treated with an equivalent volume of 0.9 % saline solution (WKY). The treatment lasted for 6 weeks. Body weight and systolic blood pressure (SBP) were measured every week with the tail-cuff plethysmograph method using DB128 noninvasive blood pressure system (Bejing Zhishuduobao Biological Technology Co. LTD, China) [12]. After 6 weeks, blood samples were collected from femoral artery and immediately processed for serum by centrifugation at 4000*g* for 10 min. Serum samples were frozen and maintained at -20 ℃ until analysis. Then the rats were sacrificed. Kidney tissues were dissected. Parts of these organs were frozen and maintained at -80 ℃ until analysis. Meanwhile, parts of

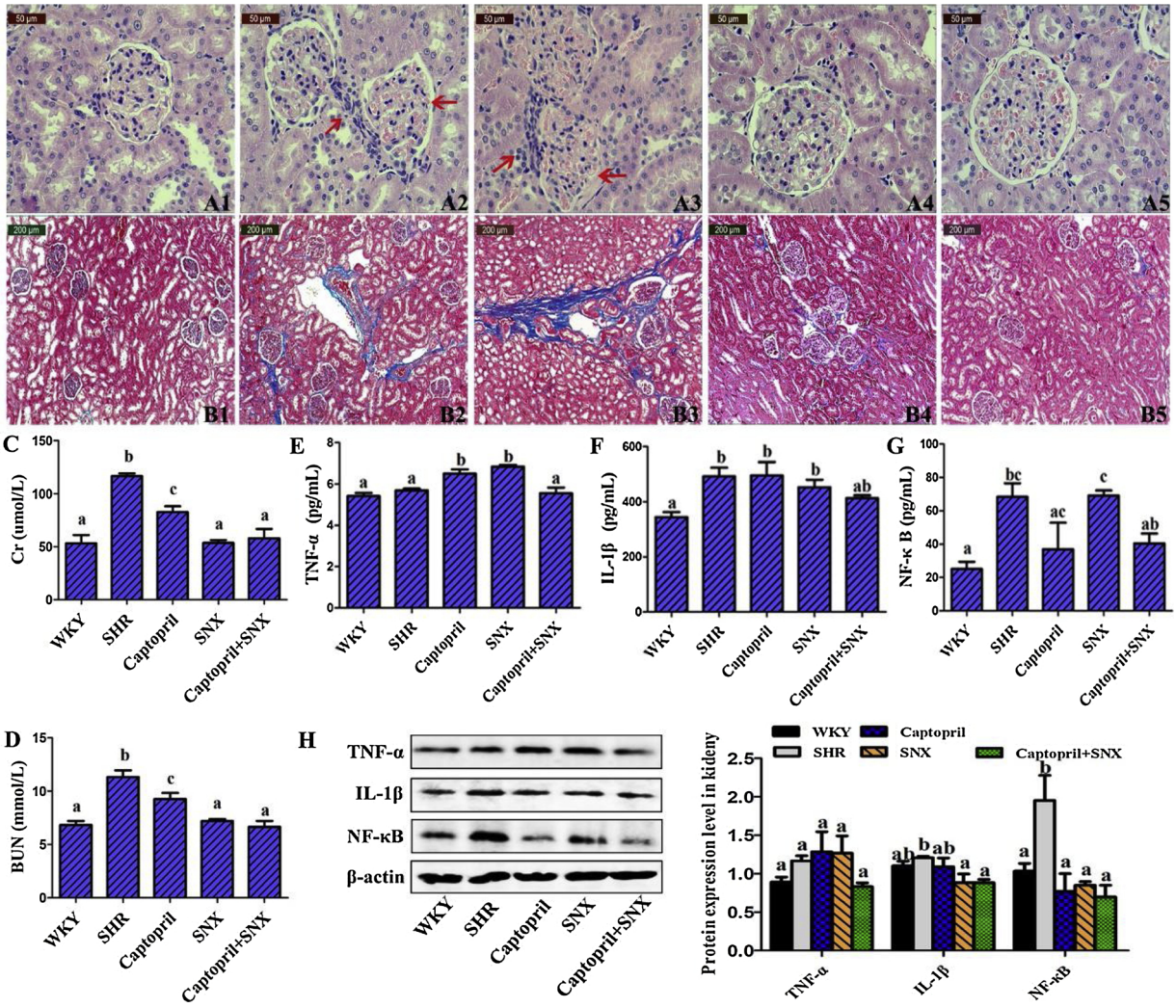

Fig. 4. Effects of drug combination on renal functional and inflammation in rats. (A) Histopathological examination of the renal among different experimental groups using H&E staining (400×). (B) Representative histological changes in renal sections using Masson's trichrome staining (original magnification, 100×). Arrows indicated inflammatory cells. Groups listed as follows, (1) WKY group, (2) SHR group, (3) Captopril group, (4) SNX group and, (5) Captopril + SNX group. The levels of (C) Cr, (D) BUN (E) TNF-α, (F) IL-1β and (G) NF-κB in serum and (H) inflammatory cytokine-associated proteins in renal from different groups. The results were presented as the means ± standard error (n = 6). Different letters meant significant differences between two groups. Letters shared in common between or among the groups indicated no significant differences ($p < 0.05$).

them were fixed in 4 % paraformaldehyde for Masson staining and hematoxylin and eosin (HE) staining analysis.

2.4. Histopathological examination

For histopathological examination, formalin preserved aorta and kidney tissues were processed consecutively for dehydration and clearing by using graded alcohol series and xylene, respectively. Then the dehydrated tissues were embedded in paraffin blocks. 5 μm thickness sections were cut from paraffin-embedded aorta and kidney tissues, and stained with Mayer's hematoxylin and eosin. Moreover, the kidney tissues slices were also stained with Masson's trichrome staining for collagen detection. Collagen will be stained blue, nucleus stained dark brown, while muscle fiber stained red in Masson's trichrome. Aorta and renal pathological changes were detected by morphological analysis under an optical microscope (Leica, DM500). The graphics were captured by a digital camera (Leica, ICC50 W). The aortic wall thickness was analyzed using the LAS V4.8 (Leica, Germany).

2.5. Biochemical analyses

Serum levels of urea nitrogen (BUN), creatinine (Cr), high-density lipoprotein cholesterol (HDLC), low-density lipoprotein cholesterol (LDLC), total cholesterol (TC), triglyceride (TG), nitric oxide (NO) and total nitric oxide synthase (TNOS) were measured by the detection kits according to the manufacturer's instructions (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China).

2.6. Measurement of endothelial nitric oxide synthase (eNOS), bradykinin, Ang II and endothelin 1(ET-1) levels

Serum samples were analyzed for AngII (Nanjing SenBeiJia Biological Technology Co., Ltd. China), eNOS, bradykinin and ET-1 (Quanzhou Ruixin Biological Technology Co., Ltd. China) with rats ELISA kits according to the manufacturer's instructions. Each value of the sample was measured within the linear portion of the standard curve.

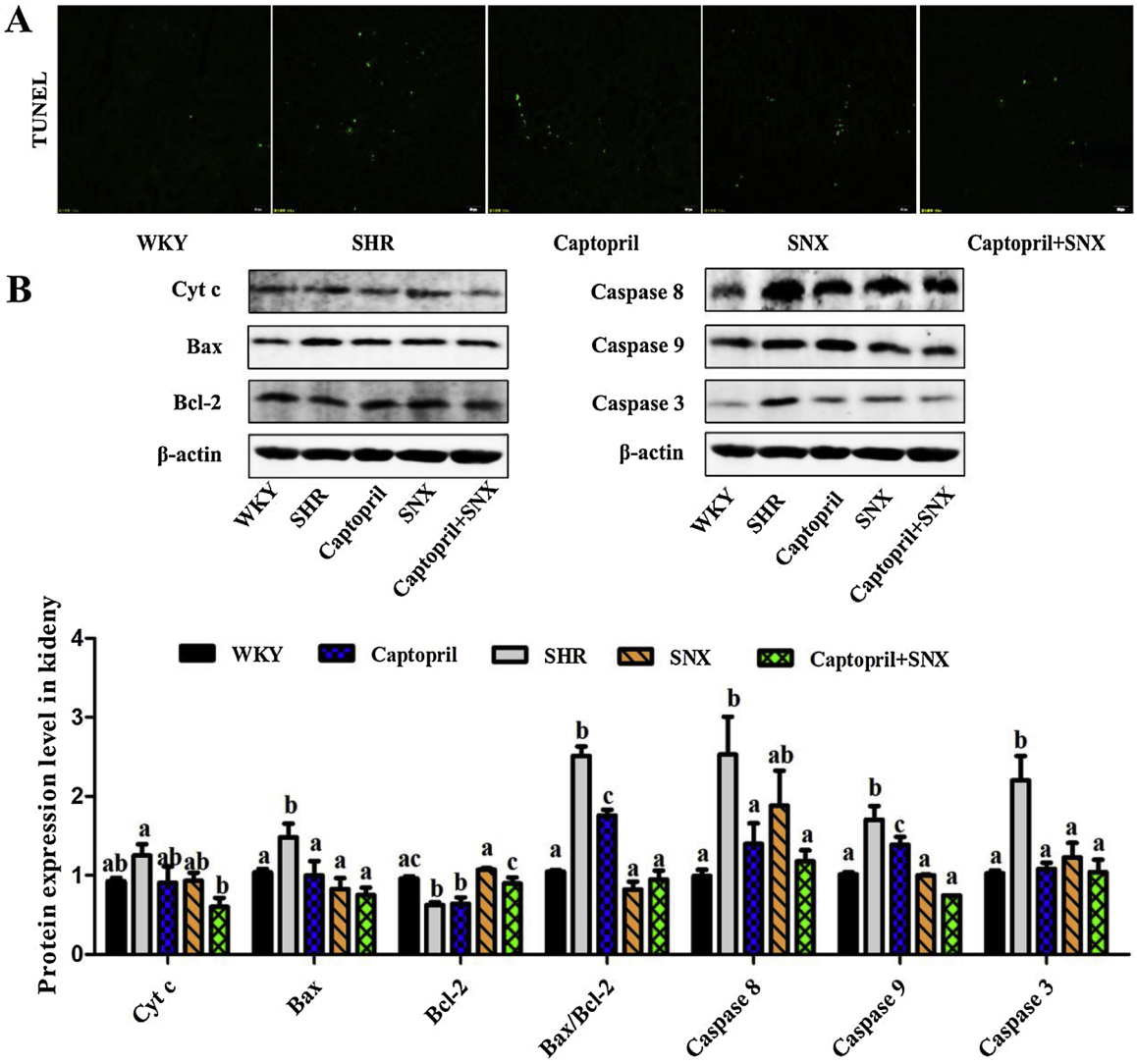

Fig. 5. Effects of drug combination on apoptosis in rats. (A) TUNEL fluorescence staining kidney sections illustrated the apoptotic nuclei. The green was labeled apoptosis. (B) Apoptosis-related proteins in renal from different groups. Different letters meant significant differences between two groups. Letters shared in common between or among the groups indicated no significant differences ($p < 0.05$).

2.7. Measurement of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and nuclear transcription factor-κB (NF-κB) levels

Serum samples were analyzed for NF-κB (Quanzhou Ruixin Biological Technology Co., Ltd. China), TNF-α and IL-1β (Multi Sciences Biotech Co., Ltd. China) with rats ELISA kits according to the manufacturer's instructions. Each value of the sample was measured within the linear portion of the standard curve.

2.8. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining

Fresh kidney tissue was fixed in 4 % paraformaldehyde, embedded in paraffin, and sliced into 4-μm sections. Induction of apoptosis was measured by TUNEL (One Step TUNEL Apoptosis Assay Kit, C1088, Beyotime, China) according to the manufacturer's instructions. TUNEL staining were analyzed with fluorescence microscope (Olympus BX53, Japan).

2.9. Western blot analyses

Kidney tissues treated with test compounds were prepared with lysis buffer containing protease/phosphatase inhibitor cocktail purchased from Sigma (St. Louis, MO, USA). The kidney tissues were lysed in 4× Laemmli sample buffer (Sigma Chemical), heated for 5 min at 100 °C. The concentrations of the proteins were tested by BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA) [13,14]. The kidney protein samples were separated by Sodium Dodecyl Sulfonate -polyacrylamide gel and then transferred onto a Polyvinylidene Fluoride (PVDF) membrane. After that, the membrane was blocked with incubation with 5 % non-fat milk buffer for 1 h at room temperature and then incubated with primary antibody for 3 h in 4 °C. The primary antibodies included TNF-α (Proteintech, USA), IL-1β (Santa Cruz, USA), NF-κB (Santa Cruz, USA), caspase 8 (Proteintech, USA), Bax (Boster, China), Bcl-2 (Boster, China), Cytochrome c (Cyt c) (Proteintech, USA), caspase 9 (Proteintech, USA), caspase 3 (Proteintech, USA), and β-actin (Cell signaling, USA). After washed with PBST buffer for three times, PVDF membrane was incubated with second antibody (LI-COR Biotechnology, USA) for 2 h. Then the expression of protein was detected by Odyssey infrared imaging system (LI-COR Biotechnology, USA).

2.10. Statistical analysis

Statistical evaluation was conducted by using SPSS 21.0 for

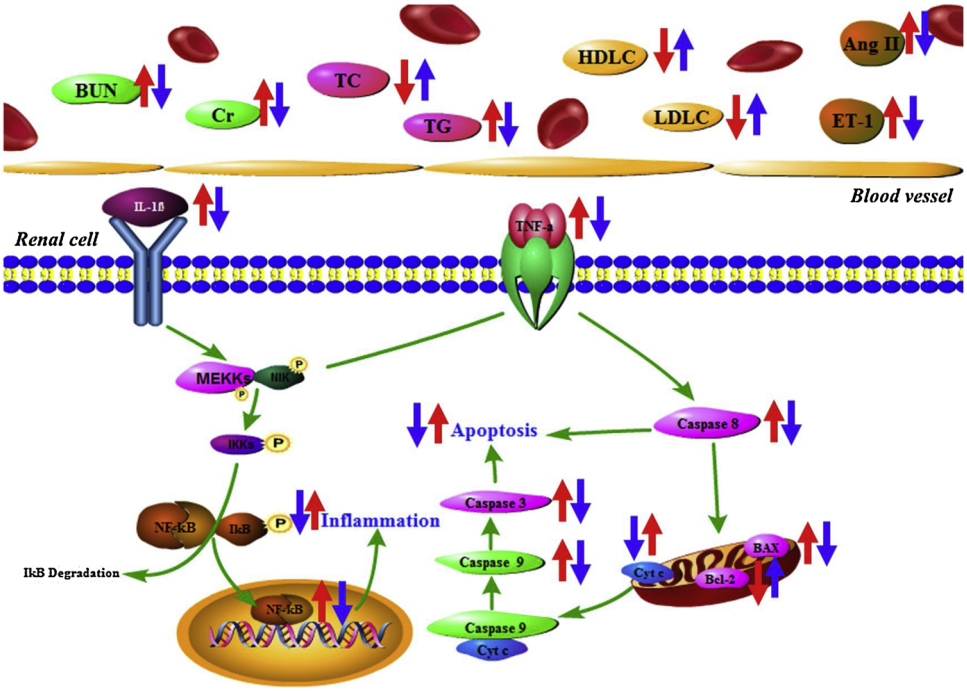

Fig. 6. Antihypertensive and renal protective mechanisms of SNX combined with captopril in SHR. Red arrows indicated differences between SHR and WKY groups, while blue arrows showed differences between captopril + SNX and SHR groups.

Windows package software. Data have been expressed as the means ± standard error mean (SEM). One-way variance analysis and Duncan multiple range test were used to determine significantly different groups. p values less than 0.05 were considered as significant differences for all statistical calculations.

3. Results

3.1. Body weight and blood pressure in rats

After a six-week observation, SBP were significantly increased in SHR group compared with WKY group. Captopril ($p < 0.01$), SNX and the combination groups ($p < 0.001$) remarkably decreased SBP after the first week administration of different drugs (Fig. 1B). The combination treatment showed more effective in the anti-hypertension and control of the weight gain.

3.2. Effects of combination on aortic wall thickness, NO/NOS, Ang II and ET-1

As we all known, aortic wall thickness was closely related to the blood pressure. As Fig. 2A–C indicated, the aortic wall thickness was increased significantly ($p < 0.001$) in the SHR group compared with that in the WKY group. Captopril + SNX group significantly reduced aortic wall thickness ($p < 0.01$). NO can maintain vascular tension and regulate blood pressure [15]. As Fig. 2D & E shown, SHR group significantly reduced the levels of NO and TNOS compared with WKY group. However, captopril group has not significant effect on NO, while the combination treatment group significantly increased the level of NO. At the same time, combination treatment group significantly increased the level of TNOS, especial for the level of eNOS (a type of nitric oxide synthase) (Fig. 2F). The combination therapy also improved the content of bradykinin and ET-1 (Fig. 2G–I). These results showed that captopril + SNX could reduce blood pressure through thinning the aortic wall and regulating NO/NOS, bradykinin and ET-1 expression.

3.3. Effects on lipid metabolism in rats

As Fig. 3 indicated, levels of TC, HDLC and LDLC were remarkably reduced in SHR group compared with WKY rats ($p < 0.05$). The combination group remarkably up-regulated the concentration of TC and HDLC ($p < 0.05$) and decreased concentration of TG ($p < 0.05$) compared with SHR group. These results denoted that combination of SNX and captopril could effectively ameliorate the lipid metabolic parameters and improve blood-lipid profiles.

3.4. Combination of SNX and captopril prevented kidney injury in SHR

Histological changes in the kidneys of rats treated with different therapeutic regimens were examined using HE and Masson's trichrome staining. As Fig. 4A & B shown, SHR promoted mononuclear cell infiltration and fibrosis compared with the normotensive WKY rats. Captopril group did not show remarkable effect. However, captopril + SNX group decreased mononuclear cell infiltration and fibrosis compared with SHR group. Meanwhile, Cr and BUN levels in SHR group were significantly increased compared with WKY group. Combination therapy was more effective in reducing the levels of Cr and BUN than captopril used alone (Fig. 4C & D).

3.5. Combination of SNX and captopril regulated inflammation and apoptosis

As Fig. 4E–G indicated, levels of inflammatory factors such as IL-1β and NF-κB were remarkably increased in SHR group compared with WKY rats ($p < 0.05$). Captopril and the combination group decreased the level of NF-κB compared with SHR group. In order to determine the potential mechanism of SNX-enhanced captopril for renal protection, western blot was used to analyze the protein levels of related inflammatory factors such as TNF-α, IL-1β and NF-κB in renal tissues. As Fig. 4H shown, they were increased in SHR group compared with WKY group. Both captopril and captopril + SNX reduced the level of NF-κB in renal tissues.

TUNEL assay was used to detect cell apoptosis. As Fig. 5A shown,

the green spots in SHR group were more than those in WKY group. Combination group lowered the number of points compared with SHR group. This study also explored the molecular mechanism of renal apoptosis, and further detected the expression of anti-apoptotic proteins Bcl-2, Cyt c, caspase 8, caspase 9 and caspase 3, as well as pro-apoptotic protein Bax. The protein levels of caspase3, 8, 9 and Cyt c significantly increased in the SHR group compared with WKY group. After six weeks treatment with captopril, SNX and captopril + SNX, expression of apoptosis related protein was markedly reduced compared with SHR group. On the other hand, the level of the anti-apoptotic protein Bcl-2 decreased markedly in the renal of SHR group and significantly increased in the combination group (Fig. 5B).

4. Discussion

Recently, the comorbidities in the hypertension made controlling blood pressure more difficult. According to 2017 ACC/AHA guidelines, 16 % hypertensive patients suffered from chronic kidney disease [3]. Meanwhile, the guidelines recommended that angiotensin converting enzyme inhibitor should be used in hypertension and chronic kidney disease patients [16]. Captopril, as an angiotensin converting enzyme inhibitor has been widely used for the therapy of arterial hypertension and cardiovascular diseases. As previous reports, it might induce acute renal failure in premature infants [17] and made functional renal insufficiency [18]. SNX was a famous classical recipe in traditional Chinese prescription composed of *Chuanxiong* and *Danggui*. Major pharmacological effects of *Danggui* extract or its components include anti-inflammatory, immunomodulatory, anti-cardiovascular and kidney protective [19]. *Chuanxiong* has been used as a traditional Chinese medicine in China for thousands of years. *Chuanxiong* have been found to possess various pharmacological effects on blood and cardiovascular, and to display numerous bioactivities such as anti-inflammation [20]. In this study, we tried to explore the combination of SNX and captopril on blood pressure and renal protection in SHR.

As a result, after a six-week treatment, the combination treatment showed more effective in the lowing blood pressure and control of the weight gain than the monotherapy displayed. SHR group significantly reduced the levels of NO and TNOS compared with WKY group. However, captopril group has not significant effect on NO. The combination group significantly increased the level of NO. At the same time, combination treatment group significantly increased the level of TNOS, especial for the level of eNOS (a type of nitric oxide synthase). As we known, the gaseous signaling molecules performed a key role in vasorelaxation. NO is an effective anti-hypertensive signaling molecule. When L-arginine is converted to citrulline by eNOS, NO is produced [21] and causes smooth muscle relaxation by the activation of soluble guanylate cyclase, followed by the accumulation of cyclic guanosine monophosphate (cGMP) [22]. NO as a kind of gas maintained vascular tension and regulated blood pressure serving as a target to prevent the development of hypertension and kidney disease [15]. Therefore, new strategies to increase NO signaling are suggested to have therapeutic potential in treating cardiovascular disease, including hypertension [23]. Therefore, NO plays an important role in the synergies between captopril and SNX.

At the same time, the combination therapy improved the content of bradykinin and ET-1, which was beneficial to the mitigation of hypertension [24]. In addition, abnormal lipid metabolism was one of the most harmful metabolic disorders and increased the risks for cardiovascular disease [25]. In this study, the combination therapy alleviated this dyslipidemia profile and regulated the levels of TG, TC and HDLC back to normal, which indicated that the combination therapy effectively ameliorated the lipid metabolic parameters and improved blood-lipid profiles.

According to the histological examination, SHR promoted mononuclear cell infiltration and fibrosis compared with the normotensive WKY rats. Captopril group did not show remarkable effect. However, captopril + SNX group decreased mononuclear cell infiltration and fibrosis compared with SHR group. At the same time, inflammatory markers were detected in renal tissue and serum. Levels of IL-1β and NF-κB were remarkably increased in SHR group renal tissue compared with WKY rats ($p < 0.05$). Captopril + SNX group significantly reduced these inflammatory markers in renal tissue ($p < 0.05$). Cr and BUN levels in SHR group were significantly increased compared with WKY group. Combination therapy was more effective in reducing the levels of Cr and BUN than captopril used alone (Fig. 4C & D). As previous research, inflammation [26] and apoptosis [27] played important roles in the development of renal injury. Inflammatory cytokines such as interleukin and TNF-α may lower the endothelium-dependent vasodilation via down-regulating eNOS and damaging the endothelium cells [28], which in kidneys contributed to local tissue injury in hypertension. Additionally, numerous studies have shown that NF-κB pathway contributed to transcriptional upregulation of inflammatory factors in the pathogenesis of renal dysfunction [4]. Apoptosis also plays a key role in the renal injury [29]. The mitochondrial apoptosis pathway plays an important role in the process of apoptosis [30]. In the apoptotic pathway, Bcl-2 family proteins and caspases are the main regulatory mediators [31]. The Bcl-2 protein family includes anti-apoptotic protein (Bcl-2) and pro-apoptotic protein (Bax). Bcl-2 leads to the destruction of mitochondrial membrane potential and the release of apoptotic factors such as cytochrome c into the cytoplasm, leading to the activation of caspases which are cysteine-dependent aspartate-directed proteases, which ultimately leads to apoptosis [32,33]. Caspases, as initiators and executioners of cell apoptosis [30]. Caspase 3 mediated mitochondrial apoptosis is one of the molecular mechanisms of renal cell apoptosis [34]. From what has been discussed above, NF-κB pathway and Caspase-mediated apoptosis pathways correlated with renal injury. In this study, Combination therapy significantly increased the level of Bcl-2 in kidney tissues, and remarkably decreased the protein expression of IL-1β, NF-κB, Bax, Cyt c, caspase 3, 8 and 9 in kidney tissues compared with SHR group. In particular, the captopril + SNX group performed better on Cytc, Bcl-2, and caspase 9 than the captopril group. At the same time, the number of tunel-positive cells decreased. The renoprotective effect of captopril + SNX was confirmed by ameliorating the expression of apoptosis and inflammation related proteins (Fig. 6).

In conclusion, the present study has demonstrated that the anti-hypertensive mechanism and renal protective effect of SNX combined with captopril in SHR. Based on these data, the combination group ($p < 0.001$) reduced blood pressure, which was better than the captopril group did ($p < 0.01$). Meanwhile, the combination group significantly reduced aortic wall thickness ($p < 0.05$). Lowering blood pressure was achieved by adjusting NO/NOS, ET-1 and dyslipidemia. In addition, combined therapy has a good protective effect on renal tissue. Combination therapy was more effective in reducing the levels of Cr and BUN than captopril used alone. At the protein levels, the captopril + SNX group performed better on Cyt c, Bcl-2, and caspase 9 than the captopril group. We hypothesized that the underlying renoprotective mechanisms of combinatorial treatment based on reducing the release of inflammatory factors and inhibition the expression of apoptotic markers. These results provided a rationale for future clinical use of SNX combined with captopril in the antihypertensive and renal protective functions in hypertension.

Statement of ethics

This animal study was approved by the Institutional Animal Care and Use Committee of China, and institutional guidelines for animal welfare and experimental conduct were followed.

Author contributions

Miss Li Yang, Mr Hao Xiang, Mr Guanyu Lu and Miss Yijia Wang

contributed in the experimental design, pharmacological experiments, data analysis, manuscript preparation for this study.

Professor Shuli Man, Changxiao Liu and Wenyuan Gao contributed in the experimental design, data analysis and manuscript preparation.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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