Radix *Cyathula officinalis* Kuan inhibits arterial remodeling in spontaneously hypertensive rats

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Abstract. There is still no resolution for arterial remodeling related with hypertension, though hypertension treatment has access to a number of pharmacological agents. The present study aimed at investigating the prevention of *Cyathula officinalis* Kuan's roots (*C. officinalis* Kuan) against in arterial remodeling *in vitro*. Spontaneously hypertensive rats (SHRs) were intragastrically administered 3, 6 or 12 g/kg *C. officinalis* Kuan or normal saline or enalapril (2.5 mg/kg) once a day for 8 weeks. Hematoxylin and eosin were used to measure blood pressure and stain carotid and arota. The serum concentration of nitric oxide (NO) was measured by NO assay kit (nitrate reductase method). The endothelin-1 transcriptional level, endothelial NO synthase of endothelium as well as angiotensin II receptor type 1 (AT1R) of aorta and carotid was tested by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and the protein level in aorta was also measured by western blotting. The blood pressure in SHR+enalapril, SHR+3 g/kg, SHR+6 g/kg and SHR+12 g/kg *C. officinalis* Kuan groups was significantly decreased at 4, 6 and 8 weeks post-treatment compared with SHR group. Different doses of *C. officinalis* Kuan and enalapril treatment showed aortic wall thinness and strengthened NO serum level, but made no impact on the transcriptional level of AT1R in aorta or endothelial NO synthase in carotid. It is suggested by such results that therapy by *C. officinalis* Kuan is able to fight against arterial remodeling, thus may provide a new means to treat arterial remodeling caused by hypertension.

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Introduction

The contribution of hypertension to mortality and morbidity in people's health is preventable, for its etiologic influence and its growing influence in stroke, kidney failure and heart attack (1). Based on the reports from World Health Organization (WHO), high levels of blood pressure, even when just suboptimal, have responsibility for 49% ischaemic heart disease and 62% cerebrovascular disease (2). The rise of BP within a patient with hypertension results from control mechanisms for blood pressure, such as vascular resistance of periphery, volume of circulating blood and cardiac output. It is a crucial task to choose the appropriate therapy for every patient (3).

During the past years, invasive and non-invasive techniques have brought improved vascular changes within experimental animals and hypertensive patients (4). In both animal and human models, there is an association between hypertension and aortic remodeling (5), which is featured by structural vascular alterations and destroyed endothelium-dependent vasodilation (6). Therefore, endothelium is essential to the vascular structure and tone (7). A declined aortic diameter within hypertensive subject in middle age may also make sense to increase pulse pressure via strengthening particular impedance, which contradicts the traditional phenotype of hypertensive aortic featured by degenerated and calcific vascular wall and increased aortic diameter (8).

To account for various mechanisms of blood pressure, scientists have developed the therapy of targeted anti-hypertension. Even though anti-hypertensive drugs, like calcium-channel blockers, receptor blockers of angiotensin II (Ang II) and inhibitors of angiotensin-converting enzyme (ACE), have extreme application in clinical treatment, there is no resolution for vascular changes induced by hypertension (9). Thus, it is necessary to develop new therapeutic tactics and drugs for vascular remodeling related with hypertension. With the characteristics of 'multi-target', composition of many compatible herbs and multiple compounds in one prescription, conventional Chinese herbs have achieved a good acceptance in China that attempts to decrease side effects and promote efficacy (10). *Cyathula officinalis (C. officinalis)*, with family of Amaranthaceae family, belongs to an herbaceous plant perennially with wide distribution in tropical regions of Africa and Asia, and especially

in Korea, Vietnam and China. *C. officinalis* Kuan's roots, *C. officinalis* Kuan, in Chinese called 'Chuan Niu Xi', have functions to remove blood stasis and restore menstrual flow, ease joint movement, as well as induce diuresis for treatment of stranguria (11). It is often applied as emmenagogue, atonic, antiarthritic, anti-fertility agent and diuretic to nourish kidneys and liver, fortify muscles and bones, and activate circulation (12). *C. officinalis* Kuan has been extracted with diverse active compounds in biology, such as palmitic acids, hyterocyclic compounds and phytoecdysteroids (13,14), whose biological attributes have been featured. Nevertheless, the anti-hypertensive attributes of *C. officinalis* Kuan have attracted little attention.

In the present study, we made efforts to evaluate the impacts of *C. officinalis* Kuan on the arterial remodeling in spontaneously hypertensive rats (SHRs). The results indicated that *C. officinalis* Kuan could improve the arterial remodeling by decreasing endothelin-1 (ET-1) and increasing endothelial nitric oxide synthase (eNOS) and ATIR expression.

Materials and methods

Animal treatments. Male rats, 12-weeks-old with spontaneous hypertension (SHR) (245-285 g) were obtained from the Shaanxi Jiahe Phytochem Co., Ltd. (Xian, China). SHR were separated into 5 groups randomly with 8 rats in each group: SHR treated by 0.9% saline were considered to be a model of hypertension (SHR); SHR in the other 4 groups were administered with 3, 6 and 12 g/kg *C. officinalis* Kuan or 2.5 mg/kg enalapril. The rats had a dark/light cycle of 12/12 h at fixed temperature of 22-23°C with available water and food freely. The administration was once a day for eight weeks. Monitoring of blood pressure was once a week using a tail BP Series Automatic non-invasive blood pressure measuring system (BP-300A; Chengdu Techman Software Co., Ltd., Chengdu, China) during the experimental period. Animal Care and Use Committee of Shanghai Putuo People's Hospital approved this study according to the guidelines on Ethical Care for Experimental Animals.

Histological assessment. At the end of the experiments, the rats were euthanized with an overdose of chloral hydrate. The aorta of the rats was harvested, and fixed with 10% formalin, dehydrated and embedded into paraffin. Next, sections with thickness of 4 μm were cut, and then stained with hematoxylin and eosin (H&E). The Olympus BX51 microscope with the camera of Olympus DP71 CCD from Olympus (Tokyo, Japan) was used to capture digital images (magnification, x200). A blinded manner was used to perform analysis on all images.

Measurement of serum NO level. The serum concentration of nitric oxide (NO) was measured by Nitric Oxide assay kit (Nitrate reductase method, A012; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with instructions of the manufacturer.

RNA extraction and analysis on quantitive reverse transcription-polymerase chain reaction (qRT-PCR). Whole RNA was

extracted from aorta by snap-freezing and samples of carotid by RNAiso Plus and PrimeScript reagent kit of reverse reaction (DRR037A) (both from Takara, Dalian, China) was used to carry out reverse transcription reaction on RNA in accordance with manufacturer's instructions. Quantitative analysis on the change of expression level was conducted by SYBR Premix Ex Taq (DRR041A; Takara) in ABI 7500 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primer sequences of PCR were: ET-1 forward, 5'-TGTTCCCT AACCTGTCTTC-3' and reverse, 5'-ACACTCCCTAAGGAC TTTC-3'; eNOS forward, 5'-CTTTCGGAAGGCGTTTGAC-3' and reverse, 5'-AACTCTTGTGCTGCTCAGG-3'; Ang II receptor type 1 (AT1R) forward, 5'-CTCTGTTCTACGGCT TTC-3' and reverse, 5'-CTTCTGTCAGGGCATTAC-3'; GAPDH forward, 5'-GTCGGTGTGAACGGATTTG-3' and reverse, 5'-TCCCATTCTCAGCCTTGAC-3'. The change in expression of mRNA within rats treated by saline, *C. officinalis* Kuan or enalapril was assessed by the $2^{-\Delta\Delta Cq}$ method.

Western blotting. Whole protein was isolated out of snap-frozen aorta samples using radioimmunoprecipitation buffer, supplemented with protease inhibitor (Beyotime Institute of Biotechnology, Shanghai, China). The concentration of protein was estimated employing the assay kit of bicinchoninic acid (Thermo Fisher Scientific, Inc.). Equivalently quantitive protein (30 μg) was divided subsequently on 12% SDS-PAGE gels, and then was moved onto membranes of nitrocellulose (EMD Millipore, Billerica, MA, USA). Following blocking, these membranes were immunoblotted overnight in 4°C with first antibodies: Anti-ET-1, anti-eNOS, anti-ATIR and anti-GAPDH. Horseradish peroxidase-conjugated second antibodies were used to incubate membranes after they were washed (1:1,000; Beyotime Institute of Biotechnology) for 1 h at 37°C. Tris-buffered saline including Tween-20 of 20% was used to wash these membranes (Amresco, LLC, Solon, OH, USA). Detection for signals employed an improved system of chemiluminescence (Pierce, Rockford, IL, USA) and their determination employed software of ImageJ version 1.46 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The quantitive values are in mean ± SD. GraphPad Prism software, version 5.0 (GraphPad Software, Inc., San Diego, CA, USA) was used to analyze nonlinear regression of each curve for dose-response. Calculation of data used one-way analysis on variance (ANOVA) and analysis on statistical calculations used SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA). Comparison among data from various groups used one-way ANOVA. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

C. officinalis Kuan therapy reduces blood pressure in SHR. Monitoring of blood pressure was conducted fortnightly at indicative time. In week 0, different doses of *C. officinalis* Kuan (3, 6 and 12 g/kg) or 2.5 mg/kg enalapril therapy did not show any impact on blood pressure in comparison to group of SHR (Table I). After 2 week, the blood pressure was 161.34±4.38, 181.50±3.15, 181.08±5.43 and

Table I. The blood pressure in SHR with enalapril or *C. officinalis* Kuan treatment.

| Groups	0 week	2 weeks	4 weeks	6 weeks	8 weeks
SHR	185.67±6.31	187.91±4.89	191.48±6.51	193.88±3.31	195.51±4.88
SHR+enalapril	189.64±5.83	161.34±4.38[b]	154.84±4.17[b]	155.31±4.38[b]	154.29±4.38[b]
SHR+3 g/kg	182.85±4.95	181.50±3.15[a]	181.74±3.21[b]	178.28±3.17[b]	177.63±5.49[b]
SHR+6 g/kg	186.81±3.74	181.08±5.43[a]	175.38±4.29[b]	172.00±4.11[b]	167.21±6.11[b]
SHR+12 g/kg	181.34±5.84	176.46±4.11[b]	170.93±7.17[b]	162.15±4.46[b]	158.50±3.94[b]

[a]P<0.05; [b]P<0.01 vs. SHR.
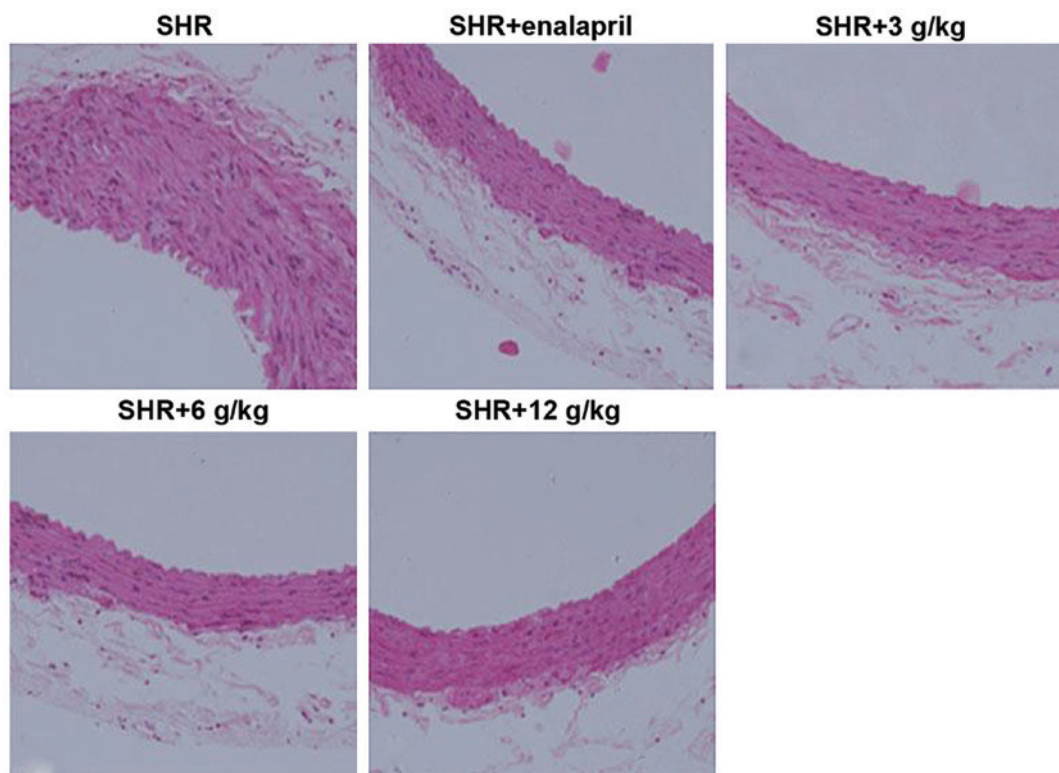
Figure 1. *C. officinalis* Kuan inhibits the thickness of aorta in SHR. After treatment of SHR with enalapril or different doses of *C. officinalis* Kuan (3, 6 or 12 g/kg), the arterial remodeling was evaluated using hematoxylin and eosin staining. *C. officinalis*, *Cyathula officinalis*; SHR, spontaneously hypertensive rat.

176.46±4.11 mmHg for enalapril, 3, 6 and 12 g/kg *C. officinalis* Kuan treatment, respectively, compared with SHR with the blood pressure of 187.91±4.89 mmHg. At the 8th week, there were no differences in the blood pressure between 12 g/kg *C. officinalis* Kuan and enalapril treatment. These results suggest that *C. officinalis* Kuan treatment significantly reduced the blood pressure of SHR.

C. officinalis Kuan treatment inhibits arterial remodeling in SHR. It has been suggested previously that an evaluation of arterial alterations may offer valuable information on hypertensive damage of organs in people. In the present study, it evaluated the prevention of *C. officinalis* Kuan from arterial remodeling employing staining with H&E. As Fig. 1 shows the aorta's medial thickness within the SHR+enalapril, as SHR+3 g/kg, SHR+6 g/kg and SHR+12 g/kg, *C. officinalis* Kuan group was significantly low compared with that of SHR

group, with obvious decrease in SHR+6 g/kg *C. officinalis* Kuan group.

C. officinalis Kuan treatment increases NO and eNOS expression and decreases ET-1 and AT1R expression in SHR. As shown in Fig. 2A, the serum level of NO in enalapril and different doses of *C. officinalis* Kuan treatment was significantly increased in comparison to SHR. Measurement of expression shown by ET-1, eNOS and AT1R within aorta used qRT-PCR and western blotting. Different doses of *C. officinalis* Kuan or enalapril treatment significantly decreased the transcriptional level of ET-1 and AT1R, while increased the transcriptional level of eNOS in the aorta of SHR (Fig. 2B and C). Whereas different doses of *C. officinalis* Kuan treatment significantly reduced the protein level of ET-1 as well as raised eNOS in the aorta of SHR significantly, but had no effect on the protein expression of AT1R (Fig. 2D). Moreover, the transcriptional
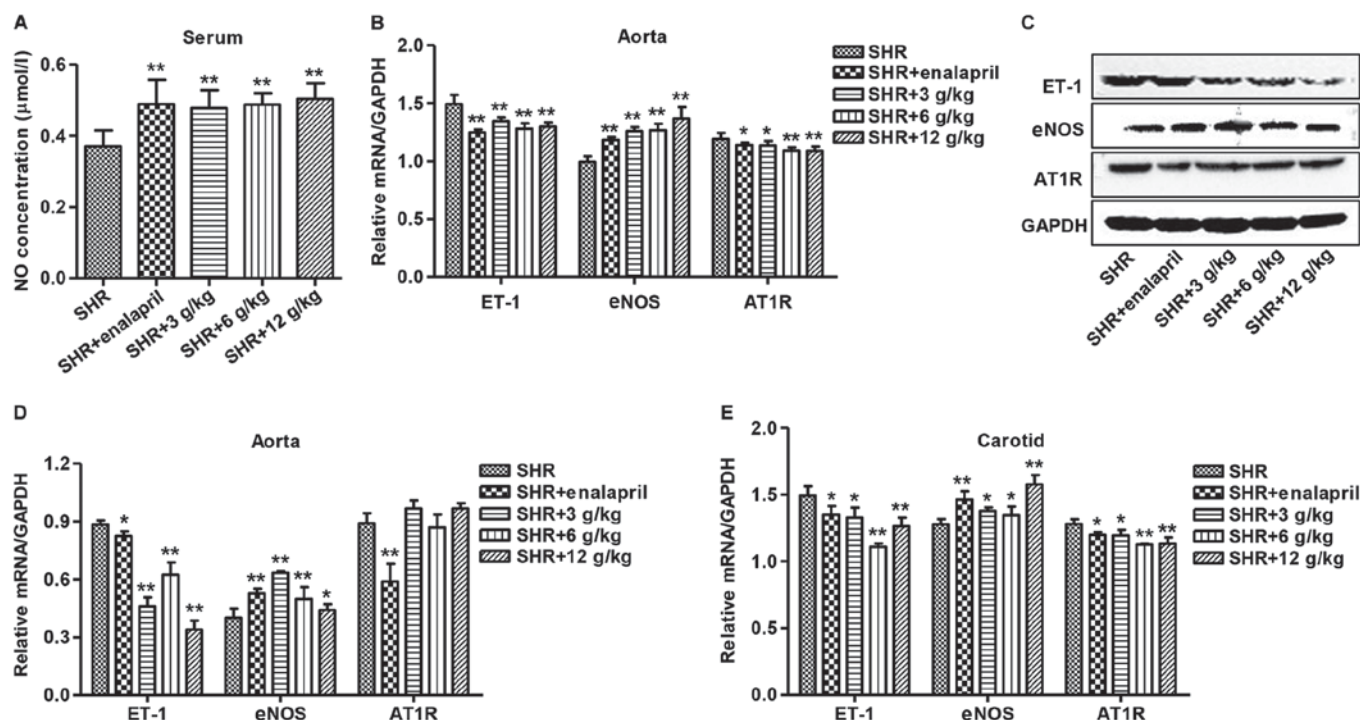
Figure 2. Effect of *C. officinalis* Kuan on serum NO and expression of ET-1, eNOS and AT1R in SHR. After treatment of SHR with enalapril or different doses of *C. officinalis* Kuan (3, 6 or 12 g/kg). (A) The serum NO concentration was measured by NO assay kit (nitrate reductase method), (B) the protein and mRNA expression, (C) ET-1, AT1R and eNOS in aorta employed qRT-PCR, and (D) western blotting for measurement and protein in ET-1, eNOS and AT1R in carotid was measured by western blotting. (E) Western blotting for measurement and protein in ET-1, eNOS and AT1R in carotid was measured by western blotting. *C. officinalis*, *Cyathula officinalis;* NO, nitric oxide; ET-1, endothelin-1; eNOS, endothelial nitric oxide synthase; AT1R, angiotensin II receptor type 1; SHR, spontaneously hypertensive rat. *P<0.05 and **P<0.01.

level of ET-1 and AT1R was significantly decreased in the carotid of SHR with enalapril or different doses of *C. officinalis* Kuan treatment, which showed increased transcriptional level of eNOS (Fig. 2E).

Discussion

Although it was demonstrated in contemporary pharmacological studies that diverse pharmacological activities were possessed by *C. officinalis* Kuan, containing immunostimulant, antitumor, analgesic, anti-inflammatory, eliminating blood stasis, anti-aging, inducing diuresis to treat stranguria, recovering menstrual flow (15,16), it is still unknown whether *C. officinalis* Kuan would affect arterial change. Within this study, we evaluated the impact of *C. officinalis* Kuan in the procedure of arterial change induced from hypertension and demonstrated that *C. officinalis* Kuan inhibited the blood pressure and arterial ET-1 and AT1R expression as well as increased serum NO level and arterial eNOS expression in SHR. This report is the first to show administration of *C. officinalis* Kuan improves the arterial remodeling, by decreasing blood pressure, ET-1 and AT1R expression and increasing the NO and eNOS expression.

Hypertension, a major public health problem, affecting up to one billion people worldwide (17) and exhibiting aortic remodeling including aortic hypertrophy, collagen accumulation and impaired endothelium dependent vasorelaxation (18), among which the main adaptive mechanisms are rearranged formations of extracellular matrix and vascular remodeling to increase blood pressure chronically

and growing mortality and morbidity (19,20), characterized in part by the proliferation and hypertrophy of vascular smooth muscle cells (21). Hypertensive vascular remodeling is contributed by the increased vascular cell, inflammation, fibrosis and hypertrophy (22). After *C. officinalis* Kuan administration for 2 weeks, SHR group had a significant decline in aorta's medial thickness and blood pressure, which was in line with the effect of enalapril in SHR. Enalapril is an orally anti-hypertensive agent with efficacy, affecting risk factors on cardiovascular and preventing decrease within renal function as well as other organ injury positively (23). *C. officinalis* Kuan presented significant prevention of both vascular function and structure from remodeling, indicating the relation between beneficial impact of *C. officinalis* Kuan and the influence to blood pressure.

In the present study, it was revealed by us that hypertension's pathogenesis is related to NO activity, whereas *C. officinalis* Kuan may have a hypertensive function that was progressed via elevating the NO level in serum, as well as preventing endothelial impacts, which is in accord with our study that anti-hypertension role is to improve NO production (24). NO belongs to a crucial vasodilator, which is indispensable for maintaining regular blood pressure. Besides, activity of impaired NO takes responsibility for hypertension pathophysiology (25). Hypertension has a pathological feature as the dysfunctional relaxation dependent on endothelium (26). Endothelial vascular cells in the sub-type of M are activated by ACh, releasing NO, and finally inducing vascular vasodilator (27), resulting in decreased average arterial pressure, sympathetic activity

and heart rate within rats through activated adenosine A2A receptors as well as reduced M1 receptor and ACh levels (28). Accumulating evidence suggests that dysfunctional eNOS, enhanced activity of xanthine oxidase, increased NADPH oxidase activity, and decreased antioxidant defense during the aging process are linked to dysfunction of the endothelium and consequent development of hypertension (29). In the present study, it was discovered that *C. officinalis* Kuan could significantly increase the expression of eNOS in SHR, mimicking the effect of enalapril.

ET-1 affects hypertension. In addition to the impact on people by raising blood pressure, myocardial hypertrophies and vascular are induced by ET-1, as independent risk elements for cardiovascular mortality and morbidity (30). It has been shown that over-activated ET-1 can exacerbate both aortic and cardiac remodeling that could be corrected by ET antagonists (31,32). In the present study, it was discovered that *C. officinalis* Kuan could significantly decrease the expression of ET-1 and AT1R in SHR, and mimic the effect of enalapril. Enalapril has the ability to decrease plasma levels in Ang II through blocking its last step of activation and offering antihypertensive actions (23). AT1R stimulation regulated ACE2 and Ang-(1-7) expression in aorta of SHR (33). Less activated AT1R as well as their endocellular signaling reduce plasma levels of Ang II (4) and alters the balance of ACE2/Ang-(1-7)/ Mas axis with ACE/Ang II/AT1R axis to improve vascular remodeling (34).

This study was the first to demonstrate that *C. officinalis* Kuan significantly improved arterial remodeling in SHR through decreasing ET-1 and AT1R expression and increasing eNOS and NO expression.

Acknowledgements

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