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Journal of Traditional and Complementary Medicine

journal homepage: <http://www.elsevier.com/locate/jtcme>

Original Research article

Vasculoprotective effects of *Centella asiatica*, *Justicia gendarussa* and *Imperata cylindrica* decoction via the NOXs-ROS-NF- κ B pathway in spontaneously hypertensive rats

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ARTICLE INFO

Article history:

Received 15 December 2018

Received in revised form

6 June 2019

Accepted 22 June 2019

Available online 24 June 2019

Keywords:

Indonesian herbal medicine

Vascular remodeling

Oxidative stress

Hypertension

ABSTRACT

Background and aim: *Centella asiatica*, *Justicia gendarussa* and *Imperata cylindrica* decoction (CJID) is efficacious for hypertension. NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NOX)-induced reactive oxygen species (ROS) generation modulates nuclear factor kappa B (NF- κ B) activation and thus mediates hypertension-induced vascular remodeling. This research aims to investigate the anti-remodeling effect of CJID through the mechanism of NOXs-ROS-NF- κ B pathway in spontaneously hypertensive rats (SHRs).

Experimental procedure: CJID was orally administered once a day for five weeks in SHRs and normotensive-WKY (Wistar Kyoto) rats. All rats were sacrificed at the end of study and different assays were performed to determine whether CJID ameliorates vascular remodeling in SHRs, such as histological examination; lactate dehydrogenase (LDH), nitric oxide (NO), malondialdehyde (MDA) and superoxide dismutase (SOD) assays; superoxide and hydrogen peroxide (H₂O₂) generation assays, immunohistochemistry and immunofluorescence assays. Changes in levels of inducible nitric oxide synthase (iNOS), NF- κ B-p65, NF- κ B inhibitor alpha/I κ B α (inhibitory kappa B- alpha), phosphorylation of I κ B α (p-I κ B α) and NOX1, NOX2, NOX4 in the thoracic aorta were determined.

Results: Vascular remodeling indicators, media thickness, collagen and elastic accumulation in the thoracic aorta, of SHRs-treated CJID were attenuated. Redox homeostasis, aortic superoxide and hydrogen peroxide generation were decreased in SHRs-treated group. Aortic iNOS, p-I κ B α , NF- κ B-p65 and NOX1, NOX2, NOX4 expressions were suppressed.

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

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Conclusions: CJJ treatment diminishes oxidative stress response in the thoracic aorta of SHR's via regulation of NOXs-ROS-NF- κ B signaling pathway. These findings indicate that CJJ possess protective effect against hypertension-induced vascular remodeling in SHR's.

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List of abbreviations

ANOVA one-way analysis of variance

a.u arbitrary units

B2P2TOOT Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional Tawangmangu (Research and Development of Medicinal Plant and Traditional Medicine)

CJID *Centella asiatica*, *Justicia gendarussa* and *Imperata cylindrica* decoction

DCFH-DA 2',7'-dichloro-dihydrofluorescein diacetate

DHE dihydroethidium

FDA food and drug administration

H₂O₂ hydrogen peroxide

I κ B α inhibitory kappa B- alpha

iNOS inducible nitric oxide synthase

KCl potassium chloride

LDH lactate dehydrogenase

LSD least significant difference

MDA malondialdehyde

NF- κ B nuclear factor kappa B

NO nitric oxide

NOX NADPH (nicotinamide adenine dinucleotide phosphate) oxidase

O₂^{•-} superoxide radical

PBS phosphate buffered saline

p-I κ B α phosphorylation of inhibitory kappa B- alpha

RIPA radioimmunoprecipitation assay

ROS reactive oxygen species

SHRs spontaneously hypertensive rats

SOD superoxide dismutase

VSMCs vascular smooth muscle cells

WKY Wistar Kyoto

1. Introduction

Vascular remodeling in hypertension and its complex multifactorial process contribute to the major cause of mortality induced by cardiovascular disease. This alteration process encompasses media layer thickening, extracellular matrix deposition, along with proliferation and migration of vascular smooth muscle cells.¹ Furthermore, two cardinal signs of hypertension-induced vascular pathology are vascular reactivity and vascular stiffness.² Abundant evidence denotes that increasing blood pressure leads to the generation of reactive oxygen species and an activation of redox signaling,³ and in turn promotes endothelial damage⁴ and vascular injury.⁵ NADPH oxidases, a major source of ROS-generation, are regulated at low concentration.⁶ Their high activities or decreased activation of antioxidant system results in oxidative stress in pathological situations. ROS modulates certain cellular process to maintain vascular tone and integrity.^{7,8} To date, several NOX isoforms in the vascular wall are identified: NOX1, NOX2 and NOX4. Each NOX isoform plays a distinct roles in regulating ROS-mediated signaling.⁹ The increase regulation of vascular NOXs modulates oxidative stress and leads to vascular remodeling. Furthermore, the increased level of MDA and LDH and the lowered activity of superoxide dismutase can disturb the body system in controlling its redox status. The disruption of redox status will trigger NF- κ B activation.¹⁰ Taken together, accumulating evidence indicates that ROS, NOX and NF- κ B are the important pathways in hypertension-induced vascular remodeling.

Medicinal plants *Centella asiatica* leaves, *Justicia gendarussa* leaves and *Imperata cylindrica* roots are known as an Indonesian traditional medicine to treat hypertension.¹¹ These plants have rich substantial compounds where a large body of pharmacological studies denote whether their active compounds have a critical function in the cardiovascular system. *Centella asiatica* has the

potential to protect vascular wall modifications in chronic venous hypertension and preserve the vascular endothelial cells,^{12,13} as well as having antioxidant and anti-inflammatory effects,^{14,15} and cardiomyopathy protection.¹⁶ *Justicia gendarussa* has anti-inflammatory^{17,18} and antioxidant functions.¹⁹ The rhizome of *Imperata cylindrica* has been proven as a vasodilator,^{20,21} anti-inflammatory agent²² and as an antioxidant.²³ Treatment with these herbs may attenuate oxidative stress, and subsequently contributing to management of hypertension-induced vascular remodeling.

Our previous study has demonstrated that a formulation of these plants inhibited left ventricular hypertrophy in SHR's via the pathway of oxidative stress diminution through the NOXs suppression.¹¹ The herbs formulation has been traditionally used in treating hypertension both in *jamu* healthcare services and by the Javanese people in Indonesia. However, it is still unclear whether CJID can inhibit vascular remodeling that is caused by hypertension. Therefore, this present study aims to determine if CJID can prevent hypertension-induced vascular remodeling in SHR's. Furthermore, we sought to clarify if its oxidative stress diminution is mediated via the NOXs-ROS-NF- κ B pathway.

2. Materials and methods

2.1. Preparation of herbs

CJID was prepared by combining 3 herbs at the following ratio: 5 g *C. asiatica* leaves, 5 g *J. gendarussa* leaves, and, 3 g *I. cylindrica* root, as it is traditionally made. All dried form of herbs was supplied by the Research and Development of Medicinal Plant and Traditional Medicine (B2P2TOOT/Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional Tawangmangu) Ministry of Health, Indonesia (document number YK.01.03/2/615/2017). However, the herbs were validated by Ms. Dyah Subositi,

M.Sc, an expert in the plant taxonomy who works at B2P2TOOT. The herb mixture was dissolved in 1 ml distilled water according to the standard volume for rat's oral administration. For extraction, the dried herb solution was placed in a covered-bottle and then to be heated above the boiling water at 90 °C for 30 min. Afterward, the herbs solution was filtered using filter paper and the filtrate was freshly administered to the rats. Based on human to rat dose calculation (FDA provided), and the ratio of CJI administration (5 g, 5 g, and 3 g), each rat was orally administered by 13.3 mg of *C. asiatica*, 13.3 mg of *J. gendarussa* and 8 mg of *I. cylindrica*.²⁴

2.2. Animals

The animal experimental protocols were approved and conducted in accordance to the recommendations from Taiwan legislations on the ethical use and care of laboratory animals. The study procedure was authorized by the Animal Review Board of the Kaohsiung Medical University, Kaohsiung, Taiwan. Thirty six SHR and normotensive controls, Wistar Kyoto rats were purchased from National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. All rats were at 8 weeks of age, weighing 180–200 g. All rats were housed in a standard animal housing facility, 12 h dark–light cycle and received standard commercial food and drinking water ad libitum. Ambient temperatures were between 18 °C and 22 °C with humidity 45–70%. The rats were randomized into 4 groups: control groups in WKY rats (WKY CTL, n = 10) and SHRs (SHR CTL, n = 10) and treatment groups in WKY rats (WKY CJD, n = 6) and SHRs (SHR CJD, n = 10). The control groups were given distilled water. The administration was performed via gastric gavage once a day for 5 weeks.

2.3. Histological examination

Perfusion fixation was performed after rats' sacrifice. The organ perfusion was modified from previous method by Gage (2012).²⁵ Afterward, the thoracic aorta was resected and divided into 3 separate regions for each subject; one section next to aortic arch for paraffinized, and the other 2 parts were for frozen section and fresh tissue specimen. One segment of thoracic aorta was laid in a mixture of 25 mM KCl (potassium chloride) and PBS (phosphate buffered saline) relaxing buffer, and then kept in 40% neutral-buffered formaldehyde at room temperature. The ~5 μm sections were stained in hematoxylin and eosin (Muto Pure Chemicals, Japan) according to former study.²⁶ The determination of vascular wall thickening was based on previous study.²⁷ The media thickness was determined by measuring the distance from the internal elastic lamina to the external elastic lamina. For every slide, measurements from 4 different points (two points when crossed with the other two points forming a perpendicular angle) were averaged. The lumen inner diameter was defined from 2 different points (two connected lines formed a perpendicular angle). The media-to-lumen ratio was calculated based on the measured lumen inner diameter and media data. The collagen structure of the vessel tissue area was evaluated using Masson's trichrome staining (Sigma-Aldrich, USA), while Verhoff's elastic (Sigma-Aldrich, USA) staining was performed to determine elastic fiber. The sections were captured using a Nikon Eclipse TE 2000-S (Tokyo, Japan) equipped with Nikon DS Fi1 digital camera and NIS-Element F software for image analysis.

2.4. Measurement of redox status

After sacrifice, blood samples were collected from left ventricular blood. The measurement of plasmatic LDH level was done using Spotchem EZ SP 4430 (ARKRAY Inc, Kyoto, Japan). The aortic MDA (Sigma-Aldrich, USA, cat. no. MAK085) and SOD (Sigma-

Aldrich, USA, cat. no. KT-034) were evaluated according to the manufacturer's protocols. One frozen segment of thoracic aorta tissue homogenate was cut into small pieces in ice-cold RIPA (radioimmunoprecipitation assay) lysis buffer (M-PER®, Thermo Scientific, USA; cat. no. 78501) mixed with 1% Triton-X100 and then underwent sonication. Supernatant was collected from the homogenates, after, it was centrifuged at 13,000 g for 30 min at 4 °C. The MDA and SOD content were measured from the total cellular protein which were obtained from the supernatant. Both data were measured using the V–5100H spectrophotometer (BioTek Instruments, Inc., VT, USA). The MDA absorbance was determined at a wavelength of 532 nm, and SOD was at 485 nm. In the presence of free radicals, nitric oxide (NO) is easy to formulate, thus this measurement of nitrite levels in the blood plasma was used as a level of NO inactivated due to superoxide radical (O₂^{•-}). The methods of measuring NO have been described previously²⁸; the nitrite content in blood plasma was estimated with Griess reagent and determined colorimetrically. Briefly, balanced volumes of plasma and Griess reagent (sulfanilamide 1% w/v, naphthylethylenediamine dihydrochloride 0.1% w/v, and orthophosphoric acid 2.5% v/v) were stirred and incubated at room temperature for 10 min. The NO absorbance was determined at 540 nm wavelength. Nitrite was defined from the standard curve obtained using sodium nitrite as standard. A normalized process was needed to define the nitrite formed to the protein content of the respective blood plasma.

2.5. Evaluation of superoxide generation in the thoracic aorta

To evaluate in situ levels of O₂^{•-} in the thoracic aorta, we used dihydroethidium (DHE) staining (Invitrogen, USA).²⁹ The ~5 μm thoracic aorta frozen sections were incubated with 10 μM DHE at 37 °C for 30 min on polylysine glass slides in a humidified chamber avoiding light. Confocal laser-scanning microscope (Zeiss LSM 700, Carl Zeiss MicroImaging GmbH, Jena, Germany) was used to capture fluorescent images of ethidium bromide. An image-analysis software (Zeiss-Axio vision software) was operated to count the number of nuclei labeled by DHE and subsequently underwent analysis. The average fluorescence pixels value in the six randomly selected thoracic aorta fields was determined as the mean intensity fluorescence.

2.6. Evaluation of hydrogen peroxide generation in the thoracic aorta

The 2',7'-dichloro-dihydrofluorescein diacetate (DCFH-DA, Invitrogen, USA) was used to detect H₂O₂. This approved method has an advantage for the quantification of ROS in isolated thoracic aorta.³⁰ The ~5 μm thoracic aorta frozen section was loaded with 10 μM DCFH-DA and then it was incubated for 30 min at 37 °C protected from light. The 10 μM DCFH-DA was obtained from a 2 mM stock solution of DCFH-DA prepared in ethanol. The average DCF levels in six random slides were obtained after each slide was observed under laser scanning confocal microscope and its fluorescence intensity measured.

2.7. Immunohistochemistry

The procedure guidance for staining of immunohistochemistry was according to previous study with some modifications as explained by the manufacturer's (DAKO, Agilent, USA) instructions.³¹ After that, the ~5 μm paraffin-fixed thoracic aorta sections underwent orderly incubation with an antigen retrieval solution (Sigma-Aldrich, USA), and with a primary antibody (iNOS; ab15323, Abcam, USA, or phospho-IκBα (Ser32/36; 5A5; Cell Signaling, USA), or IκBα (Cell Signaling, USA), or NOX1 (NOX1 (H-75; Santa Cruz Biotech, USA), or NOX4 (Santa Cruz Biotech, USA). All

primary antibodies were at 1:100, and each slide was incubated for 18 h at 4 °C. Non-immunized goat serum were used as the primary antibodies for negative controls. Finally, the immunohistochemistry staining results were photographed with a Nikon Eclipse TE 2000-S (Tokyo, Japan). For image process and analysis, the microscope was equipped with Nikon DS Fi1 digital camera and NIS-Element F software.

2.8. Immunofluorescence assay of thoracic aorta NF- κ B-p65 nuclear translocation

The staining procedure of immunofluorescence was based on previous study.³¹ The ~5 μ m acetone-fixed thoracic aorta frozen sections were incubated for 1 h in 10% bovine serum to block non-specific protein-protein interactions. Then, the sections were orderly incubated with primary antibody (anti-NF- κ B-p65 antibody; Millipore, CA; or NOX2 (Purified Mouse anti - gp91 [phox]; BD Bioscience USA) at a 1/100 dilution for 18 h at 4 °C, and incubated with rhodamine (TRITC)-conjugated goat anti-mouse secondary antibody (Millipore, CA). For the cell nuclei stain, we used DAPI. Fluorescent pictures were photographed using Zeiss Axio Imager Z1 (Zeiss LSM 700, Carl Zeiss MicroImaging GmbH, Jena, Germany).

2.9. Statistical analysis

Statistical analysis were evaluated using one-way analysis of variance (ANOVA), and a post hoc analysis was performed using Fisher's least significant difference (LSD) test. The values are the means \pm SEM. The statistically significant was considered at *P*-value less than 0.05.

3. Results

3.1. CJID improves vascular remodeling in SHR

The ROS generation increased in hypertension was accomplished with hypertension-induced vascular damage.⁵ In this study, the index of aortic vascular remodeling was measured by calculating the media-to-lumen ratio. Hence, to determine whether CJID attenuates vascular remodeling of SHRs, we measured the media thickness of vascular wall and the lumen inner diameter in thoracic aorta using H&E staining. As shown in Fig. 1 (A and B), the media thickness of vascular wall in SHRs is significantly broadened in comparison with WKY CTL (*P* < 0.01). The increased media thickness in SHRs indicates the progressive worsening of vascular remodeling by hypertension. Treatment with CJID markedly decreases the media thickness in SHRs (*P* < 0.01). By contrast, as presented in Fig. 1C, there is no significant changes in the lumen inner diameter between SHRs and WKY, both in control and treatment groups. As expected, the media-to-lumen ratio markedly declines in SHR CJID in comparison with the control group (Fig. 1D, *P* < 0.01). These results denote that CJID improves media thickness in thoracic aorta of SHRs.

3.2. CJID enhances collagen and elastic fibers in the thoracic aorta of SHRs

The changes in thoracic aorta collagen fibers denote that hypertension contributes to the vascular structural abnormalities,³¹ as well as elastic fibers.³² In this present study, as shown in Fig. 2 (A and B), the collagen fibers are significantly lower in SHR CJID group

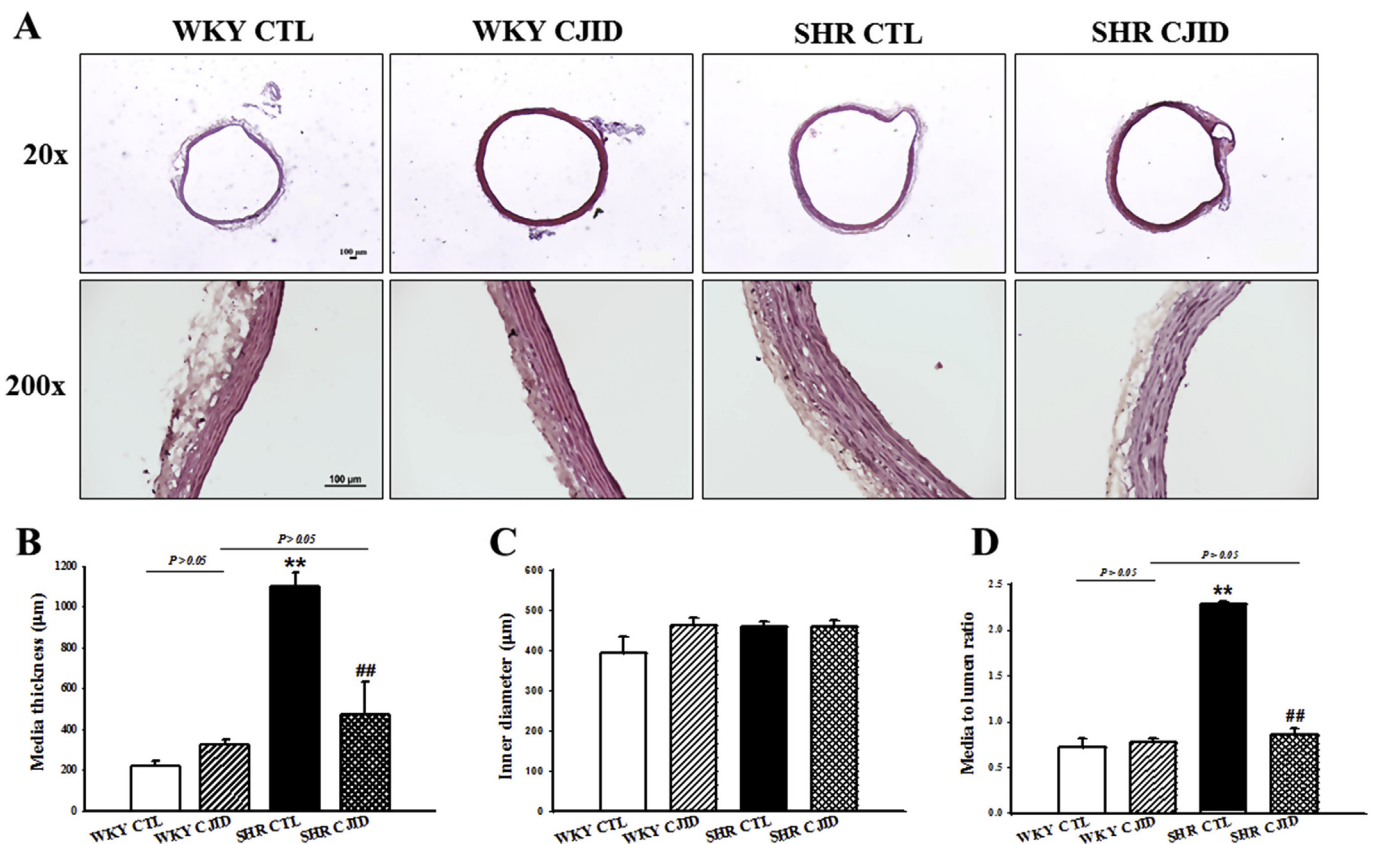


Fig. 1. Effect of CJID decoction on rat's thoracic aorta remodeling. (A) Representative images of vessel sections stained with HE staining at 20x and 200x magnification. (B) The media thickness. (C) The inner diameter. (D) The media-to-lumen ratio. Each point represents the mean \pm SEM, n = 10. ***P* < 0.01, *when compared to WKY CTL, #when compared to SHR CTL.

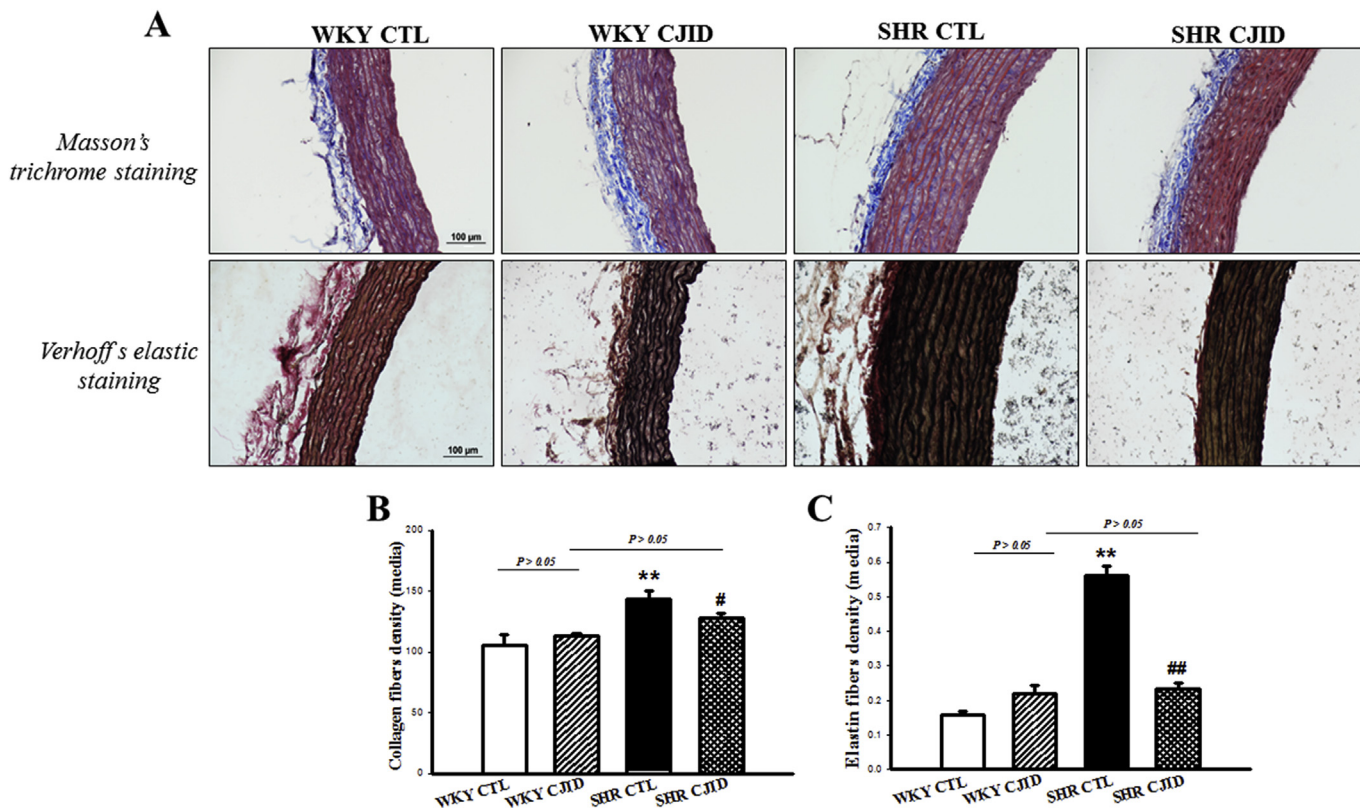


Fig. 2. Effect of CJL decoction on the vascular collagen and elastic structure. (A) Masson's trichrome collagen staining and Verhoff's elastic staining. (B) Collagen fiber density. (C) Elastic fiber density. Each point represents the mean SEM, $n = 10$. * $P < 0.05$, ** $P < 0.01$, #when compared to WKY CTL, #when compared to SHR CTL. Bar shows 100 µm.

when compared with the control ($P < 0.05$). Additionally, the elastic fibers are concomitantly similar with collagen density, CJID treatment lead to its density decrease (Fig. 2C, $P < 0.01$). CJID treatment improves collagen and elastic fibers in the thoracic aorta of SHRs.

3.3. CJID ameliorates redox homeostasis in blood plasma and aorta of SHRs

The delicate interaction between oxidant and antioxidant systems play an important role in maintaining body health and function.³³ As shown in Fig. 3 (A and B), compared to the control groups, CJID treatment attenuates the increase of plasmatic LDH ($P < 0.05$), and aortic MDA level in the thoracic aorta homogenates ($P < 0.05$) of SHRs. Likewise, the plasmatic NO level increases significantly by the administration of CJID (Fig. 3C, $P < 0.01$), and SOD level in the thoracic aorta homogenates (Fig. 3D, $P < 0.05$) of SHRs. These findings suggest that CJID improves redox homeostasis in SHRs.

3.4. CJID attenuates oxidative stress in the thoracic aorta of SHRs

To quantify the generation of oxidative stress locally in the vascular system, we measured aortic superoxide production with DHE staining. And for the detection of hydrogen peroxide production, we used DCFH-DA staining. As visualized in Fig. 4, the quantification of superoxide generation in the thoracic aorta in WKY rats; control and CJID-treated is 31.2 ± 5.3 a.u. (arbitrary units) and 29.2 ± 2.2 a.u. respectively. In comparison with the control of SHRs, CJID-treated decreases the fluorescence level of DHE. The quantification denotes the fluorescence level of SHRs control to be 57.0 ± 5.6 a.u., and CJID-treated is 34.7 ± 4.2 a.u. ($P < 0.01$).

Furthermore, the DCFH-DA fluorescence level of the thoracic aorta in WKY rats is 34.1 ± 6.1 a.u. in the control and 28.5 ± 3.1 a.u. in CJID-treated. The fluorescence level is decreased in SHRs by CJID treatment, as the fluorescence value is 53.5 ± 6.6 a.u. in the control, and 29.1 ± 4.2 a.u. in CJID-treated ($P < 0.01$). The DCFH-DA fluorescence level is significantly increased in SHRs than in WKY rats ($P < 0.05$). These results show that CJID decreases oxidative stress markers in the thoracic aorta of SHRs.

3.5. CJID suppresses the expression of iNOS in the thoracic aorta of SHRs

It should be noted that hypertension is causing vasoactive system imbalance and inducing NO production decline. Hypertension is ascribed to reduce NO production which is caused by up-regulation of iNOS in hypertensive state.³⁴ We opted for iNOS expression to evaluate iNOS-mediated NO production. It is found that iNOS expression is higher in SHR CTL (Fig. 5A and B, $P < 0.01$) in comparison with the WKY groups. In addition, as shown in Fig. 5B, CJID treatment decreases the expression iNOS in comparison with the control of SHRs ($P < 0.01$). As predicted, iNOS down-regulation leads to increase NO production in CJID-treated SHRs.

3.6. CJID suppresses $\text{I}\kappa\text{B}\alpha$ expression in thoracic aorta of SHRs

The activation of NF- κ B requires phosphorylation of $\text{I}\kappa\text{B}$ through formation NF- κ B– $\text{I}\kappa\text{B}$ complex that is mainly detected in the cytoplasm.³⁵ Afterward, we determined the expression of $\text{I}\kappa\text{B}\alpha$ in the thoracic aorta of rats. As shown in Fig. 5C, administration of CJID to SHRs for 5 weeks significantly suppresses the expression of p- $\text{I}\kappa\text{B}\alpha$ in the thoracic aorta of SHRs ($P < 0.05$). CJID is also efficient in

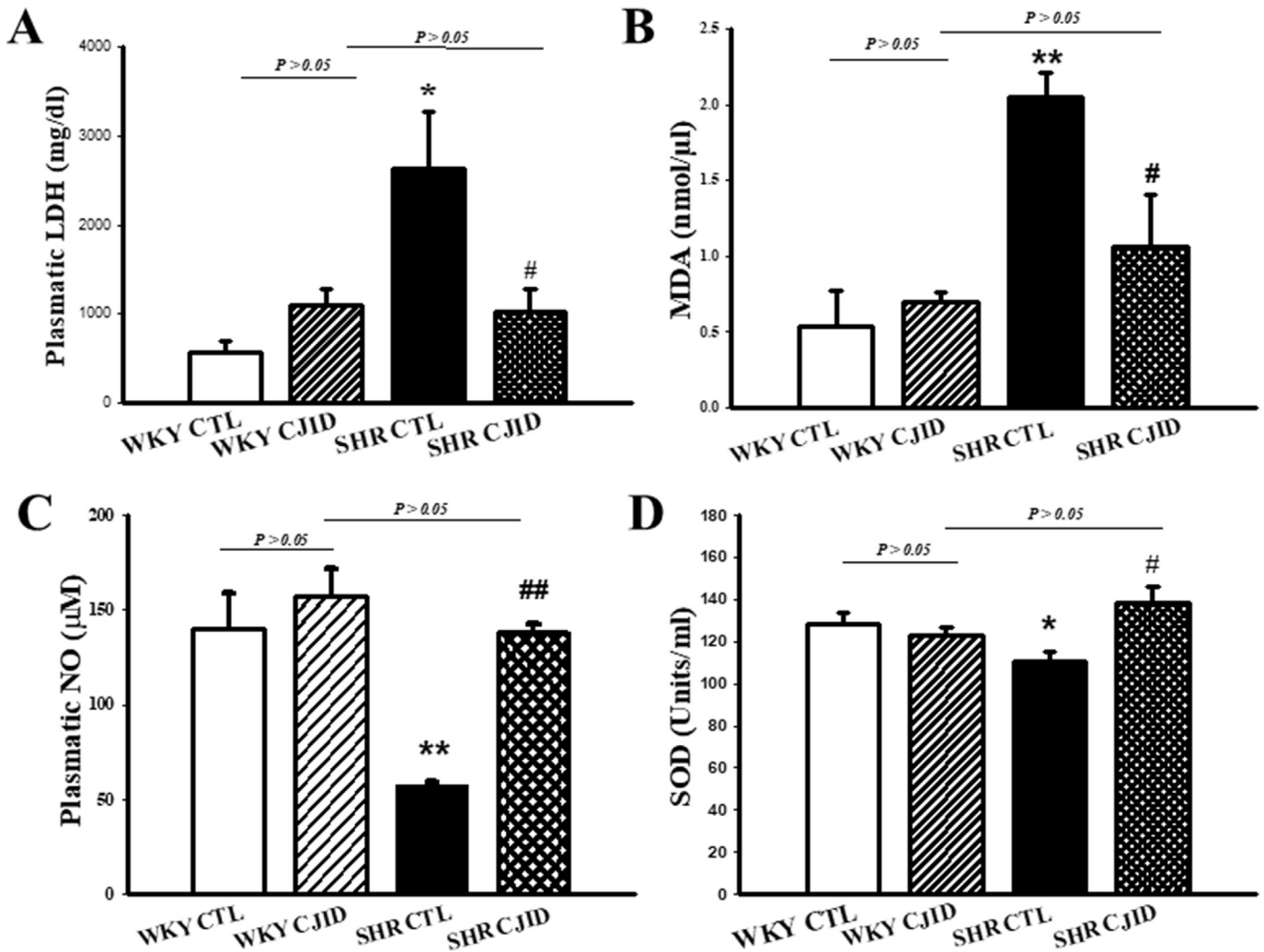


Fig. 3. CJID decoction improves redox status. (A) The blood plasma level of LDH. (B) MDA content in thoracic aorta. (C) The plasmatic NO activity level. (D) SOD level in thoracic aorta. Each point represents the mean \pm SEM, $n = 10$. * $P < 0.05$, ** $P < 0.01$, *when compared to WKY CTL, #when compared to SHR CTL.

reducing p-I κ B α and I κ B α ratio in the thoracic aorta of SHRs ($P < 0.05$). The results show that CJID attenuates the phosphorylation of I κ B α and then maintains the NF- κ B–I κ B complex in SHRs.

3.7. CJID inhibits NF- κ B nuclear translocation in thoracic aorta of SHRs

Elevation of oxidative stress modulates NF- κ B and thus stimulates its nuclear translocation, which may further lead to vascular injury.³⁶ As presented in Fig. 6A, fluorescence visualization under confocal microscope defines that NF- κ B-p65 is detained in thoracic aorta of SHRs. Hypertension causes NF- κ B-p65 to be unconstrained from NF- κ B-I κ B complex to go to the nuclei. Therefore, a high intensity level of reddish-blue color is found in the nuclei of vascular tissue. These results show that hypertension modulates NF- κ B nuclear translocation in SHRs. As shown in Fig. 6B, the intensity of magenta (reddish-blue) color was reduced. This means that NF- κ B-p65 nuclear translocation is diminished by CJID treatment.

3.8. CJID prevents the expressions of NOX1, NOX2 and NOX4 in thoracic aorta of SHRs

Vascular cells express several NOXs being NOX1, NOX2 and

NOX4 the most abundant in all layers of vascular tissue.³⁷ These NOXs are crucial to impede the generation of molecular oxygen to various ROS. Thus, we evaluated whether CJID inhibits the expression of NOX1, NOX2 and NOX4. As presented in Fig. 7, CJID treatment in SHRs causes NOX1 and NOX4 expressions to decrease significantly compared with the control group ($P < 0.01$). In addition, the NOX2 expression is increased as shown with the intensity of yellowish-blue color staining in the nuclei of vascular tissue. It means that the expression of NOX2 is suppressed in SHR CJID in comparison to that of the control group (Fig. 8, $P < 0.05$). These results denote that CJID possesses the ability to scavenge oxidative stress in aorta of SHRs.

4. Discussion

The current study demonstrates that oral administration of CJID decoction in SHRs improves vascular structure by reducing media thickening. As presented in the results section, the redox hemostasis is improved. There are decreases in plasmatic LDH and aortic MDA levels, increased in plasmatic NO and aortic SOD in SHRs, as well as reduction in the expressions of NOX1, NOX2, NOX4, NF- κ B and p-I κ B α .

Vascular remodeling is a major pathological change associated

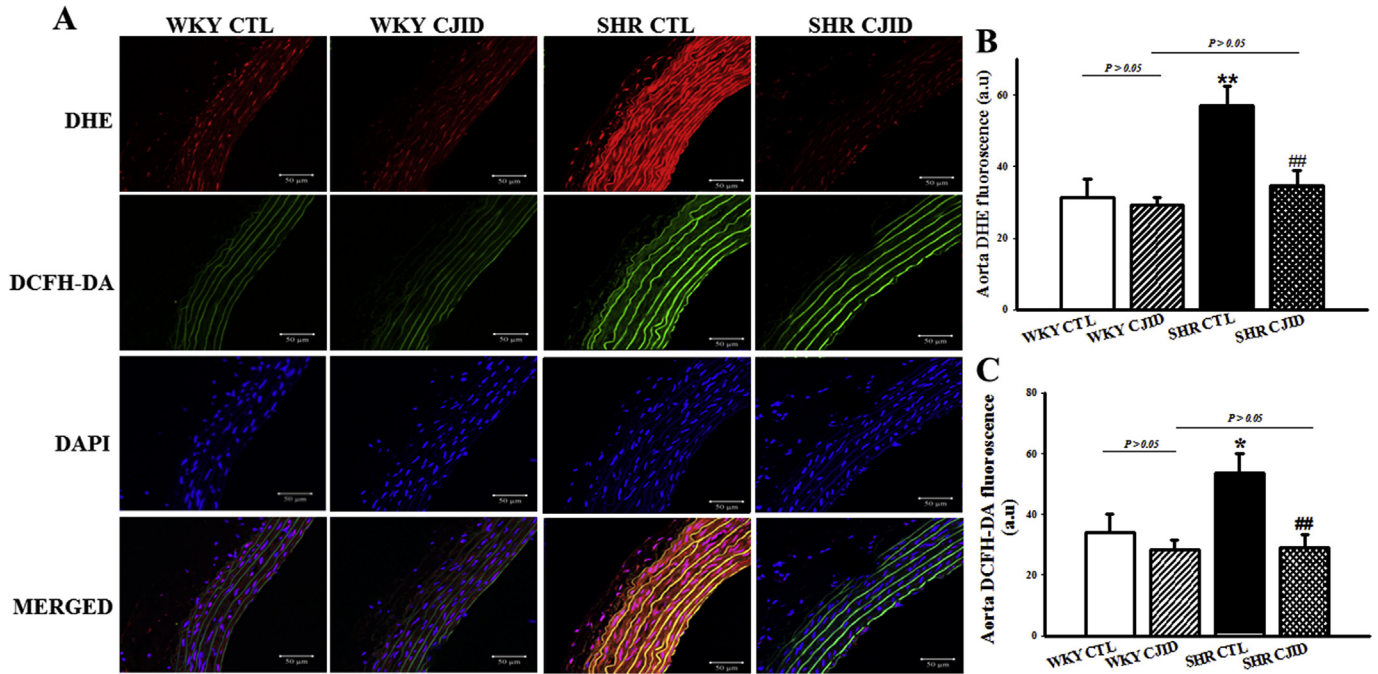


Fig. 4. Effect of CJl decoction on superoxide and hydrogen peroxide generations through confocal microscopic observations (200x) of nuclear thoracic aorta tissue. After 5 weeks treatment of. (A) Anion superoxide was detected by DHE staining and H₂O₂ was detected by DCFH-DA staining from 4 groups including WKY CTL, WKY CJID, SHR CTL, SHR CJID, (B) DHE fluorescence of thoracic aorta tissue, and (C) DCFH-DA fluorescence of thoracic aorta tissue. **P* < 0.05, ***P* < 0.01, *when compared to WKY CTL, #when compared to SHR CTL. Bar shows 50 μm.

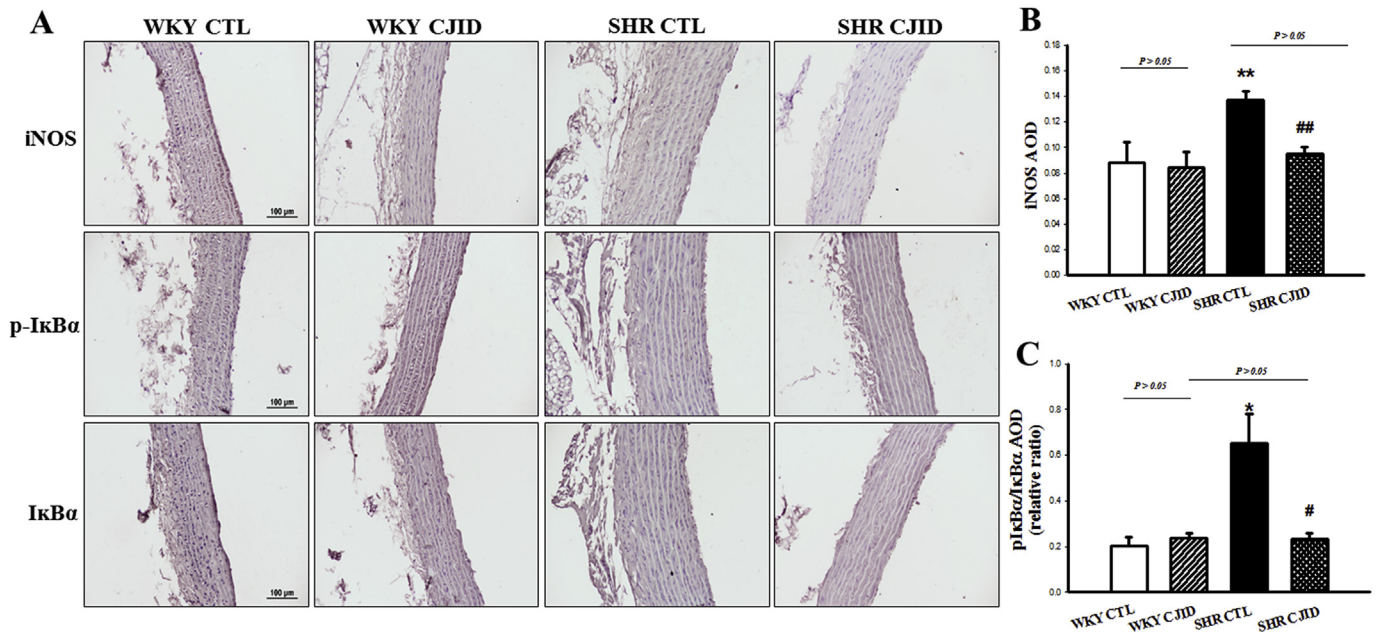


Fig. 5. CJl decoction down-regulated iNOS expression, p-IκBα and IκBα expressions in thoracic aorta. (A) The representative immunohistochemistry staining observations (200x) of thoracic aorta. (B) The iNOS expression. (C) The p-IκBα expression. (D) The IκBα expression. **P* < 0.05, ***P* < 0.01, *when compared to WKY CTL, #when compared to SHR CTL. Bar shows 100 μm.

with hypertension and is an important predictor of worsening vascular function.⁶ Increased arterial pressure induces restructuring of cellular and non-cellular components in the vascular wall. The key remodeling process is controlled by the endothelium as it functions to respond to the changes in hemodynamic and humoral states. It also facilitates the signals to underlying vascular smooth muscle cells (VSMCs). In hypertension, the changes of VSMCs

growth/apoptosis, contraction/relaxation, migration and differentiation, impaired production and degradation of extracellular matrix and stimulation of inflammatory responses result in structural remodeling.³⁸ Findings from the current study indicate that CJID prevents media thickness of the vascular wall in SHRs. By contrast, there is no significant difference among these four group's inner diameter. However, the media thickness and media-to-lumen ratio

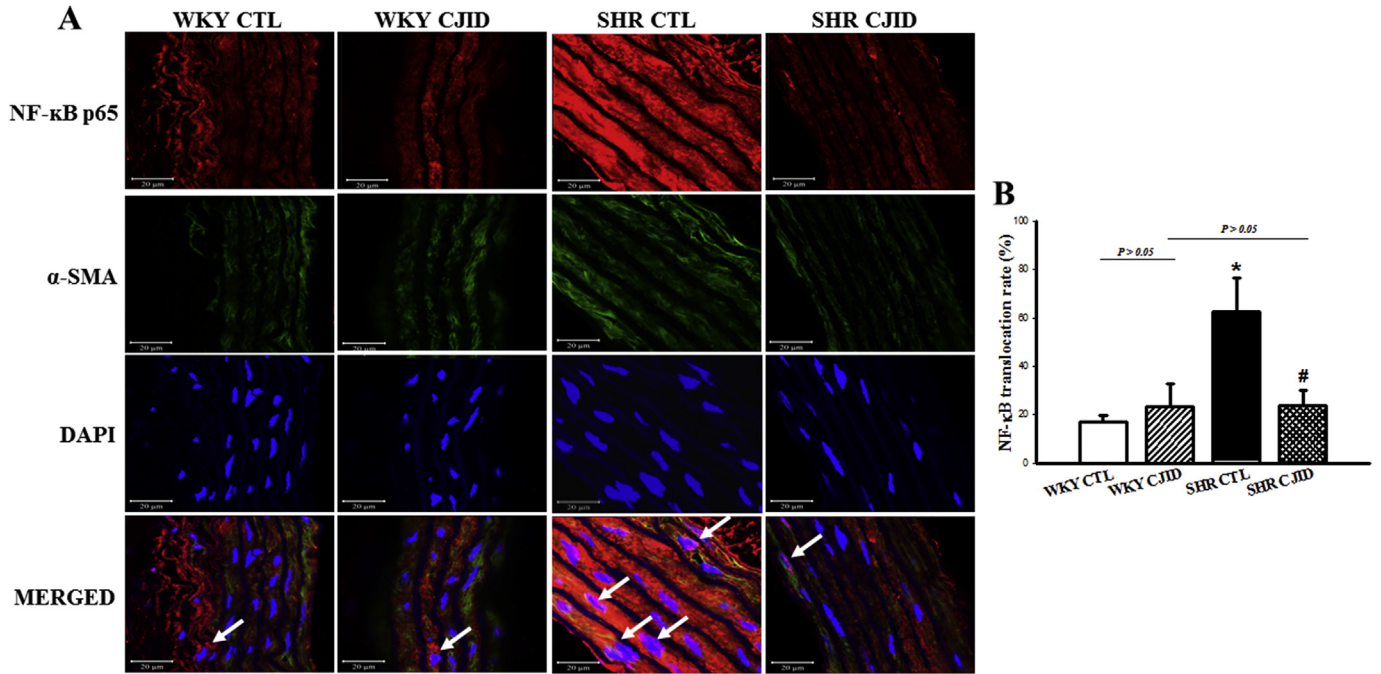


Fig. 6. Effect of CJJ decoction on thoracic aorta NF-κB-p65 nuclear translocation (A) The representative confocal immunofluorescence staining observations (600x) of thoracic aorta. Section were counterstained with rhodamine-labeled mouse antibody. Arrows show nuclear translocation. (B) NF-κB-p65 nuclear translocation in the thoracic aorta wall. The red color represent NF-κB-p65 staining, the blue color represent nuclei staining, the green color represent α-sma and the magenta color (reddish-blue) represent nuclear translocation. Data are presented as mean ± SEM, n = 10. **P* < 0.05, *when compared to WKY CTL, #when compared to SHR CTL. Bar shows 20 μm.

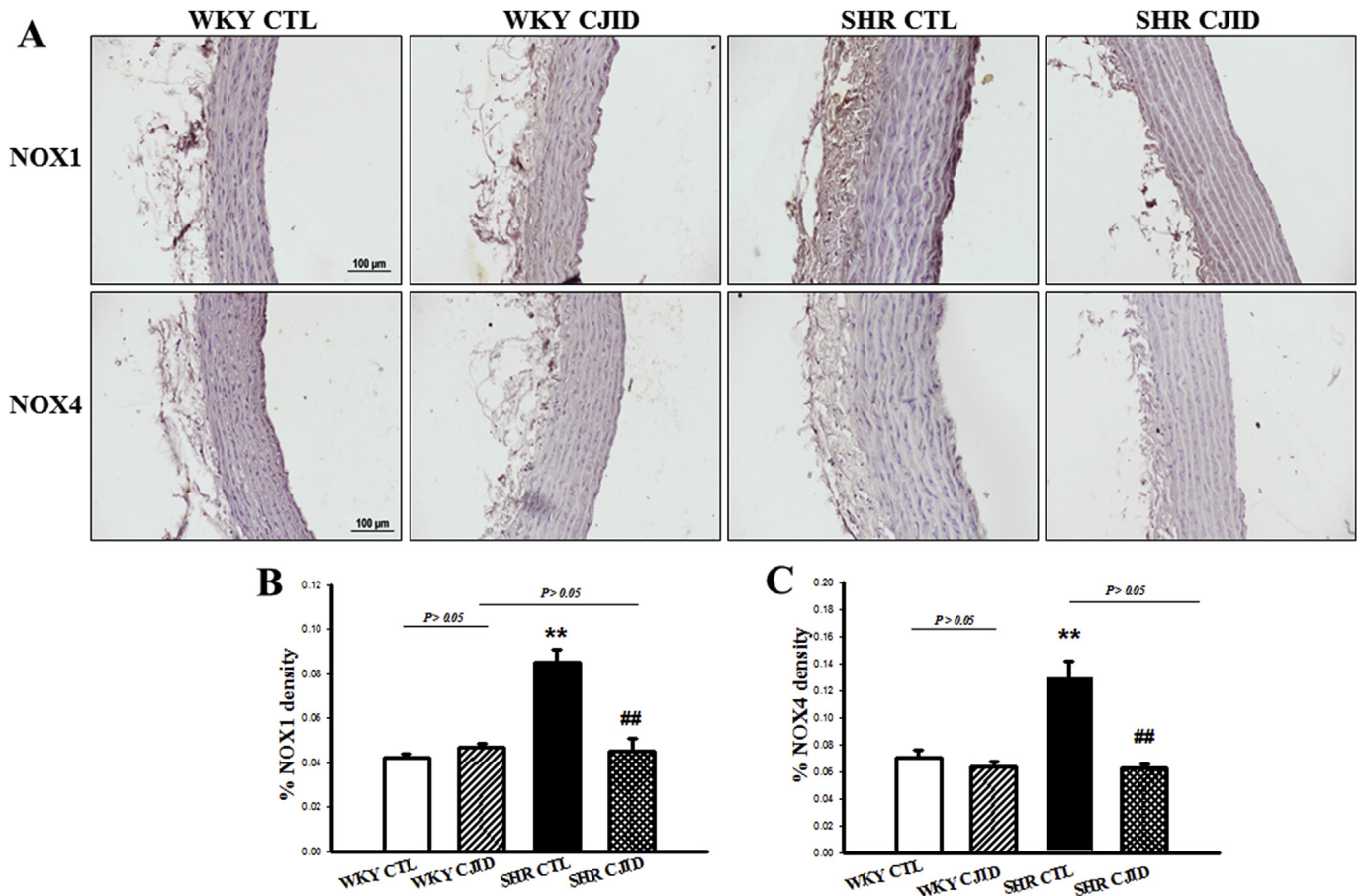


Fig. 7. Effect of CJJ decoction on the protein expression levels of NOX1 and NOX4 in the thoracic aorta by immunohistochemistry analysis. (A) The expression levels of NOX1 and NOX4 in the thoracic aorta. (B) The expression level of NOX1 in the thoracic aorta. (C) The expression level of NOX4 in the thoracic aorta. ***P* < 0.01, * when compared to WKY CTL, #when compared to SHR CTL. Bar shows 100 μm.

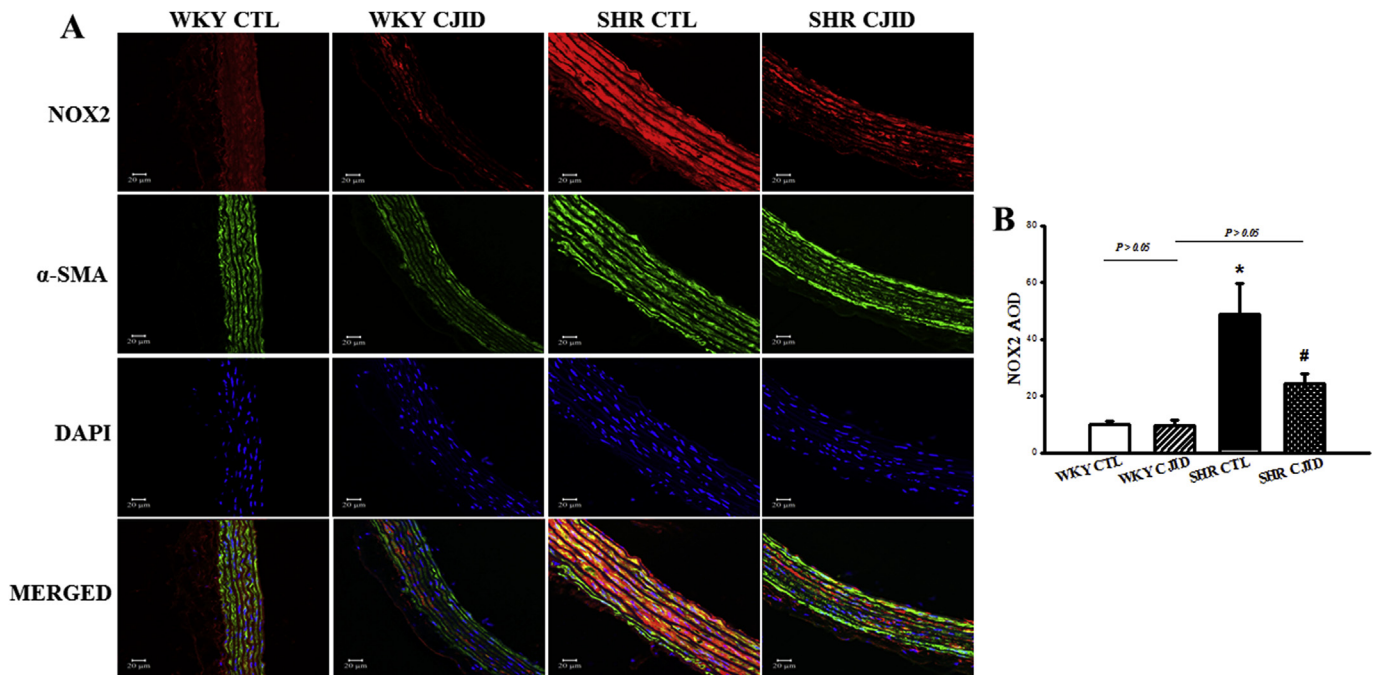


Fig. 8. Effect of CJI decoction on the protein expression levels of NOX2 in the thoracic aorta. (A) The representative confocal immunofluorescence staining observations (200x) of thoracic aorta. Sections were counterstained with rhodamine-labeled mouse antibody. (B) The expression level of NOX2 in the thoracic aorta. The red color represent NOX2 expression, the blue color represent nuclei staining, the green color represent α -SMA and the yellow color represent NOX2 expression. * $P < 0.05$, *when compared to WKY CTL, #when compared to SHR CTL. Bar shows 20 μ m.

are markedly reduced by CJID treatment. The aortic vascular remodeling is measured by calculating the index of the media-to-lumen ratio.³⁹

Vascular remodeling encompasses the reorganization of the matrix scaffolding. Accordingly, a process of active proteolysis and re-synthesis of certain components like collagen and elastic fibers are involved.⁴⁰ The increased arterial pressure causes vessel structure alteration. It results in an elevation of the ratio of the width of the wall to the width of the lumen that is caused by either an increase in muscle mass or disorganization of cellular and extracellular matrix components. In vessel wall, the elastin and collagen determine the passive mechanical properties of the large arteries. Elastin is organized into elastic fibers in the medial layer of large elastic arteries. In hypertension, as the pressure increases, they straighten as they begin to bear the load and are mostly straight at physiologic pressures. The increased blood pressure results in arterial stiffness precede changes in the elastin arterial wall structure. In this context, the increased elastin amounts-induced by high blood pressure contributes in the increased of vessel stiffness.⁴¹ In this study, CJID treatment caused the accumulation of arterial collagen and elastin to be reduced in SHRs, whereas the decrease of elastin density led the alteration of extracellular matrix components in the thoracic aorta of SHRs. Otherwise, the onset of hypertension is associated with increased matrix metalloproteinases (MMPs) activities suggesting that vascular remodeling-induced hypertension is linked to MMPs stimulation. The main function of MMPs, a family of zinc dependent endopeptidases, is to degrade and deposit structural proteins within the extracellular matrix (ECM). In hypertension, the MMPs activation leads to VSMCs proliferation, cell-to-cell tight junction protein alteration and vascular leakage, elastin and collagen derangement, and vascular dysfunction.⁴²

To investigate the underlying mechanism of this vascular remodeling, we further identified redox homeostasis by determining levels of LDH and NO in the blood plasma and levels of MDA

and SOD in the thoracic aorta. LDH is an intracellular enzyme that catalyzes the alteration of pyruvic acid to lactic acid during the process of glycolysis. Investigations have shown that LDH activity elevates in hypertension. LDH level closely correlates with accelerated NO consumption in plasma, and affects vasodilatory responses. LDH serves as the important marker seen in oxidative stress because it can be detected at the first time in the oxidative stress process. Therefore, LDH is an appropriate biochemical marker that express the severity of and the occurrence of complications in hypertension.⁴³

Our study shows that the level of plasmatic LDH increased and plasmatic NO level is reduced in SHRs. Treatment with CJID diminished LDH level in the blood plasma, but not NO level. In addition, it is assumed that the decrease of NO production in hypertension is related with the up-regulation of iNOS expression.³⁴ Our findings support the hypothesis that CJID leads to down-regulation of iNOS expression and NO production.

In the oxidative damage, polyunsaturated lipids are an essential marker for cellular injury. ROS causes cell membrane lipid bilayer to be disrupted and to generate unsaturated aldehyde, MDA.⁴⁴ In the current study, we found that there were higher levels of oxidants in SHRs which could be revealed in the levels of MDA in the thoracic aorta. Moreover, treatment of CJID improved redox homeostasis in SHRs which was shown in the higher levels of endogenous antioxidant SOD in the thoracic aorta of SHRs.

Our study is in line with previous literature showing that hypertension results in ROS generation.⁴⁵ In hypertension, the pathophysiology of vascular remodeling is notably affected by the redox-dependent pathways.⁴⁶ It was characterized that there was an elevated fluorescence intensity of DHE and DCFH-DA in the thoracic aorta of SHRs. These intensities were reduced by CJID treatment. This means that CJID declined superoxide generation and hydrogen peroxide production in the thoracic aorta of SHRs. It was outlined that increased systemic blood pressure induces ROS generation, and the major source of ROS, NADPH oxidase is

involved in the pathogenesis of vascular damage in hypertension.³⁷ As is well known, NOXs are abundantly expressed by vascular tissue. Several vascular NADPH oxidases consist of NOX1, NOX2 and NOX4 to be appealed for the oxidative mechanisms involved in the development of vascular remodeling. In this current study, our findings denote that the NOXs expression in the thoracic aorta of SHR was attenuated by administration of CJID.

We show that hypertension modulates an elevation of ROS production, which further modulated p-I κ B α and NF- κ B expressions. As proposed, the evidence we found points to Lassègue and Griendling (2010) that oxidative stress triggers NF- κ B activation by increasing the phosphorylation of I κ B α and the degradation of cytoplasmic I κ B α .⁴⁷ So that, inhibition of NF- κ B activation suppresses vascular remodeling. This current study demonstrates that CJID prevents vascular remodeling by reducing NF- κ B-p65 activation in the thoracic aorta of SHRs. As hypothesized, CJID treatment inhibits oxidative stress in vivo through the inhibition of NF- κ B activation.

A formulation containing *Centella asiatica*, *Justicia gendarussa* and *Imperata cylindrica* has been shown to exhibit anti-hypertension and prevention of left ventricular hypertrophy in SHRs. This Indonesian traditional medicine has been used for years by traditional healers in Indonesia to treat hypertension. Researches have indicated that the active ingredients of CJI are potential; it not only prevents hypertension-induced heart disease but also inhibits vascular remodeling.^{11,48,49} The oral administration of CJI decoction for five weeks reduced systolic blood pressure in SHR by 24.2% at the end of the study, and the average blood pressure lowering was at 51.3 points.¹¹ CJI contains so many active ingredients that function as radical scavenging activities. Rich content of polyphenols from CJI show an important antioxidant activity. These major active compounds including triterpenoids (asiaticoside, asiatic acid, madecassic acid and lupeol), flavonoids (quercetin, kaempferol and astragalol), lignans (Graminone B), and ascorbic acid are mainly found in CJI.^{50–52} Altogether, these compounds are potentially responsible for the antioxidant actions seen in SHRs.

However, some limitations should be noted in the current study. We have no observations on blood pressure and heart rates among the rats. As we mentioned previously, there have been certain studies concerning these functions. These studies claimed that there were anti-hypertension properties to be found in these herbs. Future endeavors are still needed to improve the study design to verify the profound pathway of CJI, which may be preventing hypertension-induced vascular remodeling.

5. Conclusions

Our findings propose an inhibition of hypertension-related vascular remodeling in SHRs through potential regulation of NOXs-ROS-NF- κ B signaling. Administration of CJI decoction to SHRs improves vascular structure through preventing NF- κ B activation and thus scavenging abundant ROS via suppressing NOX1, NOX2 and NOX4 expressions. Our study confirms that CJI serves as a genuine antioxidant ingredients against vascular damage caused by hypertension via NOXs-ROS-NF- κ B signaling pathway. Further data collection is required to determine exactly how CJI prevents hypertension-induced vascular remodeling. CJID is a novel candidate for vasculoprotective medicines for hypertensive vascular diseases patients. We might establish further animal and clinical studies to substantiate these important findings.

Funding

This study was supported in part by research grants from the

Ministry of Science and Technology (MOST 106-2320-B-037-010-MY3), Kaohsiung Medical University Chung-Ho Memorial Hospital (KMUH 105-5R37 and KMUH 106-6R45), Chi Mei Medical Center and Kaohsiung Medical University Research Foundation (108 CM-KMU-002), and in part by the Indonesian DIKTI scholarship 2014 under Contract 160.5/E4.4/2014. We deeply appreciate the excellent technical support from Ms. I-Chun Hsieh and Ms. Li –Ying Chen.

Conflicts of interest

The authors declare no conflicts of interest.

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