รายงานวิจัยฉบับสมบูรณ์

Investigation antihypertensive mechanism of hesperidin in nitric oxide-deficient and 2K-1C hypertensive rats

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สัญญาเลขที่ RSA6080005

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยขอนแก่น

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. และ มหาวิทยาลัยวิทยาลัยขอนแก่น

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กิตติกรรมประกาศ

งานวิจัยเรื่อง Investigation antihypertensive mechanism of hesperidin in nitric oxide-deficient and 2K-1C hypertensive rats การตรวจสอบกลไกต้านความดันเลือดสูงของสารเฮสเพอริดินในหนูความดันเลือดสูงที่พร่องไนตริก ออกไซด์และ 2K-1C ได้รับทุนสนับสนุนจากสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยขอนแก่น ภายใต้ทุนทุนพัฒนานักวิจัย ปี 2560 รหัสทุน RSA6080005 ผู้วิจัยจึงขอขอบพระคุณเป็นอย่างสูงมา ณ โอกาสนี้

คณะผู้วิจัย

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Abstract

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Hesperidin, a flavonoid derived from citrus fruits, possesses several beneficial effects including anti-oxidation and anti-inflammation. The aim of this study was to investigate the effects of hesperidin on cardiovascular parameters in animal models of hypertension, two-kidney, one-clipped (2K-1C) hypertensive rats and L-NAME hypertensive rats. were treated with hesperidin at 20 mg/kg or 40 mg/kg or losartan at 10 mg/kg beginning at three weeks after surgery and then continued for four weeks (n=8 / group). Hesperidin reduced blood pressure in a dose-dependent manner in hypertensive rats compared to untreated rats ($p < 0:05$). This antihypertensive effect was associated with suppression of the renin-angiotensin system (RAS) cascade that mediated oxidative stress and sympathoexcitation in 2K-1C-hypertensive rats ($p < 0:05$). In L-NAME hypertensive rats, they were treated with L-NAME (40 mg/kg); L-NAME plus hesperidin (15 mg/kg), or hesperidin (30 mg/kg), or captopril (2.5 mg/kg) for five weeks (n=8 / group). Hesperidin or captopril significantly prevented L-NAME induced hypertension and cardiac remodeling. These were associated with reducing oxidative stress and inflammatory markers and enhancing plasma nitric oxide metabolite (NOx) in L-NAME treated groups. Based on these results, it can be presumed that hesperidin has antihypertensive and cardioprotective effects. Its antihypertensive action might be associated with reducing RAS cascade-induced NOX2 over-expression and sympathoexcitation in 2K-1C hypertensive rats. Subsequently, its cardioprotective effects in L-NAME hypertensive rats may involve its antioxidant and anti-inflammatory effects.

Keywords: hesperidin; the renin-angiotensin system; sympathoexcitation; cardiovascular function and remodeling; oxidative stress; inflammation.

บทคัดย่อ

รหัสโครงการ: RSA 6080005

ชื่อโครงการ: การตรวจสอบกลไกต้านความดันเลือดสูงของสารเฮสเพอริดินในหนูความดันเลือดสูงที่พร่องไนตริก ออกไซด์และ 2K-1C

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ระยะเวลาโครงการ: 3 ปี

เฮสเพอริดินเป็นสารในกลุ่มฟลาโวนอยด์พบในผลไม้ตระกูลส้ม มีฤทธิ์ในการต้านอนุมูลอิสระและต้านการอักเสบ วัตถุประสงค์ของการศึกษานี้เพื่อตรวจสอบผลของเฮสเพอริดินต่อตัวแปรในระบบหัวใจร่วมหลอดเลือดในหนูแรทรูปแบบความดันเลือดสูง ได้แก่ หนูแรทที่ถูกชักนำให้เกิดความดันเลือดสูงด้วยสาร L-NAME และหนูแรทความดันเลือดสูงแบบ 2K-1C โดยหนูแรทความดันเลือดสูงแบบ 2K-1C ได้รับเฮสเพอริดิน 20 หรือ 40 มิลลิกรัม/กิโลกรัม หรือ losartan 10 มิลลิกรัม/กิโลกรัม ในสัปดาห์ที่ 3 หลังการผ่าตัด เป็นเวลา 4 สัปดาห์ (n=8/กลุ่ม) ส่วนหนูแรทความดันเลือดสูงที่ได้รับสาร L-NAME ได้รับ L-NAME 40 มิลลิกรัม/กิโลกรัม ร่วมกับเฮสเพอริดิน 15 หรือ 30 มิลลิกรัม/กิโลกรัม หรือ captopril 2.5 มิลลิกรัม/กิโลกรัม เป็นเวลา 5 สัปดาห์ (n=8/กลุ่ม) ผลการศึกษาพบว่าเฮสเพอริดินลดความดันเลือดในหนูแรทความดันเลือดสูงเปรียบเทียบกับหนูแรทที่ไม่ได้รับเฮสเพอริดิน ($p < 0.05$) โดยสัมพันธ์กับการยับยั้งระบบเรนินแองจิโอเทนซินซึ่งควบคุมภาวะเครียดออกซิเดชั่นและการกระตุ้นระบบประสาทซิมพาเทติก ในหนูแรทความดันเลือดสูงแบบ 2K-1C ($p < 0.05$) เฮสเพอริดินหรือ captopril ป้องกันการเกิดความดันเลือดสูงจากสาร L-NAME และการเปลี่ยนแปลงรูปแบบของหัวใจอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ซึ่งสัมพันธ์กับการลดตัวชี้วัดอนุมูลอิสระและการอักเสบ และการเพิ่มขึ้นของไนตริกออกไซด์ในพลาสมา ($p < 0.05$) จากผลการศึกษาสามารถกล่าวได้ว่าเฮสเพอริดินมีผลต้านความดันเลือดสูงและปกป้องหัวใจ โดยสัมพันธ์กับการลดการทำงานของระบบเรนินแองจิโอเทนซินซึ่งเหนี่ยวนำให้เกิดการแสดงออกของ NOX2 และการกระตุ้นระบบประสาทซิมพาเทติกในหนูแรทความดันเลือดสูงแบบ 2K-1C และผลของเฮสเพอริดินในการปกป้องหัวใจในหนูแรทความดันเลือดสูงจาก L-NAME น่าจะเกี่ยวข้องกับฤทธิ์ต้านอนุมูลอิสระและต้านการอักเสบ

คำหลัก: เฮสเพอริดิน ระบบรีนิน แองจิโอเทนซิน การกระตุ้นซิมพาเทติก การทำงานและการปรับรูปร่างของหัวใจและหลอดเลือด ภาวะเครียดออกซิเดชัน การอักเสบ

บทนำ

It is well established that activation of the renin-angiotensin system (RAS) mainly mediates the development of hypertension in an animal model of two-kidney, one-clip (2K-1C) rats (1). This was supported by several studies in that an elevation of renin (2), and angiotensin converting enzyme (ACE) activities, angiotensin II (Ang II) levels (3), as well as upregulation of angiotensin II type 1 receptor (AT_1 receptor) protein expression in the 2K-1C model were observed (4). Although Ang II, the main effector peptide of RAS, primarily causes hypertension via binding to AT_1 receptors to develop high vascular resistance and salt water retention, other important mechanisms such as Ang II-mediated oxidative stress (5) and sympathoexcitation have recently been proposed (6).

There is increasing evidence to confirm that oxidative stress is implicated in pathophysiology of 2K-1C Goldbatt hypertension (7, 8). The mRNA levels of AT_1 receptor, NADPH oxidase (NOX) subunits $p47^{phox}$ and $gp91^{phox}$ (NOX2) in renal cortex and aortic tissue of 2K-1C rats were elevated (4, 8). NOXs are primary sources of reactive oxygen species (9). NOX2 is mainly identified in endothelial cells and possibly in vascular smooth muscle cells, thus, overexpression of NOX2 in endothelial cells has been reported to promote Ang II-induced vascular oxidative stress and endothelial dysfunction in mice (10, 11). Overexpression of $gp91^{phox}$ in aortas (4) and systemic oxidative stress (12) were revealed in 2K-1C hypertension. Subsequently, up-regulation of $p47^{phox}$ protein associated with an increase in vascular superoxide ($O_2^{\bullet -}$) production and endothelial dysfunction were displayed in 2K-1C rats (12).

There is substantial evidence showing that Ang II potentiates sympathetic nerve stimulation in rat mesenteric arteries via increased noradrenaline (NA) release from presynaptic sites (13, 14). Activation of sympathetic vasomotor tone in RAS-dependent hypertension has been clearly demonstrated (15). This sympathoexcitation during renovascular hypertension is involved in the upregulation of AT_1 receptor protein in the rostral ventrolateral medulla (RVLM) of 2K-1C hypertensive animals (16). Koyama and coworkers reported the enhancement of sympathetic neurotransmission in mesenteric vascular beds of renovascular hypertensive rats (17). Moreover, Zimmerman and coworkers reported that chronic renovascular hypertension was associated with an elevation in mesenteric vascular responses to sympathetic nerve stimulation (18).

Nitric oxide (NO) is a crucial vasodilator derived from vascular endothelium to regulate vascular tone [1]. A reduction of NO production results in increased vascular resistance and high blood pressure. N^{ω}-nitro L-arginine methyl ester (L-NAME), an L-arginine analogue, is widely used as an inhibitor of nitric oxide synthase (NOS) activity to represent an animal model of hypertension. It has been reported that L-NAME-induced hypertension in rats is characterized by insufficient NO production, increased systemic oxidative stress, inflammation and endothelial dysfunction [2]. Furthermore, L-NAME-induced hypertension associated cardiovascular remodeling has also been reported in rats. For example, L-NAME (40mg/kg) administration for 4 or 5 weeks causes high blood pressure and cardiovascular remodeling including, left ventricular hypertrophy, myocardial fibrosis and thickening of vascular wall [3-5]. It is generally known that the main sequel of cardiovascular remodeling is heart failure, which is the major cause of death worldwide [6].

The initial stage of cardiac remodeling is myocardial hypertrophy because of the adaptive response to a high-pressure load to preserve cardiac function and obtain normal cardiac work. In addition, the cardiac remodeling process in L-NAME treated rats is involved in a production of myocardial fibrosis [7]. There are substantial data to show the molecular mechanism of extensive areas of cardiac fibrosis which is associated with the activation of various downstream inflammatory [8] and oxidative stress initiatives [9, 10]. For example, a high level of tumor necrosis factor (TNF-α), a pro-inflammatory cytokine, developing in response to oxidative stress in L-NAME induced hypertension has been reported [4, 11]. These inflammatory responses subsequently activate the profibrotic mediator of the transforming growth factor β1 (TGF-β1) [11]. It is well established that TGF-β1 has a key role in fibrogenesis by activating apoptosis, collagen and matrix protein synthesis [12-14]. For vascular structural changes in hypertension, it is known to be an adaptive response to an increase in wall tension [15]. This response is also related to extracellular matrix degradation of elastic fibers since the up-regulation of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) expression in vessel tissue has been confirmed in animal models of hypertension. Several lines of evidence have indicated that activation of MMP-2/9 protein expression found in the vascular remodeling process is mediated by the inflammatory cytokine, TNF-α [16-18]. Thus, it is noteworthy that natural products with high anti-oxidant and anti-inflammatory activities might be useful to alleviate cardiovascular alterations induced by nitric oxide deficiency.

Hesperidin is a flavanone glycoside, a subclass of flavonoids, abundantly found in citrus fruits such as lemon or orange peels or juices [24]. Numerous beneficial effects of hesperidin have been published. For example, the antioxidant effect of hesperidin has been reported to be able to sequester 1,1-diphenyl-2-picrylhydrazyl (DPPH) and protect cell injury-induced by paraquat and hydrogen peroxide [25], reduce plasma levels of lipid peroxidation markers and increase antioxidant enzyme activities in heart tissue in experimentally ischemic myocardial rats [26]. Hesperidin has also exhibited an anti-inflammatory effect by reducing circulating inflammatory markers, i.e. TNF-α, interleukin 6

(IL-6), and a high-sensitivity C-reactive protein (hs-CRP), in patients with type 2 diabetes [27] and suppressed inflammatory responses in lipopolysaccharide-induced RAW 264.7 cells [28]. Subsequently, a clinical study revealed that a combination of hesperidin, diosmin and troxerutin was effective to relieve the symptoms of acute hemorrhoidal disease [29]. Hesperidin can inhibit lipase activity from the porcine pancreas (19). The beneficial effect of hesperidin on microcirculation has been shown that in a combination of hesperidin and diosmin in Raynaud's syndrome treatment, capillary circulation can be improved and edema of the fingers of the hands is reduced (20). Presently, hesperidin is used as an effective supplementary treatment to relieve hemorrhoids, varicose veins, and poor circulation as in venous stasis (21, 22). Additionally, a previous study reported that hesperidin had an antihypertensive effect associated with reducing endothelial dysfunction and oxidative stress in spontaneously hypertensive rats (23). Recently, the current authors have demonstrated an antihypertensive effect of hesperidin in renovascular hypertensive rats that involved the suppression of the renin-angiotensin system [30].

Captopril is an angiotensin converting enzyme (ACE) inhibitor and commonly used as an anti-hypertensive drug [19]. Its mechanism of action is well documented to reduce angiotensin II production which subsequently suppresses renin-angiotensin-aldosterone system (RAAS)[19]. Other possible anti-hypertensive mechanisms include increase of bradykinin and prostaglandins levels [20], inhibition of superoxide production [21], and free radical scavenging effect [22]. Many studies have already reported on cardiovascular effects of captopril in nitric oxide-deficient hypertensive rats, i.e. lowering the high blood pressure, improvement of vascular function [21], and prevention of cardiovascular remodeling [23]. In L-NAME hypertensive rats, there is an evidence showing the up-regulation of angiotensin II receptor type 1 (AT1R) which mediates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression and superoxide formation [10]. This study used captopril to be a positive control agent because the L-NAME hypertension model is also involved with activation of RAAS, where captopril inhibits the RAAS.

Losartan, a selective non-peptide angiotensin AT_1 receptor antagonist, was used as a positive control in this study. It has been well established to target RAS to manage hypertension. Several studies reported other potential effects that are linked to its antihypertensive effects including, antioxidant, anti-inflammatory, and anti-proliferative effects (2, 7, 24, 25). Of special importance is that losartan can inhibit sympathetic nerve activity and AT_1 receptor expression in 2K-1C hypertensive rats (7).

There are two main aims of the present study, 1) to explore whether hesperidin could prevent L-NAME-induced hypertension and cardiovascular remodeling in rats, 2) to investigate whether hesperidin could reduce blood pressure, inhibit the RAS cascade, and suppress sympathetic nerve activity and NOXs protein expression in renovascular hypertensive rats.

วิธีการทดลอง

Part I

Animals and experimental protocols

Male Sprague-Dawley rats weighing 150-180 g were obtained from Nomura Siam International Co., Ltd., Bangkok, Thailand. They were housed at 25 ± 2°C with a 12 h dark–light cycle at the Northeast Laboratory Animal Center, Khon Kaen University, Khon Kaen, Thailand. All procedures complied with the standards for the care and use of experimental animals and were approved by the Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (AEKKU-NELAC 37/2559). After a week of acclimatization, rats were anesthetized with pentobarbital sodium (60 mg/kg, ip.) and then a silver clip (0.2 mm i.d.) was placed on to the left renal artery. The sham-operated group had the same surgical procedure but the clips were not applied on the left renal arteries. Three weeks after the surgery, the 2K-1C rats were divided into 5 groups of 8-9 rats each.

Group I Sham + vehicle or polyethylene glycol (PG) (0.5 ml/100 mg/BW; p.o.)

Group II 2K-1C + vehicle or PG (0.5 ml/100 mg/BW; p.o.)

Group III 2K-1C + hesperidin (20 mg/kg/BW; p.o.)

Group IV 2K-1C + hesperidin (40 mg/kg/BW; p.o.)

Group V 2K-1C + losartan (10 mg/kg/BW; p.o.)

Hesperidin, losartan, and PG were intragastrically administered daily for 4 weeks of the study.

Indirect measurement of blood pressures and heart rates (HR) in conscious rats

Indirect blood pressures were measured once a week for 8 weeks. Systolic blood pressures (SP) and HRs were measured in conscious rats by the tail-cuff plethysmography (IITC model 179 blood pressure analyzer) method. In brief, conscious rats were placed in a restrainer and allowed to calm prior to blood pressure measurement. The tail of each rat was placed inside the tail cuff, and the cuff was automatically inflated and released. For each rat, blood pressures and HRs were recorded as the mean values from the three measurements at 15 min intervals.

Direct measurement of blood pressures and HRs in unconscious rats

Direct blood pressure and HR were determined at the end of study. Briefly, rats were anesthetized by intra-peritoneal administration of pentobarbital-sodium (60 mg/kg). Body temperatures were monitored. A polyethylene tube was inserted into a femoral artery for blood pressure measurement. SP, diastolic blood pressures (DP), mean arterial pressures (MAP) and HRs were continuously monitored by way of pressure transducers and recorded using the Acknowledge Data Acquisition and Analysis Software (BIOPAC Systems Inc., California, USA).

Measurement of oxidative stress markers

Blood was collected from abdominal aortas, mixed with EDTA and placed on ice for plasma malondialdehyde (MDA) measurements. The concentration of plasma MDA was measured as thiobarbituric acid-reactivity (TBA) reactive substances by a spectrophotometric method as previously described (12). Productions of $O_2^{\bullet-}$ in vascular tissues were determined by lucigenin-enhanced chemiluminescence. The carotid artery was rapidly removed and placed in ice-cold saline, and connective tissues and adherent fat was cleaned off. The vessel was cut into 1 cm lengths and incubated with 1 mL oxygenated Krebs-KCl buffer and allowed to equilibrate at pH 7.4, 37 °C for 30 min. Thereafter, lucigenin was added to the sample tube and placed in a luminometer (Turner Biosystems, Sunnyvale, CA, USA). Luminometer counts were recorded every 30 s for 5 min and averaged. Vascular tissue $O_2^{\bullet-}$ production was expressed as relative light unit counts per minute per milligram of dried tissue weight.

Biochemical measurements

The concentration of plasma Ang II was measured using an Ang II Enzyme immunoassay (EIA) kit (St. Louis, MO, USA). ACE activity was evaluated in plasma using a fluorescent assay following the basic method with some modifications (26). Plasma NA was determined by HPLC with an electrochemical detector (DECADE II, Waters, Milford, MA) using commercial kits (RECIPE, Dessauerstraße 3, D-80992 Munich, Germany). Plasma nitric oxide metabolites (NOx) were assayed using an enzymatic conversion method with some modifications (12).

Vascular Function Study

Experimental protocols in isolated mesenteric vascular beds

After exsanguination, mesenteric vascular beds were carefully isolated and then placed on a stainless-steel grid in a humid chamber. The preparations were perfused with physiological Krebs' solution at a constant flow rate of 5 ml/min, using a peristaltic pump (07534-04, Cole-Palmer Instrument, Illinois, USA). Kreb's solution is composed of the following (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.8, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 1.25 and glucose 11.1. The mesenteric vascular beds were pretreated with a desensitizing agent, capsaicin (0.1 μM), for 20 min followed by a 30 min washout period to facilitate a desensitization of vanilloid receptors and to cause a diminution of sensory neurotransmitters. After the washout period, electrical field stimulation (EFS) (5-40 Hz, 90 V, 1 ms, for 30 s at 5-min intervals) was performed. Contractile responses to EFS were detected as changes in mean perfusion pressure (mmHg) using a pressure transducer and data recorded via the BIOPAC System (BIOPAC Systems Inc., California, USA). The preparations were allowed to equilibrate for 30 min before the next trial. After the resting period, NA (0.15 nmol-15 nmol) was applied to evaluate the contractile responses to exogenous NA. To determine vasoactive performance of resistance small arteries, methoxamine (5-7 μM) was added into Kreb's solution to raise tone 70-90 mmHg above baseline. Subsequently, different doses of vasoactive agents, acetylcholine (ACh, 1 nM-0.01 μM) or sodium nitroprusside (SNP, 1 nM-0.01 μM) were applied.

Experimental protocols in isolated aortic rings

The thoracic aorta was rapidly removed and cut into rings 2-3 mm long for tension measurements. They were mounted in 15 ml baths containing Krebs' solution at 37 °C and gassed with a 95% O_2 and 5% CO_2 gas mixture. Isometric contractions were recorded with a resting tension of 1 g using a transducer connected to a 4-channel bridge amplifier and a PowerLab A/D converter and a PC running Chart v5 (PowerLab System, AD Instruments, Australia). ACh (0.01 μM-3 μM) induced endothelial mediated-relaxations and SNP (0.01 μM-3 μM) were assessed by pre-contracting with phenylephrine (10 μM) and relaxations were expressed as % relaxation.

Western blot analysis

AT_1 receptor, $p47^{phox}$ and $gp91^{phox}$ protein expressions in the thoracic aorta were measured using the Western blot method following a previous publication as described with some modifications (12). The vessels were homogenized and the proteins determined by electrophoresis on a sodium dodecylsulfate polyacrylamide gel electrophoresis system. Thereafter, the proteins were electrotransfered onto a polyvinylidene difluoride membrane and blocked with 5% skimmed milk for 2 hours at room temperature before overnight incubation at 4°C with mouse monoclonal antibodies to $p47^{phox}$ and $gp91^{phox}$ (BD Biosciences, CA, USA) or rabbit polyclonal antibodies to AT_1 receptor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After the incubation period, the membranes were washed with tris-buffered saline with tween and then incubated for 2 hours at room temperature with horseradish peroxidase conjugated secondary antibody. The blots were developed in Amersham™ ECL™ Prime solution (Amersham Biosciences Corp., Piscataway, NJ, USA), and densitometric analysis was performed using an ImageQuantTM 400 (GE Healthcare Life Sciences, Piscataway, NJ, USA). The intensities of AT_1 receptor, $p47^{phox}$ and $gp91^{phox}$ bands were normalized to that of β-actin, and data were expressed as a percentage of the values determined in the control group from the same gel.

PartII

Materials and Methods

Drugs and chemicals

Hesperidin (purity $\geq$ 98%) was purchased from Chem Faces Company (Hubei, China). N(G)-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and captopril were purchased from Sigma-Aldrich Corp (St Louis, MO, USA). All other chemicals used in this study were obtained from standard companies and were of analytical grade quality.

Animals and Experimental protocols

Male Sprague-Dawley rats (body weight 220-250 g) were supplied by Nomura Siam International Co., Ltd., Bangkok, Thailand. The animals were housed in a HVAC (Heating, Ventilation and Air-Conditioning) System (25±2 °C) facility and maintained on a 12 h light and 12 h dark cycle with free access to a standard rat diet and water at the Northeast Laboratory Animal Center, Khon Kaen University. All experimental protocols in this study were in accordance with the standards for the care and use of experimental animals and the approval for all experiments were obtained from the Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (AEKKU-NELAC 37/2559).

After seven days of an acclimatization period, rats were randomly assigned to 5 groups (8/group). The control group animals received tap water and were orally administrated propylene glycol (PG, 1.5 mL/Kg) as a vehicle. L-NAME treated rats received L-NAME (40 mg/kg/day) in their drinking water and were further divided into 4 following groups; L-NAME plus PG, L-NAME plus hesperidin at dose 15 mg/kg (L-NAME+H15 group), L-NAME plus hesperidin 30 mg/kg (L-NMAE+H30 group), L-NAME group plus captopril at a dose 2.5 mg/kg (L-NAME+Cap group). Additionally, normal rats (n=5) were orally treated with hesperidin (30 mg/kg) for 5 weeks to test the hypotensive effect of hesperidin. Hesperidin and captopril were dissolved in vehicle and intragastrically administered once daily for five weeks. The doses of hesperidin and captopril used in this study were influenced by previous studies in this laboratory [10, 30].

Blood pressure measurements

To monitor blood pressure changes throughout the experimental period, systolic blood pressure (SP) was obtained in awake rats once a week for 5 weeks using tail-cuff plethysmography (IITC/Life

Science Instrument model 229 and model 179 amplifier; Woodland Hills, CA, USA). At the end of the final experimental day, rats were anesthetized with pentobarbital sodium (60 mg/kg, ip.). Then, the femoral artery was cannulated and connected to a pressure transducer for monitoring baseline values of SP, diastolic blood pressure (DP), mean arterial pressure (MAP), and heart rate (HR) using the Acknowledge Data Acquisition software (Biopac Systems Inc., Santa Barbara, CA, USA).

Collection of blood and organs

After blood pressure measurement, rats were sacrificed by exsanguination and blood samples were collected from abdominal aortas into EDTA or heparin tubes for assays of oxidative stress and inflammatory markers. The carotid arteries were rapidly excised for analysis of superoxide ($O_2^{\bullet-}$) production. The thoracic aortas and heart tissues were collected for western blotting and morphometric analysis.

Assays of vascular $O_2^{\bullet-}$ production, plasma malondialdehyde (MDA), plasma nitric oxide metabolite (nitrate/nitrite, NOx), plasma TNF-α and plasma TGF- β1 levels

The carotid arteries were cleaned from connective tissues and cut into 0.5 cm length and incubated with 1 mL oxygenated Krebs-KCl solution at pH 7.4, 37 °C for 30 minutes. Production of $O_2^{\bullet-}$ in carotid arteries was determined by lucigenin-enhanced chemiluminescence as previously described [31] with some modifications [32]. Plasma NOx was assayed using an enzymatic conversion method [33] with some modifications [32]. The concentrations of plasma TNF-α and TGF-β1 were measured using enzyme-immunoassay assay (ELISA) kits (eBioscienc, Inc., San Diego, CA, USA and ab119557, Abcam Plc, Cambridge, UK).

Morphometric analysis of thoracic aorta and heart tissue

Heart weight (HW) and left ventricular weight (LVW) were measured, and calculated as an LVW/BW ratio. Thereafter, the left ventricles and thoracic aortas were fixed with 4% paraformaldehyde and then embedded in paraffin and cut into serial 5-μm-thick sections. Each section was stained with hematoxylin and eosin (H&E) and/or Picrosirius Red. Sections were captured with a Digital sight DS-2MV light microscope (Nikon, Tokyo, Japan) or a stereoscope (Nikon SMZ745T with NIS-elements D 3.2, Tokyo, Japan). Morphometric evaluations of the sections were performed with Image J software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis of tumor necrosis factor receptor 1 (TNF-R1), TGF- β1, MMP-2 and MMP-9 protein expressions in cardiac and aortic tissues

Protein samples were prepared by homogenization of cardiac and aortic tissues in a lysis buffer (Cell Signaling Technology Inc., Danvers, MA, USA). The proteins were then electrophoresed on a sodium dodecylsulfate polyacrylamide gel electrophoresis system and transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) with 0.1% Tween 20 for 2 hours at room temperature before overnight incubation at 4°C with primary antibodies against TNF-R1, TGF-β1, MMP-2, MMP-9 or β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Thereafter, the membranes were washed three times with TBS and then incubated for 2 hours at room temperature with horseradish peroxidase conjugated secondary antibody. The protein bands were detected using Luminata™ Forte HRP detection reagent (Merck KGaA, Darmstadt, Germany) and densitometric analysis was performed using ImageQuantTM 400 (GE Healthcare Life Sciences, Piscataway, NJ, USA). The intensity of each band was normalized to that of β-actin, and data were expressed as a percentage of the values determined in the control group from the same gel.

Statistical analysis

Data are expressed as mean ± S.E.M. The differences among treatment groups were analyzed by one-way analysis of variance (ANOVA) followed by Bonferini's post-hoc test. A p-value of less than 0.05 was considered statistically significant.

ผลการทดลอง

Part I Effects of hesperidin and losartan on blood pressure in 2K-1C hypertensive rats during seven weeks of experiment

Effects of hesperidin and losartan on blood pressure in conscious rats

At the beginning of the experiments, baseline SP was similar in all experimental groups. After placing a clip on left renal artery for one week, the SP was significantly high in the 2K-1C hypertensive rats compared to those of sham-operated control rats. Then, SP was gradually increased over 3 weeks of the experiment. Treatment with hesperidin (20 and 40 mg/kg/BW) significantly reduced SP in a dose-response dependent manner (160.79 ± 7.76 and 143.96 ± 3.65 mmHg, respectively, p<0.05) compared to the untreated rats (210.57 ± 11.68 mmHg) (p<0.05). Losartan markedly reduced SP (141.11 ± 3.12 mmHg) (P < 0.05) in hypertensive rats comparing to those of hypertensive rats. However, there was no significant difference between hesperidin at dose 40 mg/kg/BW, and losartan treated-hypertensive rats (Fig. 1).

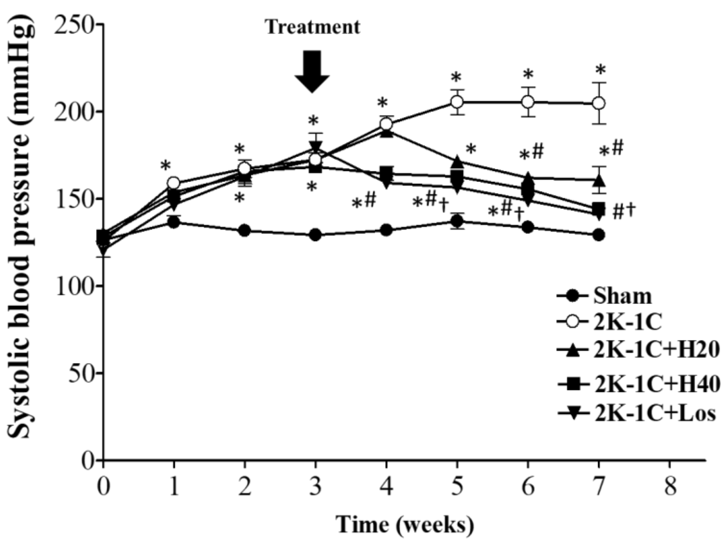

Figure 1. Effect of hesperidin and losartan on systolic blood pressure in 2K-1C hypertensive rats. Data were expressed as means ± SEM. (n = 8-9/group). *P < 0.05 vs Sham, $^{\#}P$ < 0.05 vs 2K-1C, $^{\dagger}P$ < 0.05 vs H20 (Sham = sham-operated control, Los = losartan 10 mg/kg/BW, H = hesperidin)

Effect of hesperidin and losartan on blood pressure and heart rate in anesthetized 2K-1C hypertensive rats

There were significant increases in SP, DP, MAP and HR in 2K-1C hypertensive rats comparing to sham-operated group ($P < 0.05$). Daily treatment of hesperidin for 4 weeks significantly decreased SP, DP and MAP in 2K-1C hypertensive rats comparing to that of untreated group ($P < 0.05$). Moreover, in hypertensive rats treated with losartan and hesperidin (40 mg/kg/BW) were significantly lower than those of in the hypertensive rat treatment with hesperidin (20 mg/kg/BW)($P < 0.05$). There were no difference between hesperidin at dose 40 mg/kg/BW and losartan group (table 1).

Table 1. Effects of hesperidin and losartan on blood pressure and heart rate in anesthetized 2K-1C hypertensive rats.

Parameters	Sham	2K-1C	2K-1C+H20	2K-1C+H40	2K-1C+Los
SP (mmHg)	113.54±1.78	202.48±5.50*	151.53±2.99*#	133.55±3.83*#†	121.20±9.25#†
DP (mmHg)	76.64±1.96	138.63±5.10*	105.22±3.26*#	91.29±3.59*#†	84.31±6.91#†
MAP (mmHg)	88.94±1.36	159.92±4.86*	120.66±3.10*#	105.38±3.60*#†	96.61±7.66#†
HR (beat/min)	362.75±6.90	383.25±13.70	378.89±14.03	359.54±7.98	380.75±11.51

Data were expressed as means ± SEM. (n = 8-9/group). $^{*}P < 0.05$ vs Sham, $^{\#}P < 0.05$ vs 2K-1C, $^{\dagger}P < 0.05$ vs H20 (Sham = sham-operated control, Los = losartan 10 mg/kg/BW, H = hesperidin)

Effects of hesperidin and losartan on RAS activity in 2K-1C hypertensive rats

Effects of hesperidin and losartan on ACE activity and serum angiotensin II concentrations in 2K-1C hypertensive rats

The ACE activity was significantly increased in 2K-1C hypertensive rats compared to sham-operated rats ($p < 0.05$). A reduction of ACE activity in 2K-1C hypertensive rats treated with hesperidin (at both concentration) and losartan were observed compared to untreated rats ($p < 0.05$; Fig. 2A). Furthermore, the serum Ang II was significantly high in 2K-1C hypertensive rats compare to sham rats. This high levels of serum Ang II was attenuated by hesperidin and losartan ($p< 0.05$;

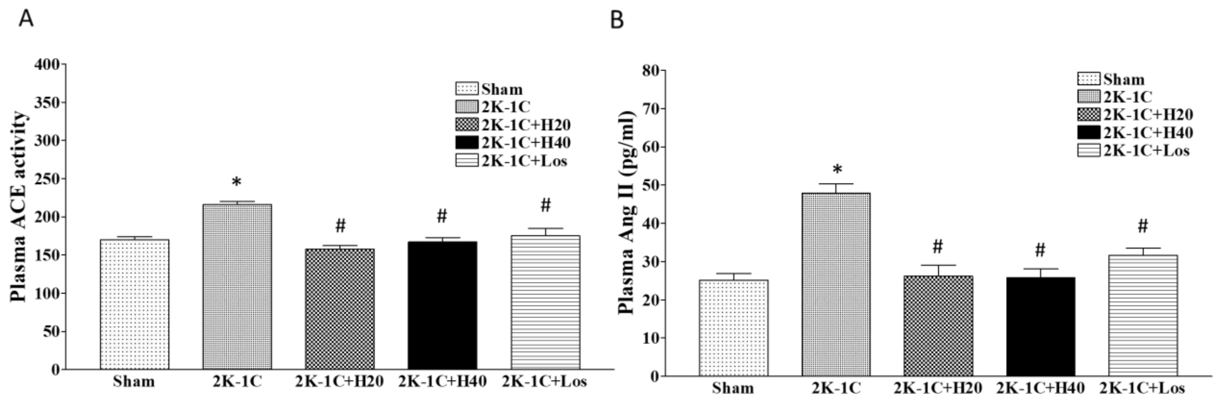

Fig. 2B).

Figure 2. Effects of hesperidin and losartan on ACE activity (A) and ANG II (B) in 2K-1C hypertensive rats. Data were expressed as means ± SEM. (n = 6-8/group). *$P < 0.05$ vs Sham, #$P < 0.05$ vs 2K-1C (Sham = sham-operated control, Los = losartan 10 mg/kg/BW, H = hesperidin)

Effects of hesperidin and losartan on AT_1 receptor protein expression

The expression of AT_1 receptor protein in aortic tissue from 2K-1C hypertensive rats was significantly upregulated when compared to sham-operated rats ($p < 0.05$). Administration of hesperidin significantly suppressed the expression of AT_1 receptor protein in 2K-1C hypertensive rats. In addition, losartan also reduced the upregulation of AT_1 receptor protein expression in 2K-1C hypertensive rats ($P < 0.05$; Fig. 3).

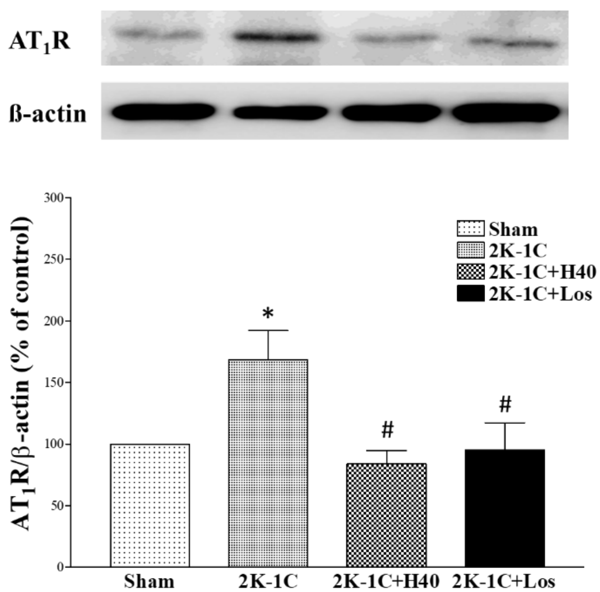

Figure 3. Effect of hesperidin and losartan on AT_1R protein expression in 2K-1C hypertensive rats. Data were expressed as means ± SEM. (n = 4/group). $^{*}P < 0.05$ vs Sham, $^{\#}P < 0.05$ vs 2K-1C (Sham = sham-operated control, Los = losartan 10 mg/kg/BW, H = hesperidin)

Effects of hesperidin and losartan on oxidative stress

Effect of hesperidin and losartan on NOX2 and $p47^{phox}$ protein expression in 2K-1C hypertensive rats

There was overexpression of NOX2 in 2K-1C hypertensive rats compared to those of control rats. Hesperidin treatment restored overexpression of NOX2 in 2K-1C rats while losartan significantly suppressed the upregulation of NOX2 in 2K-1C hypertensive rats ($P < 0.05$; Fig. 4A). Subsequently, the results also showed the upregulation of $p47^{phox}$ in 2K-1C hypertensive rats. This upregulation was significantly attenuated by hesperidin and losartan ($P < 0.05$; Fig. 4B).

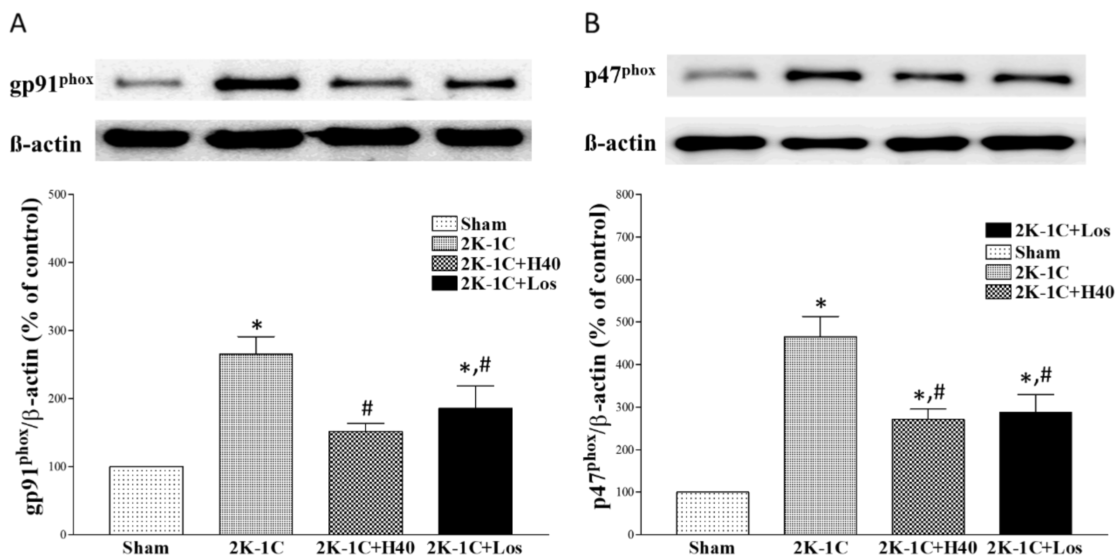

Figure 4. Effect of hesperidin and losartan on NOX2 (A) and $p47^{phox}$ (B) protein expression in 2K-1C hypertensive rats. Data were expressed as means ± SEM. (n = 4/group). $^*P < 0.05$ vs Sham, $^{\#}P < 0.05$ vs 2K-1C (Sham = sham-operated control, Los = losartan 10 mg/kg/BW, H = hesperidin)

Effects of hesperidin and losartan on vascular $O_2^{\cdot-}$ production and plasma MDA in 2K-1C hypertensive rats

There were a significantly increased vascular $O_2^{\cdot-}$ production in 2K-1C hypertensive rats compared to sham-operated rats (p<0.05). The rise of $O_2^{\cdot-}$ production was significantly reduced in 2K-1C hypertensive rats treated with hesperidin (20 and 40 mg/kg/BW) and losartan compared to untreated rats (p<0.05). However, there was no significant difference of vascular $O_2^{\cdot-}$ production among hypertensive rats treated with hesperidin and losartan, and sham-operated rats (Figure 5A). Plasma MDA levels in 2K-1C hypertensive rats was significantly higher than those of sham-operated rats (p<0.05). Hesperidin significantly reduced plasma MDA levels in 2K-1C hypertensive rats compared to those of untreated rats (p<0.05). Moreover, losartan treatment reduced plasma MDA levels in 2K-1C hypertensive rats compared to untreated rats (p<0.05) (Figure 5B).

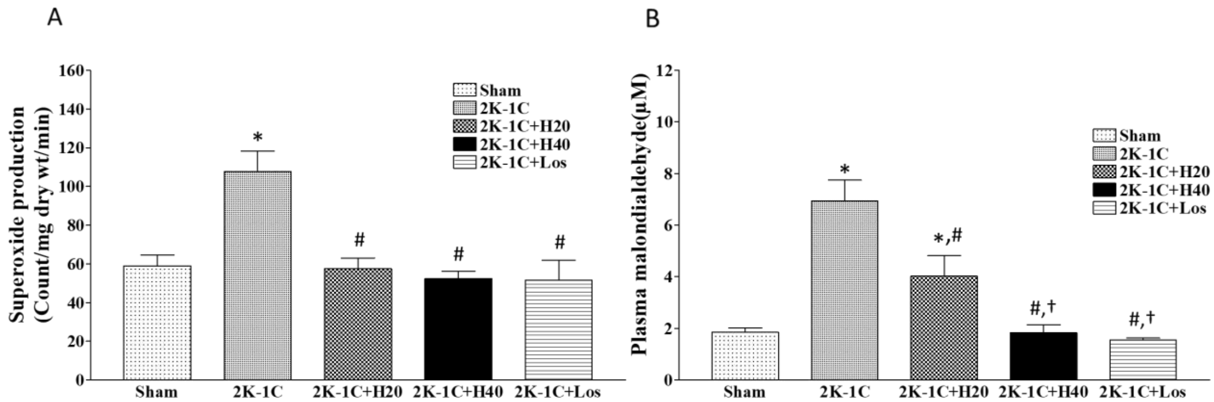

Figure 5. Effects of hesperidin and losartan on vascular $O_2^{\bullet -}$ production (A) and plasma MDA levels (B) in all experimental groups. Data are expressed as mean ± SEM (n=6-8 /group), **P* < 0.05 vs Sham, #*P* < 0.05 vs 2K-1C, †*P* < 0.05 vs H20 (Sham = sham-operated control, Los = losartan 10 mg/kg/BW, H = hesperidin)

Effects of hesperidin and losartan on vascular function in 2K-1C hypertensive rats

Effects of hesperidin and losartan on vasorelaxation responses to vasodilator agents in mesenteric vascular beds and aortic rings and plasma NO metabolites

Vasorelaxation response to ACh (0.1 μM–0.1 mM) in the mesenteric vascular bed was significantly blunted in 2K-1C hypertensive rats compared to sham-operated rats (*p*<0.05). Treatment with hesperidin at dose 40 mg/kg/BW improved the response to ACh in 2K-1C hypertensive rats compared to untreated rats (*p*<0.05). Moreover, 2K-1C hypertensive rats treated with losartan significantly improved the response to ACh compared to 2K-1C hypertensive rats (*p*<0.05; Fig. 6A). There was no significant difference in the vasorelaxation responses to SNP among groups, indicating normal vascular smooth muscle cell function (Data are not show). Endothelium-dependent vasorelaxation responses to ACh (0.01 μM–3 μM) were significantly blunted in aortic rings from 2K-1C hypertensive rats compared to sham-operated rats (*p*<0.05). Hesperidin at dose 40 mg/kg improved vascular response to ACh compared to untreated rats (*p*<0.05). Moreover, 2K-1C hypertensive rats treated with losartan had a significant improvement of the response to ACh compared to 2K-1C hypertensive rats (*p*<0.05) (Fig. 6C). In addition, vasorelaxation response to SNP, an NO donor, did not differ significantly among groups (Data are not show).

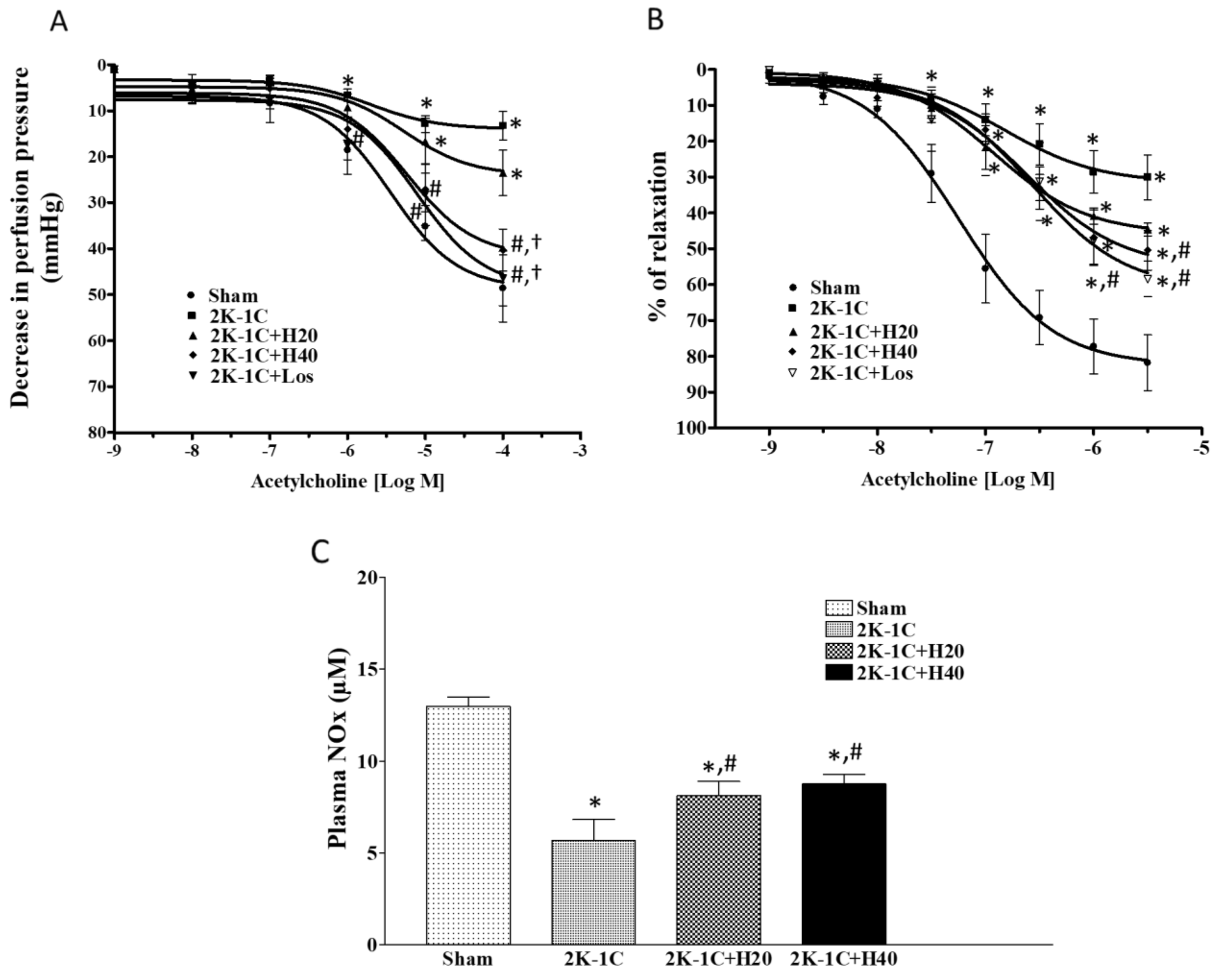

Figure 6. Effects of hesperidin and losartan on vascular responses to exogenous acetylcholine in mesenteric vascular beds **(A)**; exogenous acetylcholine in thoracic aorta **(B)** and plasma NOx **(C)**. Data are expressed as mean ± SEM (n=5-6 /group), **P* < 0.05 vs Sham, $^{\#}P$ < 0.05 vs 2K-1C, $^{\dagger}P$ < 0.05 vs H20 (Sham = sham-operated control, Los = losartan 10 mg/kg/BW, H = hesperidin)

Effects of hesperidin and losartan on contractile responses to EFS and exogenous NE in mesenteric vascular beds and plasma NE concentration

EFS at 5-40 Hz produced an increased in perfusion pressure that was frequency-dependent vasoconstriction in all preparations. A significant increase in contractile responses to EFS was observed in the mesenteric vascular bed isolated from 2K-1C hypertensive rats compared to the responses in sham-operated rats (p<0.05). Contractile response to EFS in 2K-1C hypertensive rats-treated with hesperidin and losartan were reduced comparing to those of hypertensive rats (p<0.05)

(Fig. 7A). However, the contractile response to exogenous NE (0.1 μM–0.1 mM) was not different among groups (Data are not show). An increase in plasma NE concentrations was found in 2K-1C hypertensive rats (p<0.05). The elevation of plasma NE concentrations was decreased in hesperidin treated rats at dose 40 mg/kg/BW compared to those of untreated rats (p<0.05). Moreover, losartan treatment reduced plasma NE concentrations in 2K-1C hypertensive rats compared to untreated rats (p<0.05) (Figure 7B).

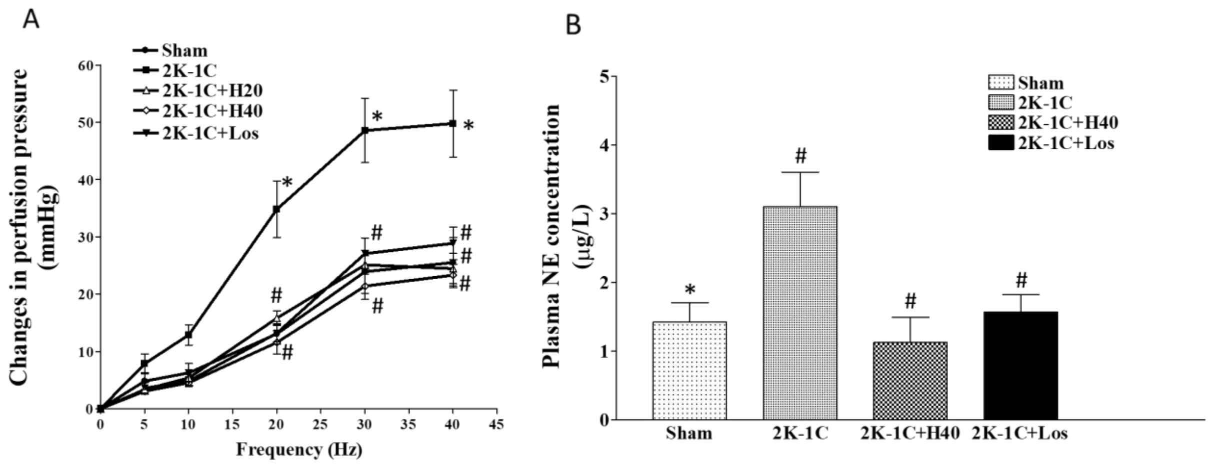

Figure 7. Effects of hesperidin and losartan on contractile responses to sympathetic nerve stimulation **(A)** and plasma NE concentrations **(B)**. Data are expressed as mean ± SEM (n=5-6 /group). *P < 0.05 vs Sham, $^{\#}P$ < 0.05 vs 2K-1C. (Sham = sham-operated control, Los = losartan 10 mg/kg/BW, H = hesperidin)

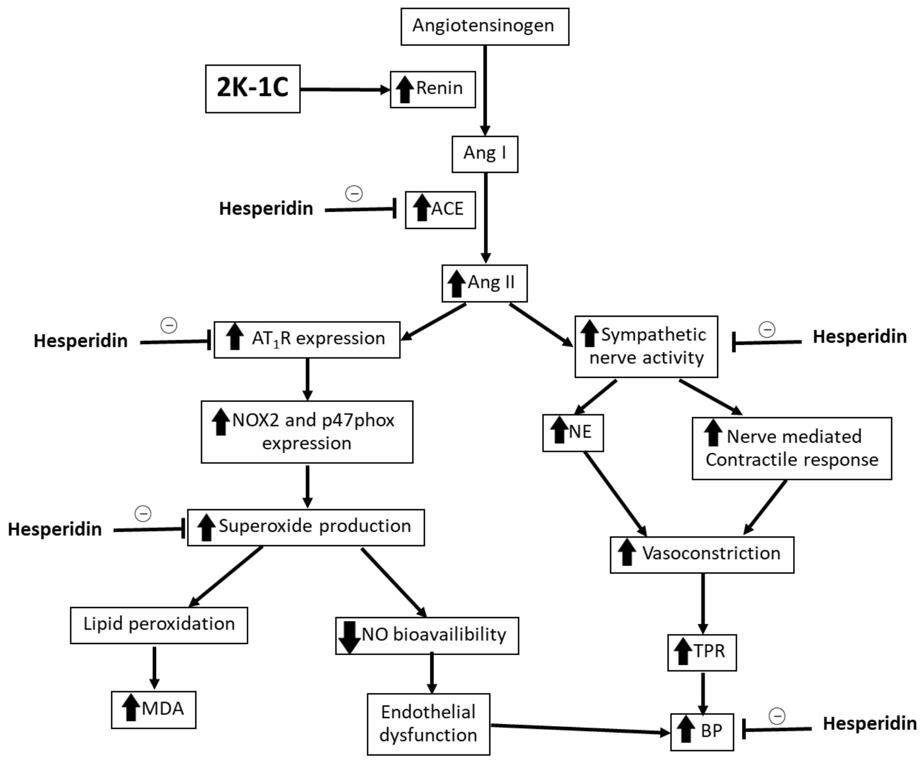

Figure 8. Mechanism of action of hesperidin

Part II

Effects of hesperidin and captopril on blood pressure in conscious rats

There were no significant differences in systolic blood pressure of all rats at the beginning of the study. Administration of L-NAME caused a gradual increase in SP of all rats compared to control rats (SP at 5th week, 200.21±6.52 vs. 122.14±1.75 mmHg, p < 0.01, Figure 9). Co-administration of L-NAME and hesperidin at doses of 15 or 30 mg/kg (2.5 mg/kg) significantly partially prevented L-NAME-induced high blood pressure in a dose dependent manner compared to those of untreated rats (SP at 5th week, 177.50±3.91 and 162.74±2.82 mmHg, p < 0.05). Captopril also partially alleviated L-NAME induced hypertension (152.19±5.01 mmHg) compared with untreated rats (p < 0.05). In addition, captopril produced greater preventive effect on SP than hesperidin (15 and 30 mg/kg).

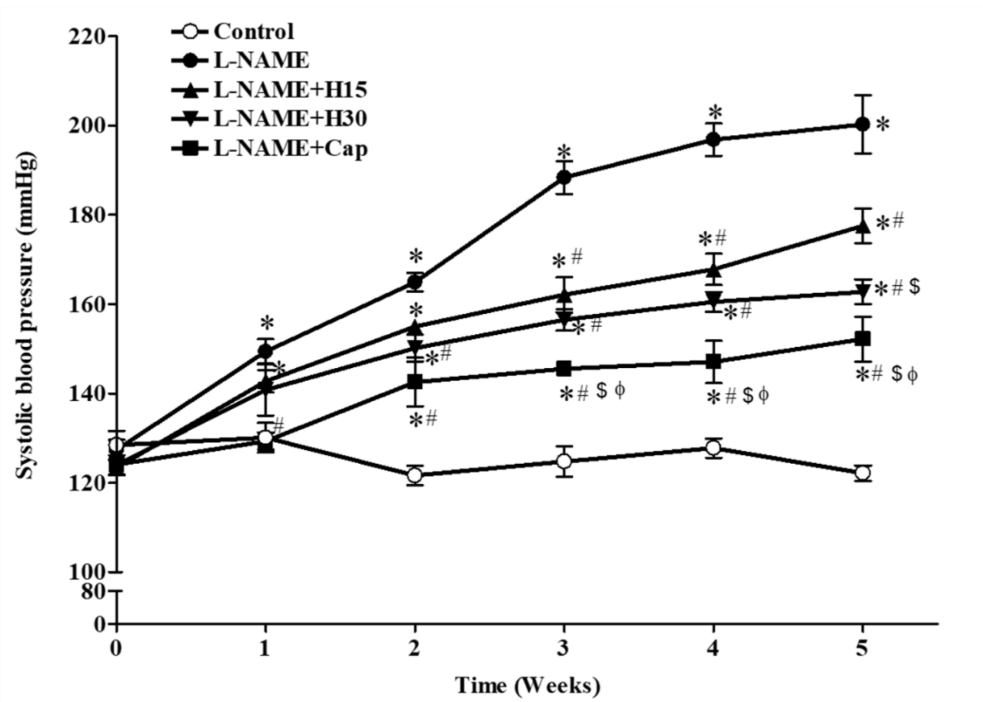

Figure 9. Time-course changes in systolic blood pressures of all experimental groups. Data are expressed as mean ± S.E.M (n = 7-8)/ group, * p < 0.05 vs. control, $^{\#}$ p < 0.05 vs. L-NAME, $^{\$}$ p < 0.05 vs. L-NAME + hesperidin (15 mg/kg), $^{\phi}$ p<0.05 vs. L-NAME + hesperidin (30 mg/kg) group.

Effects of hesperidin and captopril on SP, DP, MAP and HR in anesthetized rats

Blood pressure data received from the indirect blood pressure measurement method were consistent with the values from the direct method since L-NAME treated rats exhibited high blood pressure including high SP, DP, MAP, and high HR compared to those of control rats ($p < 0.05$, Table 1). Hesperidin at doses of 15 and 30 mg/kg significantly decreased SP, DP and MAP in a dose-dependent manner compared to the untreated group ($p < 0.05$). Similarly, captopril reduced the development of hypertension induced by L-NAME compared to untreated rats ($p < 0.05$). Hesperidin at a dose 30 mg/kg, however, also affected the elevation of HR compared to untreated rats ($p < 0.05$, Table 2). Furthermore, hesperidin had no effect on blood pressure in normotensive rats (SP=122.29 ± 4.05 mmHg, n =4).

Table 2. Effects of hesperidin and captopril on blood pressure and heart rate in anesthetized rats.

Parameters	Control	L-NAME	L-NAME+H15	L-NAME+H30	L-NAME+Cap
SP (mmHg)	120.92 ± 2.27	205.88 ± 3.19 *	179.38 ± 16.51 $^{*\#}$	154.07 ± 4.88 $^{*\#\$}$	140.14 ± 7.06 $^{\#\$}$
DP (mmHg)	72.68 ± 3.31	141.65 ± 5.73 *	114.13 ± 16.57 $^{*\#}$	86.89 ± 5.74 $^{*\#\$}$	91.48 ± 7.36 $^{\#\$}$
MAP (mmHg)	88.76 ± 2.47	161.41 ± 4.01 *	135.88 ± 16.00$^{*\#}$	109.28 ± 5.39 $^{*\#\$}$	107.70 ± 6.27 $^{\#\$}$
HR (beat/min)	367.86 ± 11.90	419.30 ± 11.96 *	391.93 ± 14.35	351.44 ± 13.47 $^{\#\$}$	384.28 ± 17.31

SP: systolic blood pressure; DP: diastolic blood pressure; MAP: mean arterial pressure; HR: heart rate. Values are mean ± S.E.M (n = 7-8/group), * $p < 0.05$ vs. control, $^{\#}$ $p < 0.05$ vs. L-NAME, $^{\$}$ $p < 0.05$ vs. L-NAME+H15.

Effects of hesperidin and captopril on left ventricular (LV) morphometry and fibrosis

Rat body weights did not differ among all experimental groups. After 5 weeks of L-NAME administration, HW, LVW and LVW/BW ratios were significantly increased compared to those of control rats. Co-administration of L-NAME and hesperidin or captopril significantly decreased those values when compared with the untreated group (Table 3). Morphometric analysis of hearts showed that chronic administration of L-NAME significantly increased LV wall thickness and LV muscle fiber cross-sectional area (CSA) compared with the normal control group ($p < 0.05$, Table 2). Hypertensive rats that received hesperidin or captopril had significantly reduced wall thicknesses and CSA of the LV compared to untreated rats ($p < 0.05$) (Table 3, Figure 10A). LV fibrosis was significantly increased in L-NAME-treated rats compared to those of the normal control rats ($p < 0.05$). Hesperidin or captopril treatment significantly prevented L-NAME-induced LV fibrosis compared to untreated rats ($p < 0.05$) (Figure 10B).

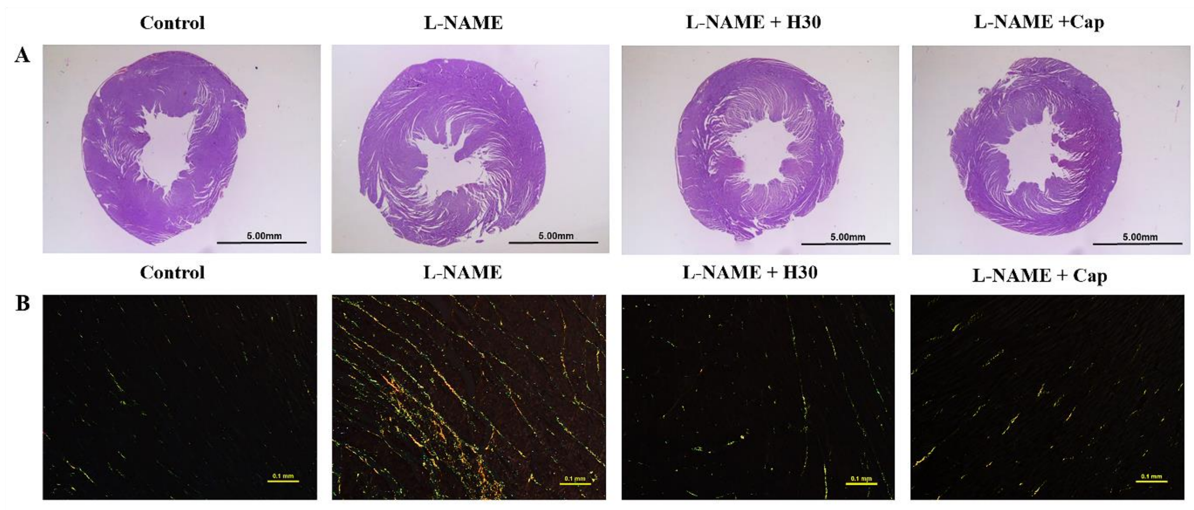

Figure 10. The histology and morphology of LV from control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. Representative images of LV sections, (A) stained with hematoxylin and eosin under stereomicroscopes and (B) stained with picrosirius red under polarized light microscope using a 20x objective lens.

Table 3. Effect of hesperidin and captopril on the cardiac mass indices and cardiovascular structural modifications in left ventricle and thoracic aorta.

Cardiac mass indices			
Groups	Body weight (g)	Heart weight/BW (mg/g)	LVW/BW (mg/g)
Control	434 ± 6.8	3.14 ± 0.17	2.06 ± 0.10
L-NAME	413 ± 16.9	4.21 ± 0.26*	3.04 ± 0.18*
L-NAME+H30	406 ± 9.7	3.11 ± 0.23$^{\#}$	2.23 ± 0.17$^{\#}$
L-NAME+Cap	401 ± 9.7	3.12 ± 0.18$^{\#}$	2.07 ± 0.12$^{\#}$

Left ventricle			
Groups	LV wall thickness (mm)	LV CSA (mm^2)	LV fibrosis (%)
Control	2.72 ± 0.05	57.58 ± 1.05	0.69 ± 0.04
L-NAME	3.28 ± 0.04*	72.42 ± 0.51*	2.72 ± 0.15*
L-NAME+H30	2.90 ± 0.06$^{\#}$	61.12 ± 1.75$^{\#}$	0.92 ± 0.09$^{\#}$
L-NAME+Cap	2.79 ± 0.09$^{\#}$	59.87 ± 1.63$^{\#}$	1.00 ± 0.06$^{\#}$

Thoracic aorta structural modifications				
Groups	Wall thickness (μm)	CSA (x10^3 μm^2)	VSMCs (cells/CSA)	Collagen deposition (% area fraction)
Control	106.39 ± 1.02	579.00 ± 15.16	1298.00 ± 73.64	15.78 ± 0.70
L-NAME	150.58 ± 2.09*	810.50 ± 18.64*	2013.71 ± 51.62*	31.32 ± 1.00*
L-NAME+H30	127.11 ± 2.90$^{*,\#}$	617.95 ± 18.65$^{\#}$	1540.16 ± 46.88$^{*,\#}$	24.84 ± 0.69$^{*,\#}$
L-NAME+Cap	129.91 ± 6.50$^{*,\#}$	658.38 ± 40.22$^{\#}$	1671.78 ± 24.90$^{*,\#}$	23.68 ± 0.63$^{*,\#}$

LV: left ventricular, LVW: left ventricular weight, BW: body weight, CSA: cross sectional area, VSMCs: vascular smooth muscle cells. Values are expressed as mean ± S.E.M, (n = 6/group). * $p < 0.05$ when compared to control group, and $^{\#}$ $p < 0.05$ when compared to L-NAME group.

Effect of hesperidin and captopril on vascular morphology

Vascular wall hypertrophy was observed in thoracic aortas collected from L-NAME hypertensive rats (Figure 11A) with significant increases in vascular wall thickness, CSA and smooth muscle cells numbers compared to those of control rats ($p < 0.05$; Table 3, Figure 11A). Moreover, the relative amounts of collagen depositions (Figure 11B) in the aortic walls of L-NAME hypertensive rats were also clearly observed ($p < 0.05$; Table 3, Figure 11B). Hesperidin or captopril treatment partially prevented the vascular structural abnormalities in aortas induced by L-NAME ($p < 0.05$).

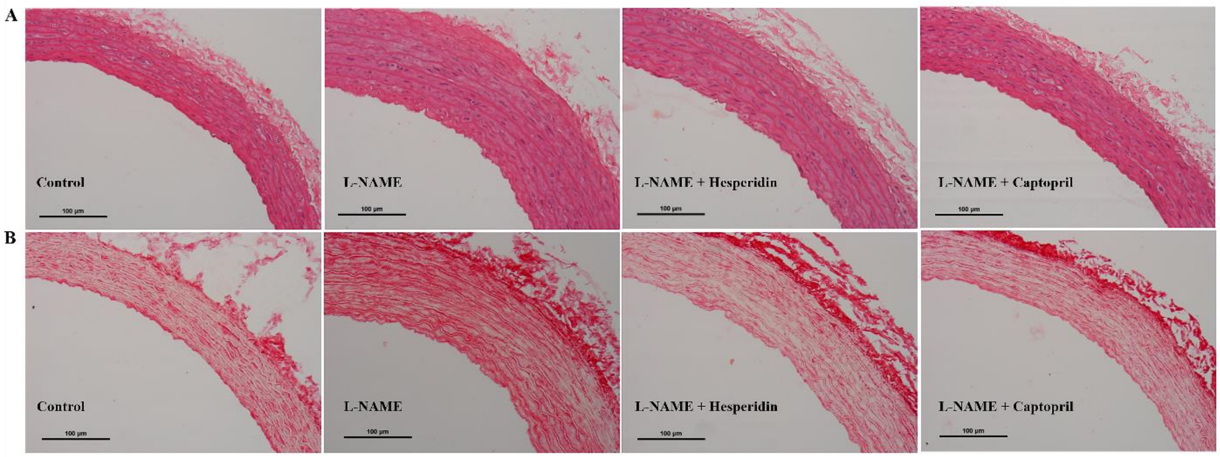

Figure 11. The histology and morphology of thoracic aorta from control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. Representative images of aortic sections, (A) stained with hematoxylin and eosin and (B) stained with picrosirius red under light microscope using a 20x objective lens.

Effects of hesperidin and captopril supplementation on oxidative stress markers, plasma nitric oxide metabolites (NOx) levels in L-NAME treated rats

L-NAME treated rats showed a significant increase in production of vascular $O_2^{\bullet -}$ (263.26 ± 11.20 vs. 71.42 ± 15.97 count/mg dry wt/min, $p < 0.001$) and plasma MDA levels compared to control groups (10.24 ± 0.4 vs. 3.11 ± 0.27 μM, $p < 0.05$). When treated with hesperidin or captopril the elevations of vascular $O_2^{\bullet -}$ and plasma MDA were mitigated compared to those of untreated rats (7.91 ± 0.92, 4.83 ± 0.74 and 3.88 ± 0.25 count/mg dry wt/min and 138.86 ± 28.75, 97.28 ±16.67 and 92.14 ± 12.90 μM, $p < 0.05$) (Figure 12A and B). In addition, low levels of plasma NOx were found in L-NAME hypertensive rats compared with control rats (3.49 ± 1.0 vs. 10.17 ± 0.95 μM, $p < 0.05$). These low levels of plasma NOx were improved by hesperidin or captopril supplementation (4.38 ± 1.15, 7.48 ± 1.03 and 8.48 ± 1.21 μM , $p < 0.05$) (Figure 12C).

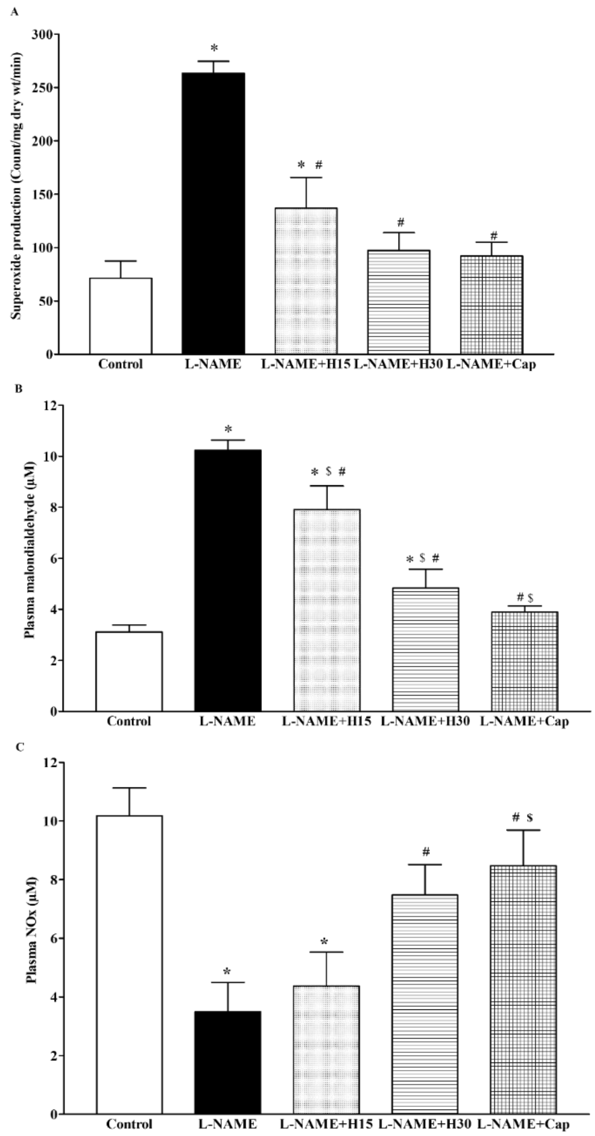

Figure 12. Effects of hesperidin and captopril supplementation on vascular $O_2^{\bullet -}$ production (A), plasma MDA (B) and plasma NOx (C) levels in control, L-NAME, L-NAME + hesperidin (15mg/kg), L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (5 mg/kg) groups. Data are expressed as mean ± S.E.M (n = 7-8)/group, * $p < 0.05$ vs. control, $^{\#}$ $p < 0.05$ vs. L-NAME group, $^{\$}$ $p < 0.05$ vs. L-NAME+H15.

Effects of hesperidin and captopril on protein expression of TNF-R1 and TGF- β1 in heart tissues and concentrations of TNF-α and TGF- β1 in plasma

Overexpressions of TNF-R1 and TGF- β1 proteins were found in heart tissues collected from the hypertensive group compared to the control group ($p < 0.001$). Interestingly, supplementation with hesperidin and captopril partially reversed these protein up-regulations ($p < 0.01$; Figure 13A and B). These results were consistent with the results in that high levels of plasma TNF-α and TGF-β1 were observed in L-NAME hypertensive rats compared to those of control rats (168.49 ± 13.05 vs. 24.21 ± 8.51 pg/mL and 23.54 ± 3.91 vs. 4.90 ± 0.50 ng/mL, $p < 0.01$). Administration of hesperidin or captopril attenuated these high levels of plasma TNF-α (58.23 ± 14.71 or 20.97 ± 6.97 pg/mL) and TGF- β1 (5.23 ± 0.32 or 4.79 ± 0.55 ng/mL, $p < 0.05$) in hypertensive rats (Figure 13C and 13D).

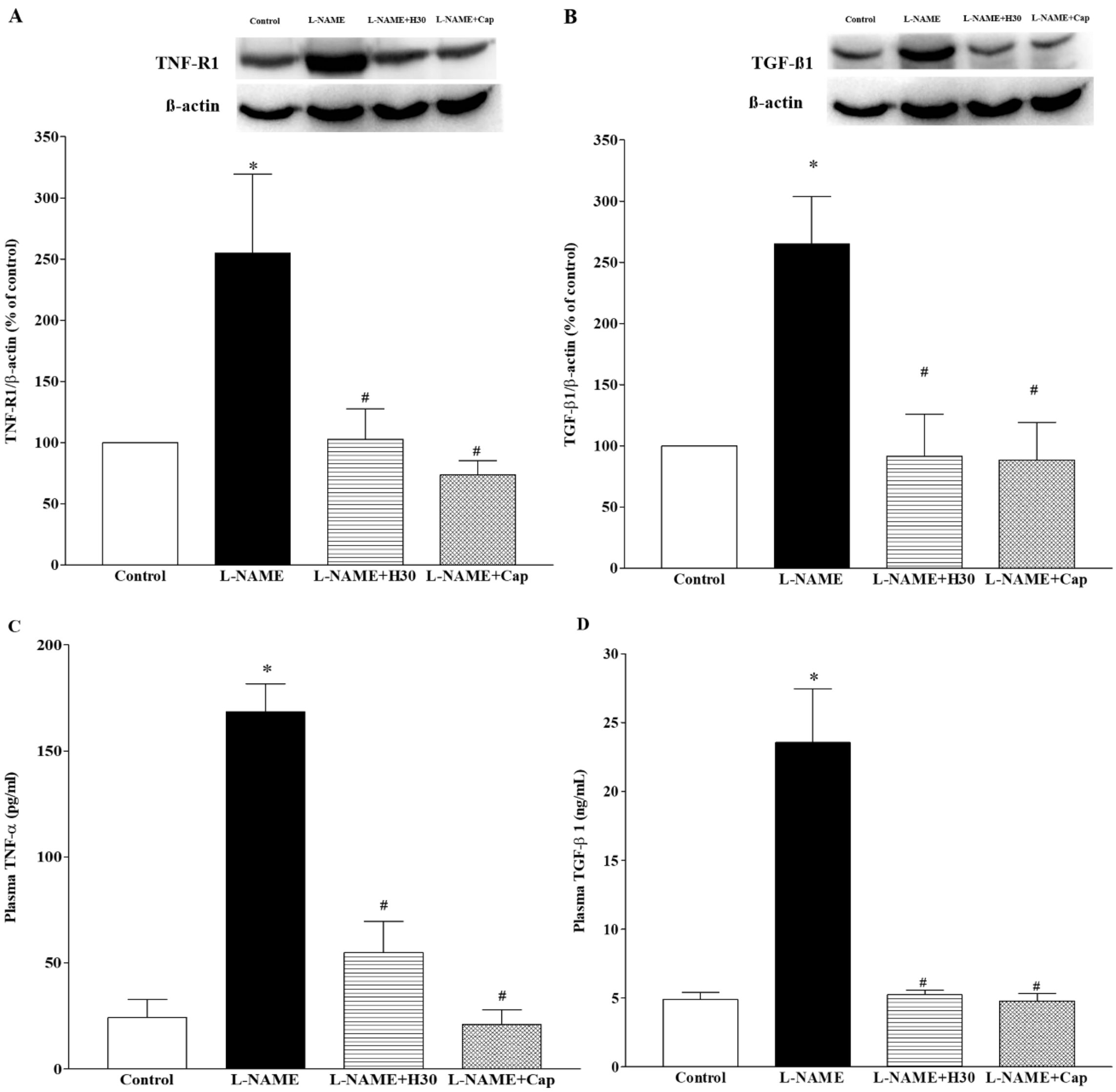

Figure 13. Effects of hesperidin and captopril on protein expression of TNF-R1 (A) and TGF- β1 (B) in heart tissue and on concentrations of plasma TNF-α (C) and TGF- β1 (D) collected from control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. The

top panel shows representative bands of TNF-R1 (A) and TGF- β1 (B) protein expression in heart tissues. Values are mean ± S.E.M (n=4 for each group), * $p < 0.05$ vs. control, # $p < 0.05$ vs. L-NAME group.

Effects of hesperidin and captopril on protein expression of MMP-2 and MMP-9 in aortic tissue

A significant increase in of MMP-2 and MMP-9 protein expression was observed in thoracic aortic tissues collected from the hypertensive group compared with the control group (Figure 14A and 14B, $p < 0.05$). Hesperidin or captopril treatment significantly suppressed the level of MMP-2 and MMP-9 protein expression compared with untreated rats, ($p < 0.05$).

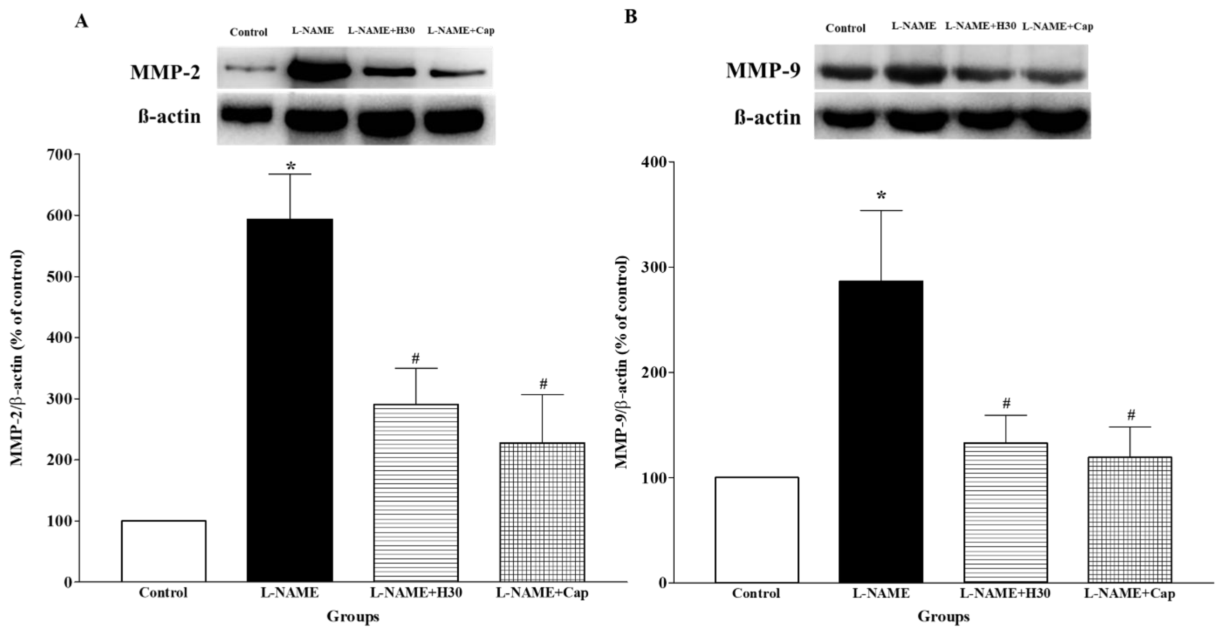

Figure 14. Effects of hesperidin and captopril on protein expression of MMP-2 (A) and MMP-9 (B) in aortic tissue collected from control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. The top panels, shows representative bands of MMP-2 (A) and MMP-9 (B) protein expression in thoracic aortas. Values are mean ± S.E.M (n=4 for each group), * $p < 0.05$ vs. control, # $p < 0.05$ vs. L-NAME group.

บทวิจารณ์

Part I

The present findings show that hesperidin reduced blood pressure in 2K-1C hypertensive rats. Hesperidin also affected RAS activation by decreasing ACE activity, plasma Ang II levels and suppressed AT_1 receptor expression in 2K-1C hypertensive rats. Increases in $O_2^{\bullet-}$ production and plasma MDA as well as $p47^{phox}$ and $gp91^{phox}$ protein overexpression were alleviated in the 2K-1C group treated with hesperidin or losartan. Hesperidin and losartan improved endothelial dysfunction and nitric oxide (NO) bioavailability in 2K-1C hypertensive rats. Sympathetic nerve activation, as evident by the enhancement of nerve-mediated contractile responses and high levels of plasma NA were shown in 2K-1C hypertensive rats. This sympathoexcitation was not found in 2K-1C rats treated with hesperidin or losartan.

High blood pressures associated with increases in plasma ACE activity and plasma Ang II were shown in 2K-1C rats in the present study. The elevation of plasma Ang II observed in this study was the consequence of RAS activation. This was supported by the findings that a partial occlusion of one renal artery can develop hyperactivation of RAS in animals (8, 27). Sawamura and Nakada demonstrated high renal levels of ANG I, II and ACE activity in the clipped-kidney rats (3). Ang II, an octopeptide product of RAS, induces high blood pressure via activation of AT_1 receptors to produce vasoconstriction, Na^+ and water reabsorption, and sympathetic nervous stimulation (28). The up regualtion of AT_1 receptors that might enhance Ang II-mediated hypertensive effects was found in this animal model. A relevant study indicated that prolonged exposure to Ang II could induce the upregulation of brain AT_1 receptors (29). This study, in renovascular hypertensive rats, it revealed the antihypertensive effect of hesperidin, which involved the suppression of the RAS pathway. This is the first evidence to report the ACE inhibiting activity of hesperidin in an animal model of hypertension. The attenuation of plasma ACE activity seems then to be related to reductions of plasma Ang II level, AT_1 receptor expression and then blood pressure. The ACE inhibitor activity of hesperidin might then be mediated by its flavonoid structure since there is evidence supporting flavonoids inhibiting ACE activity and then lowering blood pressure (30). Furthermore, a molecular docking study discovered that flavonoids were able to bind with zinc ion in the active site of ACE enzymes (31).

The upregulation of NOX subunits ($p47^{phox}$ and $gp91^{phox}$ protein expression) in aortic tissues of 2K-1C hypertensive rats as a sequential activation of circulating Ang II was observed in this study.

This was associated with increases in oxidative stress markers including plasma MDA levels and vascular $O_2^{\bullet-}$ production. These current findings were supported by other studies performed in the 2K-1C hypertension model. For example, overexpression of p47^{phox} protein was demonstrated to be responsible for oxidative stress in RAS- dependent hypertension in rats (12). Wei and coworkers, indicated that Ang II can increase reactive oxygen species production through activating NADPH oxidases (32). It is now accepted that high $O_2^{\bullet-}$ production promotes the development of hypertension since $O_2^{\bullet-}$ can rapidly react with NO to form the potent cytotoxic peroxynitrite. It has been clearly shown that peroxynitrite causes oxidative damage to endothelial cells and decreases NO bioavailability (33). Jung and coworkers indicated that NO bioavailability was reduced because of activation of the NOXs and $O_2^{\bullet-}$ production in the 2K-1C model (34). The impairment of endothelium-dependent vasodilation is supported by reducing the vascular response to ACh but not to SNP and was clearly shown in the present study. This was consistent with a reduction of NO metabolites observed in 2K-1C hypertensive rats. Another possible antihypertensive mechanism of hesperidin proposed in this study involved its antioxidant effect. Hesperidin reduced p47^{phox} and gp91^{phox} expression, $O_2^{\bullet-}$ production and plasma MDA levels in 2K-1C hypertensive rats which was associated with the improvement of vascular function and plasma NOx level. There are substantial data to indicate the antioxidant property of hesperidin (35). Yamamoto and coworkers firstly reported the antihypertensive effect of hesperidin in spontaneously hypertensive rats associated with improved vascular function and a reduction of oxidative stress (23). In RAS-dependent hypertension in rats, this study indicated that the antihypertensive effect of hesperidin might involve the antioxidant property or a sequential suppression of RAS cascade or both.

Sympathetic activation is an important factor to stimulate and maintain high blood pressure in renovascular hypertensive rats (36). Sympathoexcitation was confirmed in renovascular hypertension as indicated by a significant rise in low-frequency SP during the mid-developmental and maintenance phases of the hypertension in 2K-1C hypertensive rats (37). A recent study by Nishi and coworkers showed sympathetic vasomotor hyperactivity and baroreflex dysfunction which contributed to the development and maintenance of renovascular arterial hypertension (8). The present results demonstrated that there was an enhancement of contractile responses to sympathetic nerve stimulation without affecting the response to exogenous NA in 2K-1C rats, suggesting the augmentation of NA release from pre-junctional sites. This result could be supported with high plasma NA in 2K-1C rats. In addition, there is evidence to support findings that an increase in the vasoconstrictive response to peri-arterial nerve stimulation was uncovered in isolated mesenteric vascular beds from 2K-1C rats (18). The authors suggested that this enhancement was partially mediated by Ang II-induced facilitation of NA release (18). In fact, Ang II increases sympathetic nerve activity via activation of the AT_1 receptor in the rostral RVLM, a pressor area of the medulla, and activates sympathetic outflow to enhance NA release from sympathetic nerve terminals (6). Furthermore, treatment with hesperidin suppressed the nerve-mediated contractile response in 2K-1C hypertensive rats, which was relevant to a decreased plasma NA level. An intriguing possibility is that hesperidin inhibited the RAS cascade and subsequently reduced oxidative stress and sympathoexcitetation.

In the present study, losartan was used as a positive control and was able to decrease blood pressure, RAS activation, oxidative stress, and improve vascular function in the 2K-1C hypertensive rats. The antihypertensive actions of losartan as an AT_1 receptor antagonist in 2K-1C hypertensive rats were strongly documented (38). Losartan also caused a significant reduction of brain AT_1 receptors with attenuation of the brain and plasma oxidative stress in renovascular hypertensive rats (39).

In conclusion, the results of this present study indicate that hesperidin reduced blood pressure via suppressing RAS cascade mediated-NOX overexpression and sympatoexcitation or it directly reduced oxidative stress (Figure 8). It could therefore be suggested that hesperidin might potentially be further developed as a complimentary agent in the treatment of hypertension.

Part II

This study demonstrates that rats that received L-NAME developed hypertension and cardiovascular remodeling. Hesperidin mitigated the high blood pressure and cardiac remodeling by reducing left ventricular hypertrophy and fibrosis associated with down-regulations of TGF-β1 and TNF-R1 protein expression and a reduction of plasma levels of TGF-β1 in L-NAME induced hypertension in rats. Vascular remodeling, including vascular hypertrophy and increased collagen deposition induced by L-NAME in rats was inhibited by hesperidin supplementation. This was consistent with the decreased protein expression of MMP-2 and MMP-9 in aortic tissue. Furthermore, hesperidin that prevented cardiovascular remodeling-induced by L-NAME in the present study was linked to the reduction of an inflammatory cytokine, oxidative stress markers and enhanced NO availability.

It was found that chronic treatment of L-NAME produced the development of NO-deficient hypertension as well as cardiovascular remodeling. These remodelings included increases in LVW/HW ratio, LV wall thickness, LV CSA, LV fibrosis, aortic wall thickness, aortic cross-sectional areas, aortic smooth muscle cell numbers and collagen deposition. It has been well accepted that chronic inhibition of NO synthase using L-NAME results in NO depletion, increased vascular tone and high blood pressure [34]. Several studies demonstrated that cardiovascular remodeling occurred after chronic treatment with L-NAME (40 mg/kg) for 5 weeks [4, 10, 35]. The mechanisms involved in cardiac remodeling in an animal model of nitric oxide deficient hypertension are still unclear, however, two possible mechanisms related to hemodynamics and non-hemodynamic aspects have been described [36]. Hemodynamic overload in hypertension provoked left ventricular hypertrophy because of the adaptive response to conserve cardiac output [37]. A reduction of NO is one of several non-hemodynamic factors that participate in cardiac remodeling because when NO is suppressed, hypertensive cardiac remodeling through the cyclic guanosine monophosphate/ protein kinase G (cGMP/PKG) pathway is initiated to inhibit fibrotic synthesis [38]. It is well documented that vascular remodeling in hypertension occurs in response to long-term modifications of hemodynamic conditions [39, 40]. Furthermore, numerous studies reported that vascular remodeling is characterized by increases in wall thickness, CSA and smooth muscle cell numbers in L-NAME hypertensive rats [3, 4, 41]. In this present study, hesperidin partially inhibited the development of hypertension as well as cardiovascular remodeling induced by chronic L-NAME treatment. These effects may have involved an increase in NO bioavailability, reductions of oxidative stress and inflammation as further possibilities.

Oxidative stress is one of the important mechanisms of L-NAME induced hypertension since L-arginine analogues activate eNOS uncoupling leading to an overwhelming vascular superoxide generation [42] by the fact that superoxide can rapidly react with nitric oxide to form peroxynitrite [43]. This reaction results in reducing nitric oxide bioavailability [44]. In the present study, increases in plasma MDA levels and vascular superoxide production were accompanied by decreased plasma NOx levels that were observed in the L-NAME hypertensive rats. Hesperidin alleviated L-NAME induced oxidative stress and thus increased NO bioavailability in an increase in plasma NOx level. A large number of studies have confirmed that hesperidin has strong antioxidant activity [26, 45]. Hesperidin exhibits its antioxidant properties with two main mechanisms including, directly scavenging reactive oxygen species [46] and boosting cellular antioxidant defense [25]. Thus, this is one of the possible mechanisms of the cardiovascular protective effects of hesperidin in this study that might have involved its antioxidant capability that resulted in increased NO bioavailability, which reduced vascular resistance.

There is substantial evidence to support that inflammation is one of pathologies that occur in L-NAME hypertensive rats [47, 48]. Results of the current study proved that as in the previous studies, there were increases in the levels of the proinflammatory cytokine, TNF-α, in plasma and expression of TNF-α protein in heart tissue of L-NAME hypertensive rats. Myocardial TGF-β protein expression was also observed in L-NAME hypertensive rats. It is well established that TGF-β plays and an important role in responses to inflammation to activate fibrogenesis, which is the important pathological process for cardiac remodeling [49, 50]. The present study has also shown that hesperidin attenuated cardiac remodeling accompanied by decreased systemic and heart inflammation in L-NAME hypertensive rats. The protein expression of TGF-β in cardiac tissue was also down-regulated in the hesperidin supplemented group. The anti-inflammatory effect of hesperidin has been clearly revealed in both cellular and animal models. In human umbilical vein endothelial cells, hesperidin significantly suppressed TNF-α [51]. Li and coworkers demonstrated that hesperidin decreased the production of IL-1β, IL-6, and TNF-α in a rat model of rheumatoid arthritis [52]. Thus, the current results confirmed that the cardiprotective effect of hesperidin was associated with its great anti-inflammatory effect.

Additionally, vascular remodeling with collagen deposition was associated with overexpression of MMP-2 and MMP-9 in aortic tissue in L-NAME hypertensive rats as shown in this study. Several studies described that MMPs play an important role in physiological processes that contribute to hypertension-induced maladaptive arterial changes and sustained hypertension [53, 54]. Overexpression of MMP mediated vascular remodeling was stimulated by oxidative stress and inflammatory cytokines [54]. Del Mauro and coworkers demonstrated that MMP-2 and MMP-9 activity was a pathologic process in L-NAME induced morphometric alterations in the aorta [55]. Interestingly, the authors of the present study first reported L-NAME induced hypertension and vascular remodeling in rats in which there was up-regulation of MMP-2 and MMP-9 protein expression in response to oxidative stress. Hesperidin prevented vascular remodeling induced by L-NAME associated with down-regulation of MMP-2 and MMP-9. This effect might be involved in its antioxidant and anti-inflammatory effects, which further inhibited MMPs activation and collagen degradation.

Captopril was used as a positive control to prevent the development of hypertension and cardiovascular remodeling. It was found that captopril prevented L-NAME-induced the development of hypertension and cardiovascular remodeling. These findings are supported by the previous studies that captopril prevented high blood pressure, left ventricular hypertrophy and vascular remodeling induced by L-NAME in rats [56, 57]. Captopril also reduced oxidative stress and inflammatory markers and suppressed protein expressions of TNF-R1, TGF-β1 and MMPs. An antioxidative effect of captopril in the present study might associate with two main mechanisms, direct and indirect effects. Captopril contains free sulfhydryl groups that directly scavenging oxygen free radicals [58] or it suppresses AT1R-mediated NADPH oxidase expression and superoxide production [10]. It has been demonstrated that captopril improved ventricular hypertrophy in rats by suppressing MMP-2 and MMP-9 expression [59]. In addition, anti-inflammatory effect of captopril in animal model of hypertension has been reported [60]. Results showed the greater effect of captopril on SP comparing hesperidin that may involve the potent ACE inhibitor capacity of captopril or the different titration doses between two these agents.

In conclusion, the findings of this study indicated that hesperidin had cardiovascular protective effects by preventing L-NAME-induced development of hypertension and cardiovascular remodeling in rats. These effects were affirmed by reducing oxidative stress and inflammation.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) พร้อมแจ้งสถานะของการตีพิมพ์ เช่น submitted, accepted, in press, published

มีผลงานตีพิมพ์ในวารสารวิชาการนานาชาติจำนวน 4 เรื่อง

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2. Wunpathe C, Potue P, Maneesai P, Bunbupha S, Prachaney P, Kukongviriyapan U, et al. Hesperidin Suppresses Renin-Angiotensin System Mediated NOX2 Over-Expression and Sympathoexcitation in 2K-1C Hypertensive Rats. The American journal of Chinese medicine. 2018:1-17. Impact factor =3.51 Q=1. published
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2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
- เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
- เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

  สร้างนักวิจัยใหม่จำนวน 2 คนคือ

  1. ดร.จุฑามาศ วันเพ็ชร
  2. นางสาวประภัสสร โพธิ์ตื

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

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Hesperidin Suppresses Renin-Angiotensin System Mediated NOX2 Over-Expression and Sympathoexcitation in 2K-1C Hypertensive Rats

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Abstract: Hesperidin, a flavonoid derived from citrus fruits, possesses several beneficial effects including anti-oxidation and anti-inflammation. The aim of this study was to investigate the effects of hesperidin on the renin-angiotensin system (RAS) cascade that mediated oxidative stress and sympathoexcitation in two-kidney, one-clipped (2K-1C) hypertensive rats. 2K-1C hypertension was induced in male Sprague-Dawley rats. Hypertensive rats were treated with hesperidin at 20 mg/kg or 40 mg/kg or losartan at 10 mg/kg beginning at three weeks after surgery and then continued for four weeks ($n = 8-9$/group). Hesperidin reduced blood pressure in a dose-dependent manner in hypertensive rats compared to untreated rats ($p < 0.05$). Increased plasma angiotensin converting enzyme (ACE) activity and angiotensin II levels, as well as, upregulated AT_1 receptor protein expression in aortic tissues were attenuated in hypertensive rats treated with hesperidin. Hesperidin suppressed oxidative stress markers and NADPH oxidase over-expression, and restored plasma nitric oxide metabolites in 2K-1C rats. This was associated with improvement of the vascular response to acetylcholine in isolated mesenteric vascular beds and aortic rings from 2K-1C rats treated with hesperidin ($p < 0.05$). Enhancement of nerve-mediated vasoconstriction related to high

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plasma noradrenaline in the 2K-1C group was alleviated by hesperidin treatment ($p < 0.05$). Furthermore, losartan exhibited antihypertensive effects by suppressing the RAS cascade and oxidative stress and improved vascular dysfunction observed in 2K-1C rats ($p < 0.05$). Based on these results, it can be presumed that hesperidin is an antihypertensive agent. Its antihypertensive action might be associated with reducing RAS cascade-induced NOX2 over-expression and sympathoexcitation in 2K-1C hypertensive rats.

Keywords: Hesperidin; 2K-1C Hypertensive Rats; Renin-Angiotensin System; Sympathoexcitation.

Introduction

Hesperidin is a bioflavonoid glycoside mostly found in oranges, lemons and other citrus fruits (Garg *et al.*, 2001). Several biological activities of hesperidin have been revealed including, anti-oxidation (Homayouni *et al.*, 2017), anti-inflammation and analgesic activity (Galati *et al.*, 1994). Hesperidin can inhibit lipase activity from the porcine pancreas (Kawaguchi *et al.*, 1997). The beneficial effect of hesperidin on microcirculation has been shown via the combination of hesperidin and diosmin in Raynaud's syndrome treatment through which capillary circulation can be improved and edema of the fingers of the hands is reduced (Zudin *et al.*, 2017). Presently, hesperidin is used as an effective supplementary treatment to relieve hemorrhoids, varicose veins, and poor circulation as in venous stasis (Cesarone *et al.*, 2006; Giannini *et al.*, 2015). Additionally, a previous study reported that hesperidin had an antihypertensive effect associated with reducing endothelial dysfunction and oxidative stress in spontaneously hypertensive rats (Yamamoto *et al.*, 2008).

It is well established that the activation of the renin-angiotensin system (RAS) mainly mediates the development of hypertension in an animal model of two-kidney, one-clip (2K-1C) rats (Ponchon and Elghozi, 1996). This was supported by several studies in that an elevation of renin (Wilcox *et al.*, 1996), and angiotensin converting enzyme (ACE) activities, angiotensin II (Ang II) levels (Sawamura and Nakada, 1996), as well as upregulation of angiotensin II type 1 receptor (AT_1 receptor) protein expression in the 2K-1C model were observed (Santuzzi *et al.*, 2015). Although Ang II, the main effector peptide of RAS, primarily causes hypertension via binding to AT_1 receptors to develop high vascular resistance and salt water retention, other important mechanisms such as Ang II-mediated oxidative stress (Bendall *et al.*, 2007) and sympathoexcitation have recently been proposed (Oliveira-Sales *et al.*, 2009).

There is increasing evidence to confirm that oxidative stress is implicated in pathophysiology of 2K-1C Goldbatt hypertension (Nishi *et al.*, 2010, 2013). The mRNA levels of AT_1 receptor, NADPH oxidase (NOX) subunits $p47^{phox}$ and $gp91^{phox}$ (NOX2) in renal cortex and aortic tissue of 2K-1C rats were elevated (Nishi *et al.*, 2010; Santuzzi *et al.*, 2015). NOXs are primary sources of reactive oxygen species (Bedard and Krause, 2007). NOX2 is mainly identified in endothelial cells and possibly in vascular smooth muscle cells, and thus, overexpression of NOX2 in endothelial cells has been reported to promote

Ang II-induced vascular oxidative stress and endothelial dysfunction in mice (Bendall *et al.*, 2007; Murdoch *et al.*, 2011). Overexpression of gp91^{phox} in aortas (Santuzzi *et al.*, 2015) and systemic oxidative stress (Boonla *et al.*, 2014) were revealed in 2K-1C hypertension. Subsequently, upregulation of p47^{phox} protein associated with an increase in vascular superoxide ($O_2^{\bullet -}$) production and endothelial dysfunction were displayed in 2K-1C rats (Boonla *et al.*, 2014).

There is substantial evidence showing that Ang II potentiates sympathetic nerve stimulation in rat mesenteric arteries via increased noradrenaline (NA) release from presynaptic sites (Campbell and Jackson, 1979; Eikenburg *et al.*, 1981). Activation of sympathetic vasomotor tone in RAS-dependent hypertension has been clearly demonstrated (Oliveira-Sales *et al.*, 2008). This sympathoexcitation during renovascular hypertension is involved in the upregulation of AT_1 receptor protein in the rostral ventrolateral medulla (RVLM) of 2K-1C hypertensive animals (de Oliveira-Sales *et al.*, 2010). Koyama and coworkers reported the enhancement of sympathetic neurotransmission in mesenteric vascular beds of renovascular hypertensive rats (Koyama *et al.*, 2010). Moreover, Zimmerman and coworkers reported that chronic renovascular hypertension was associated with an elevation in mesenteric vascular responses to sympathetic nerve stimulation (Zimmerman *et al.*, 1987).

Losartan, a selective nonpeptide AT_1 receptor antagonist, was used as a positive control in this study. It has been well established to target RAS to manage hypertension. Several studies reported other potential effects that are linked to its antihypertensive effects including, anti-oxidant, anti-inflammatory, and antiproliferative effects (Wilcox *et al.*, 1996; An *et al.*, 2010; Du *et al.*, 2012; Nishi *et al.*, 2013). Of special importance is that losartan can inhibit sympathetic nerve activity and AT_1 receptor expression in 2K-1C hypertensive rats (Nishi *et al.*, 2013).

The aim of the present study was to investigate whether hesperidin could reduce blood pressure, inhibit the RAS cascade, and suppress sympathetic nerve activity and NOXs protein expression in renovascular hypertensive rats.

Materials and Methods

Animals and Experimental Protocols

Male Sprague-Dawley rats weighing 150–180 g were obtained from Nomura Siam International Co., Ltd., Bangkok, Thailand. They were housed at 25 ± 2°C with a 12 h dark–light cycle at the Northeast Laboratory Animal Center, Khon Kaen University, Khon Kaen, Thailand. All procedures complied with the standards for the care and use of experimental animals and were approved by the Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (AEKKU-NELAC 37/2559). After a week of acclimatization, rats were anesthetized with pentobarbital sodium (60 mg/kg, ip.) and then a silver clip (0.2 mm i.d.) was placed on to the left renal artery. The sham-operated group had the same surgical procedure but the clips were not applied on the left renal arteries. Three weeks after the surgery, the 2K-1C rats were divided into five groups of 8–9 rats each.

Group I Sham + vehicle or polyethylene glycol (PG) (0.15 ml/100 mg; p.o.)
Group II 2K-1C + vehicle or PG (0.15 ml/100 mg; p.o.)
Group III 2K-1C + hesperidin (20 mg/kg; p.o.)
Group IV 2K-1C + hesperidin (40 mg/kg; p.o.)
Group V 2K-1C + losartan (10 mg/kg; p.o.)

Hesperidin, losartan, and PG were intragastrically administered daily for four weeks of the study.

Indirect Measurement of Blood Pressures and Heart Rates in Conscious Rats

Indirect blood pressures were measured once a week for eight weeks. Systolic blood pressures (SP) and heart rates (HRs) were measured in conscious rats by the tail-cuff plethysmography (IITC model 179 blood pressure analyzer) method. In brief, conscious rats were placed in a restrainer and allowed to calm prior to blood pressure measurement. The tail of each rat was placed inside the tail cuff, and the cuff was automatically inflated and released. For each rat, blood pressures and HRs were recorded as the mean values from the three measurements at 15 min intervals.

Direct Measurement of Blood Pressures and HRs in Unconscious Rats

Direct blood pressure and HR were determined at the end of study. Briefly, rats were anesthetized by intra-peritoneal administration of pentobarbital-sodium (60 mg/kg). A polyethylene tube was inserted into a femoral artery for blood pressure measurement. SP, diastolic blood pressures (DPs), mean arterial pressures (MAPs) and HRs were continuously monitored by way of pressure transducers and recorded using the Acknowledge Data Acquisition and Analysis Software (BIOPAC Systems Inc., California, USA).

Measurement of Oxidative Stress Markers

Blood was collected from abdominal aortas, mixed with EDTA and placed on ice for plasma malondialdehyde (MDA) measurements. The concentration of plasma MDA was measured as thiobarbituric acid-reactivity (TBA) reactive substances by a spectrophotometric method as previously described (Boonla *et al.*, 2014). Productions of $O_2^{\bullet -}$ in vascular tissues were determined by lucigenin-enhanced chemiluminescence. The carotid artery was rapidly removed and placed in ice-cold saline, and connective tissues and adherent fat was cleaned off. The vessel was cut into 1 cm lengths and incubated with 1 ml oxygenated Krebs-KCl buffer and allowed to equilibrate at pH 7.4, 37°C for 30 min. Thereafter, lucigenin was added to the sample tube and placed in a luminometer (Turner Biosystems, Sunnyvale, CA, USA). Luminometer counts were recorded every 30 s for 5 min and averaged. Vascular tissue $O_2^{\bullet -}$ production was expressed as relative light unit counts per minute per milligram of dried tissue weight.

Biochemical Measurements

The concentration of plasma Ang II was measured using an Ang II enzyme immunoassay (EIA) kit (St. Louis, MO, USA). ACE activity was evaluated in plasma using a fluorescent assay following the basic method with some modifications (Friedland and Silverstein, 1979). Plasma NA was determined by HPLC with an electrochemical detector (DECADE II, Waters, Milford, MA) using commercial kits (RECIPE, Dessauerstraße 3, D-80992 Munich, Germany). Plasma nitric oxide metabolites (NOx) were assayed using an enzymatic conversion method with some modifications (Boonla *et al.*, 2014).

Vascular Function Study: Experimental Protocols in Isolated Mesenteric Vascular Beds

After exsanguination, mesenteric vascular beds were carefully isolated and then placed on a stainless-steel grid in a humid chamber. The preparations were perfused with physiological Krebs' solution at a constant flow rate of 5 ml/min, using a peristaltic pump (07534–04, Cole-Palmer Instrument, Illinois, USA). Kreb's solution is composed of the following (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.8, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 1.25 and glucose 11.1. The mesenteric vascular beds were pretreated with a desensitizing agent, capsaicin (0.1 μM), for 20 min followed by a 30 min washout period to facilitate a desensitization of vanilloid receptors and to cause a diminution of sensory neurotransmitters. After the washout period, electrical field stimulation (EFS) (5–40 Hz, 90 V, 1 ms, for 30 s at 5-min intervals) was performed. Contractile responses to EFS were detected as changes in mean perfusion pressure (mmHg) using a pressure transducer and data recorded via the BIOPAC System (BIOPAC Systems Inc., California, USA). The preparations were allowed to equilibrate for 30 min before the next trial. After the resting period, NA (0.15–15 nmol) was applied to evaluate the contractile responses to exogenous NA. To determine vasoactive performance of resistance small arteries, methoxamine (5–7 μM) was added into Kreb's solution to raise tone 70–90 mmHg above baseline. Subsequently, different doses of vasoactive agents, acetylcholine (ACh, 1 nM–0.01 μM) or sodium nitroprusside (SNP, 1 nM–0.01 μM) were applied.

Experimental Protocols in Isolated Aortic Rings

The thoracic aorta was rapidly removed and cut into rings 2–3 mm long for tension measurements. They were mounted in 15 ml baths containing Krebs' solution at 37°C and gassed with a 95% O_2 and 5% CO_2 gas mixture. Isometric contractions were recorded with a resting tension of 1 g using a transducer connected to a 4-channel bridge amplifier and a PowerLab A/D converter and a PC running Chart v5 (PowerLab System, AD Instruments, Australia). ACh (0.01–3 μM) induced endothelial mediated-relaxations and SNP (0.01–3 μM) were assessed by pre-contracting with phenylephrine (10 μM) and relaxations were expressed as % relaxation.

Western Blot Analysis

AT_1 receptor, p47^{phox} and gp91^{phox} protein expressions in the thoracic aorta were measured using the Western blot method following a previous publication as described with some modifications (Boonla *et al.*, 2014). The vessels were homogenized and the proteins determined by electrophoresis on a sodium dodecylsulfate polyacrylamide gel electrophoresis system. Thereafter, the proteins were electrotransfered onto a polyvinylidene difluoride membrane and blocked with 5% skimmed milk for 2 h at room temperature before overnight incubation at 4°C with mouse monoclonal antibodies to p47^{phox} and gp91^{phox} (BD Biosciences, CA, USA) or rabbit polyclonal antibodies to AT_1 receptor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After the incubation period, the membranes were washed with tris-buffered saline with tween and then incubated for 2 h at room temperature with horseradish peroxidase conjugated secondary antibody. The blots were developed in Amersham™ ECL™ Prime solution (Amersham Biosciences Corp., Piscataway, NJ, USA), and densitometric analysis was performed using an ImageQuantTM 400 (GE Healthcare Life Sciences, Piscataway, NJ, USA). The intensities of AT_1 receptor, p47^{phox} and gp91^{phox} bands were normalized to that of β-actin, and data were expressed as a percentage of the values determined in the control group from the same gel.

Statistical Analysis

Results are reported as means ± SEM. Comparisons between groups were performed using one-way ANOVA followed by Fisher's Least Significant Difference tests. A probability value of less than 0.05 was considered statistically significant.

Results

Effects of Hesperidin and Losartan on Blood Pressure in 2K-1C Hypertensive Rats During Seven Weeks of The Experiment: Hesperidin and Losartan on Blood Pressures in Conscious Rats

At the beginning of the experiments, baseline SP was similar in all experimental groups. After placing a clip on left renal artery for one week, the SP was significantly higher in the 2K-1C hypertensive rats compared to those of sham-operated control rats. Then, SP gradually increased over three weeks of the experiment. Treatment with hesperidin with 20 mg/kg and 40 mg/kg significantly reduced SP in a dose-response dependent manner at 160.79 ± 7.76 mmHg and 143.96 ± 3.65 mmHg, $p < 0.05$, compared to the untreated rats at 210.57 ± 11.68 mmHg ($p < 0.05$). Losartan markedly reduced SP to 141.11 ± 3.12 mmHg ($p < 0.05$) in losartan hypertensive rats comparing to those of untreated hypertensive rats. There were no significant differences, however, between hesperidin at the dose 40 mg/kg and losartan treated hypertensive rats (Fig. 1).

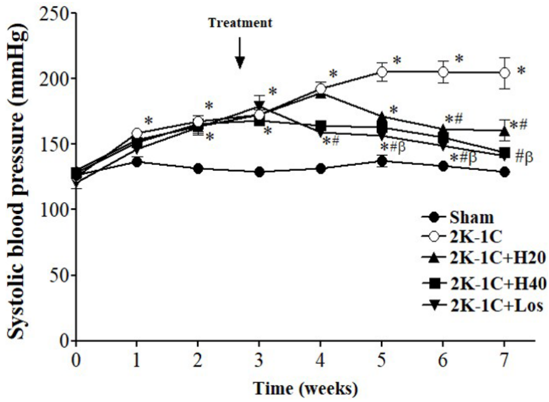


Figure 1. Effect of hesperidin and losartan on SP in 2K-1C hypertensive rats. Data are expressed as means ± SEM ($n = 8$–9/group). $^{*}p < 0.05$ vs. Sham, $^{\#}p < 0.05$ vs. 2K-1C, $^{ß}p < 0.05$ vs. H20 (Sham = sham-operated control, Los = losartan 10 mg/kg, H = hesperidin).

Effect of Hesperidin and Losartan on Blood Pressure and HR in Anesthetized 2K-1C Hypertensive Rats

There were significant increases in SP, DP, MAP and HR in 2K-1C hypertensive rats compared to the sham-operated group ($p < 0.05$). Daily treatment of hesperidin for four weeks significantly decreased SP, DP and MAP in 2K-1C hypertensive rats compared to those of untreated group ($p < 0.05$). Moreover, in hypertensive rats treated with losartan or hesperidin (40 mg/kg) were significantly lower than those of in the hypertensive rat treatment with hesperidin (20 mg/kg) ($p < 0.05$). There were no differences between hesperidin at a dose 40 mg/kg and the losartan group (Table 1).

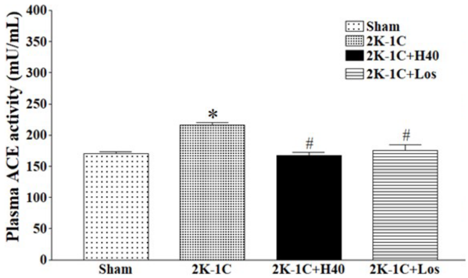
Effects of Hesperidin and Losartan on RAS Activity in 2K-1C Hypertensive Rats: Hesperidin and Losartan on ACE Activity and Plasma Ang II Concentrations in 2K-1C Hypertensive Rats

The ACE activity was significantly increased in 2K-1C hypertensive rats compared to sham-operated rats ($p < 0.05$). A reduction of ACE activity in 2K-1C hypertensive rats

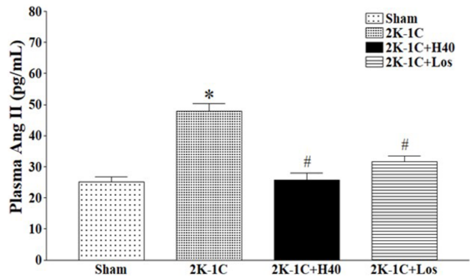
Table 1. Effects of Hesperidin and Losartan on Blood Pressure and HR in Anesthetized 2K-1C Hypertensive Rats

Parameters	Sham	2K-1C	2K-1C+H20	2K-1C+H40	2K-1C+Los
SP (mmHg)	113.54 ± 1.78	202.48 ± 5.50*	151.53 ± 2.99*#	133.55 ± 3.83*#ß	121.20 ± 9.25#ß
DP (mmHg)	76.64 ± 1.96	138.63 ± 5.10*	105.22 ± 3.26*#	91.29 ± 3.59*#ß	84.31 ± 6.91#ß
MAP (mmHg)	88.94 ± 1.36	159.92 ± 4.86*	120.66 ± 3.10*#	105.38 ± 3.60*#ß	96.61 ± 7.66#ß
HR (beat/min)	362.75 ± 6.90	383.25 ± 13.70	378.89 ± 14.03	359.54 ± 7.98	380.75 ± 11.51

Notes: Data are expressed as means ± SEM ($n = 8$–9/group). $^{*}p < 0.05$ vs. Sham, $^{\#}p < 0.05$ vs. 2K-1C, $^{ß}p < 0.05$ vs. H20 (Sham = sham-operated control, 2K-1C = two-kidney, one-clip, Los = losartan 10 mg/kg, H = hesperidin).



(A)



(B)

Figure 2. Effects of hesperidin and losartan on ACE activity (A) and Ang II level (B) in 2K-1C hypertensive rats. Data are expressed as means ± SEM ($n = 6$–8/group). $^*p < 0.05$ vs. Sham, $^\#p < 0.05$ vs. 2K-1C (Sham = sham-operated control, Los = losartan 10 mg/kg, H = hesperidin).

treated with hesperidin (40 mg/kg) and losartan were observed compared to untreated rats ($p < 0.05$; Fig. 2A). Furthermore, the plasma Ang II was significantly higher in 2K-1C hypertensive rats compared to sham rats. These high levels of plasma Ang II were attenuated by hesperidin and losartan ($p < 0.05$; Fig. 2B).

Effects of Hesperidin and Losartan on AT_1 Receptor Protein Expression

The expression of AT_1 receptor protein in aortic tissues from 2K-1C hypertensive rats was significantly upregulated when compared to sham-operated rats ($p < 0.05$). Administration of hesperidin significantly suppressed the expression of AT_1 receptor protein in 2K-1C hypertensive rats. In addition, losartan also reduced the upregulation of AT_1 receptor protein expression in 2K-1C hypertensive rats ($p < 0.05$; Fig. 3).

Effects of Hesperidin and Losartan on Oxidative Stress: Hesperidin and Losartan on NOX2 and $p47^{phox}$ Protein Expression in 2K-1C Hypertensive Rats

There was overexpression of NOX2 in 2K-1C hypertensive rats compared to that of control rats. Hesperidin treatment reduced overexpression of NOX2 in 2K-1C rats while losartan significantly suppressed the upregulation of NOX2 in 2K-1C hypertensive rats ($p < 0.05$; Fig. 4A). Subsequently, the results also showed the upregulation of $p47^{phox}$ in 2K-1C hypertensive rats. This upregulation was significantly attenuated by hesperidin and losartan ($p < 0.05$; Fig. 4B).

Effects of Hesperidin and Losartan on Vascular $O_2^{\bullet-}$ Production and Plasma MDA in 2K-1C Hypertensive Rats

There was significantly increased vascular $O_2^{\bullet-}$ production in 2K-1C hypertensive rats compared to sham-operated rats ($p < 0.05$). The rise of $O_2^{\bullet-}$ production was significantly

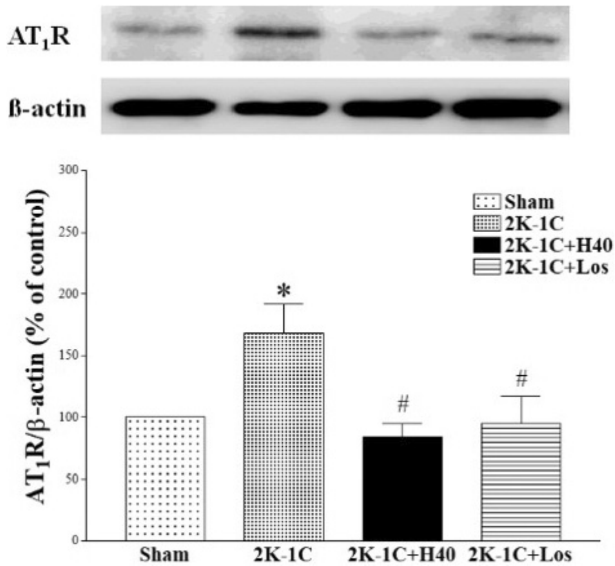


Figure 3. Effects of hesperidin and losartan on AT_1 receptor protein expression in 2K-1C hypertensive rats. Data are expressed as means ± SEM ($n = 4$/group). $^*p < 0.05$ vs. Sham, $^{\#}p < 0.05$ vs. 2K-1C (Sham = sham-operated control, Los = losartan 10 mg/kg, H = hesperidin).

reduced in 2K-1C hypertensive rats treated with hesperidin and losartan compared to untreated rats ($p < 0.05$). There were no significant differences of vascular $O_2^{\bullet-}$ production between hypertensive rats treated with hesperidin and losartan and sham-operated rats (Fig. 5A). Plasma MDA levels in 2K-1C hypertensive rats were significantly higher than

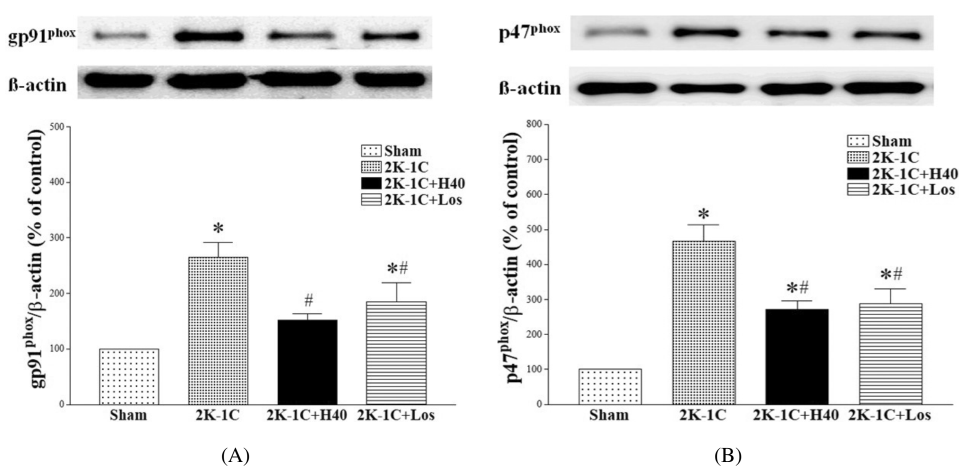
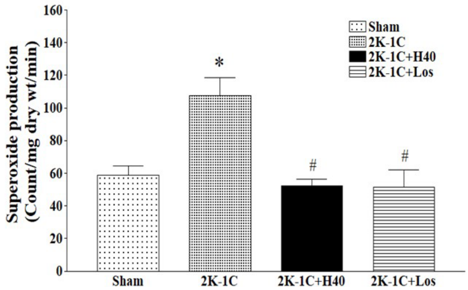
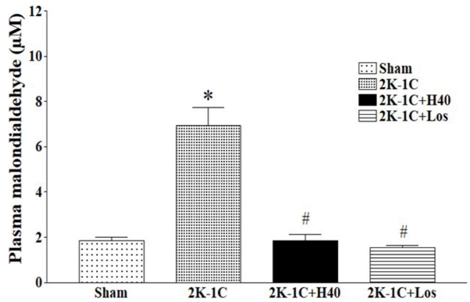


Figure 4. Effects of hesperidin and losartan on NOX2 (A) and p47^{phox} (B) protein expression in 2K-1C hypertensive rats. Data are expressed as means ± SEM ($n = 4$/group). $^*p < 0.05$ vs. Sham, $^{\#}p < 0.05$ vs. 2K-1C (Sham = sham-operated control, Los = losartan 10 mg/kg, H = hesperidin).



(A)



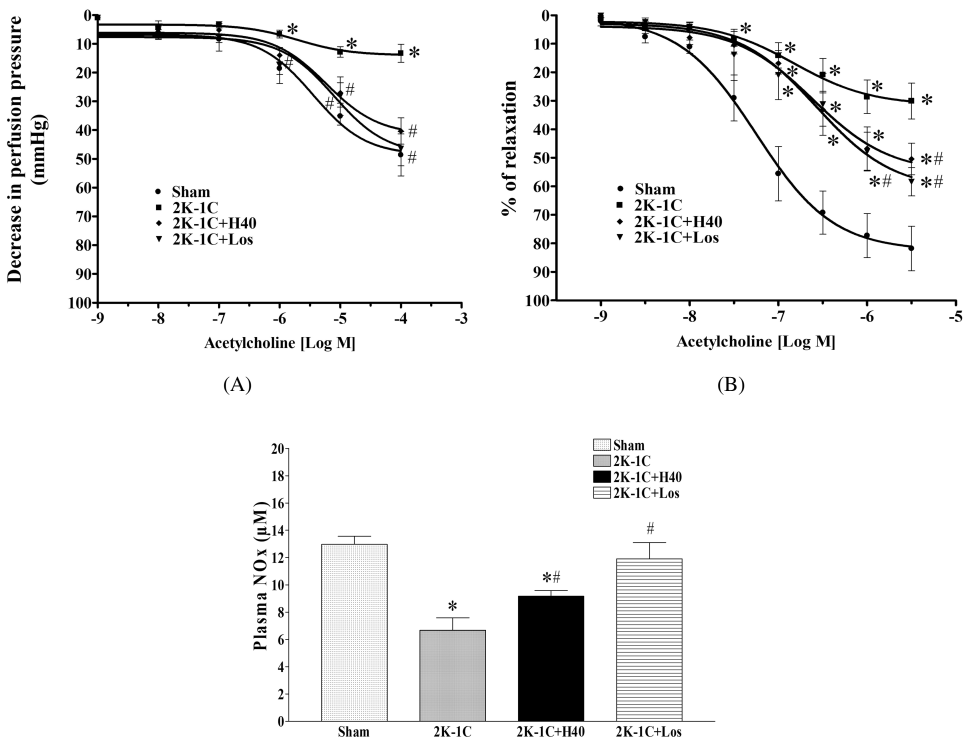
(B)

Figure 5. Effects of hesperidin and losartan on vascular $O_2^{\bullet-}$ production (A) and plasma MDA levels (B) in all experimental groups. Data are expressed as mean ± SEM ($n = 8$/group). $^*p < 0.05$ vs. Sham, $^{\#}p < 0.05$ vs. 2K-1C (Sham = sham-operated control, Los = losartan 10 mg/kg, H = hesperidin).

those of sham-operated rats ($p < 0.05$). Hesperidin significantly reduced plasma MDA levels in 2K-1C hypertensive rats compared to those of untreated rats ($p < 0.05$). Moreover, losartan treatment also reduced plasma MDA levels in 2K-1C hypertensive rats compared to untreated rats ($p < 0.05$) (Fig. 5B).

Effects of Hesperidin and Losartan on Vascular Function in 2K-1C Hypertensive Rats: Hesperidin and Losartan on Vasorelaxation Responses to Vasodilator Agents in Mesenteric Vascular Beds, Aortic Rings and Plasma NOx

The vasorelaxation response to ACh (0.1 μM–0.1 mM) in the mesenteric vascular bed was significantly blunted in 2K-1C hypertensive rats compared to sham-operated rats ($p < 0.05$). Treatment with hesperidin at a dose of 40 mg/kg improved the response to ACh in 2K-1C hypertensive rats compared to untreated rats ($p < 0.05$). Moreover, 2K-1C hypertensive rats treated with losartan significantly improved the response to ACh compared to 2K-1C hypertensive rats ($p < 0.05$) (Fig. 6A). There were no significant differences in the vasorelaxation responses to SNP among groups, indicating normal vascular smooth muscle cell function (data not shown). Endothelium-dependent vasorelaxation responses to ACh (0.01–3 μM) were significantly blunted in aortic rings from 2K-1C hypertensive rats compared to sham-operated rats ($p < 0.05$). Hesperidin at a dose 40 mg/kg improved vascular responses to ACh compared to untreated rats ($p < 0.05$). Moreover, 2K-1C hypertensive rats treated with losartan had a significant improvement of the response to ACh compared to 2K-1C hypertensive rats ($p < 0.05$) (Fig. 6B). The vasorelaxation response to SNP, an NO donor, did not differ significantly among groups (data not shown). In addition, a significant decrease in plasma NOx concentration was found in 2K-1C hypertensive rats compared to sham-operated rats ($p < 0.05$). The level of plasma NOx was significantly increased in 2K-1C rats treated with hesperidin and losartan ($p < 0.05$) (Fig. 6C).



(C)

Figure 6. Effects of hesperidin and losartan on vascular responses to exogenous ACh in mesenteric vascular beds (A); in thoracic aortas (B) and plasma nitric oxide metabolites (C). Data are expressed as mean ± SEM ($n = 6$/group). *$p < 0.05$ vs. Sham, $^{\#}p < 0.05$ vs. 2K-1C (Sham = sham-operated control, Los = losartan 10 mg/kg, H = hesperidin).

Effects of Hesperidin and Losartan on Contractile Responses to EFS and Exogenous NA in Mesenteric Vascular Beds and Plasma NA Concentration

EFS at 5–40 Hz produced an increase in perfusion pressure that was frequency-dependent vasoconstriction in all preparations. Significant increases in contractile responses to EFS were observed in the mesenteric vascular bed isolated from 2K-1C hypertensive rats compared to the responses in sham-operated rats ($p < 0.05$). Contractile responses to EFS in 2K-1C hypertensive rats-treated with hesperidin and losartan were reduced compared to those of hypertensive rats ($p < 0.05$) (Fig. 7A). The contractile responses to exogenous NA, however, (0.1 μM–0.1 mM) were not different among groups (data are not shown). An increase in plasma NA concentrations was found in 2K-1C hypertensive rats ($p < 0.05$). The elevation of plasma NA concentrations was decreased in hesperidin treated rats at a dose of 40 mg/kg compared to those of untreated rats ($p < 0.05$). Moreover, losartan treatment reduced plasma NA concentrations in 2K-1C hypertensive rats compared to untreated rats ($p < 0.05$) (Fig. 7B).

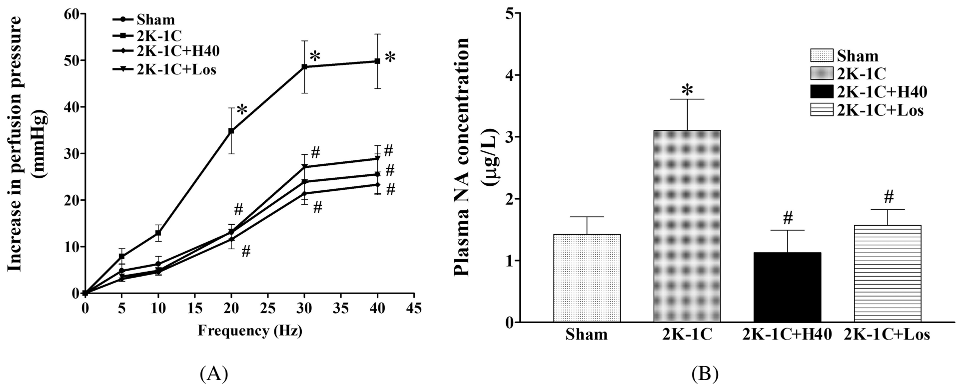


Figure 7. Effects of hesperidin and losartan on contractile responses to sympathetic nerve stimulation (A) and plasma NA concentrations (B). Data are expressed as mean ± SEM ($n = 6$/group). *$p < 0.05$ vs. Sham, $^{\#}p < 0.05$ vs. 2K-1C (Sham = sham-operated control, Los = losartan 10 mg/kg, H = hesperidin).

Discussion

The present findings show that hesperidin reduced blood pressure in 2K-1C hypertensive rats. Hesperidin also affected RAS activation by decreasing ACE activity, plasma Ang II levels and suppressed AT_1 receptor expression in 2K-1C hypertensive rats. Increases in $O_2^{\bullet -}$ production and plasma MDA as well as p47^{phox} and gp91^{phox} protein overexpression were alleviated in the 2K-1C group treated with hesperidin or losartan. Hesperidin and losartan improved endothelial dysfunction and nitric oxide (NO) bioavailability in 2K-1C hypertensive rats. Sympathetic nerve activation, as evident by the enhancement of nerve-mediated contractile responses and high levels of plasma NA were shown in 2K-1C hypertensive rats. This sympathoexcitation was not found in 2K-1C rats treated with hesperidin or losartan.

High blood pressure was associated with increases in plasma ACE activity and plasma Ang II was shown in 2K-1C rats in the present study. The elevation of plasma Ang II observed in this study was the consequence of RAS activation. This was supported by the findings that a partial occlusion of one renal artery can develop hyperactivation of RAS in animals (Romero *et al.*, 1997; Nishi *et al.*, 2010). Sawamura and Nakada demonstrated high renal levels of ANG I, II and ACE activity in the clipped-kidney rats (Sawamura and Nakada, 1996). Ang II, an octopeptide product of RAS, induces high blood pressure via activation of AT_1 receptors to produce vasoconstriction, Na^+ and water reabsorption, and sympathetic nervous stimulation (Fyhrquist *et al.*, 1995). The upregulation of AT_1 receptors that might enhance Ang II-mediated hypertensive effects was found in this animal model. A relevant study indicated that prolonged exposure to Ang II could induce the upregulation of brain AT_1 receptors (Mitra *et al.*, 2010). This study, in renovascular hypertensive rats, revealed the antihypertensive effect of hesperidin, which involved the suppression of the RAS pathway. This is the first evidence to report the ACE inhibiting activity of hesperidin in an animal model of hypertension. The attenuation of plasma ACE activity seems then to be related to reductions of plasma Ang II level, AT_1 receptor expression and then blood pressure. The ACE inhibitor activity of hesperidin might then be mediated by its flavonoid structure since there is evidence supporting flavonoids inhibiting ACE activity and then lowering blood pressure (Kameda *et al.*, 1987). Furthermore, a molecular docking study discovered that flavonoids were able to bind with zinc ion in the active site of ACE enzymes (Shafaei *et al.*, 2016).

The upregulation of NOX subunits (p47^{phox} and gp91^{phox} protein expression) in aortic tissues of 2K-1C hypertensive rats as a sequential activation of circulating Ang II was observed in this study. This was associated with increases in oxidative stress markers including plasma MDA levels and vascular $O_2^{\bullet -}$ production. These current findings were supported by other studies performed in the 2K-1C hypertension models. For example, overexpression of p47^{phox} protein was demonstrated to be responsible for oxidative stress in RAS-dependent hypertension in rats (Boonla *et al.*, 2014). Wei and coworkers indicated that Ang II can increase reactive oxygen species production through activating NOXs (Wei *et al.*, 2006). It is now accepted that high $O_2^{\bullet -}$ production promotes the development of hypertension since $O_2^{\bullet -}$ can rapidly react with NO to form the potent cytotoxic peroxynitrite. It has clearly been shown that peroxynitrite causes oxidative damage to endothelial cells and decreases NO bioavailability (Förstermann and Li, 2011). Jung and coworkers indicated that NO bioavailability was reduced because of activation of the NOXs and $O_2^{\bullet -}$ production in the 2K-1C model (Jung *et al.*, 2004). The impairment of endothelium-dependent vasodilation is supported by reducing the vascular response to ACh but not to SNP and was clearly shown in the present study. This was consistent with a reduction of NO metabolites observed in 2K-1C hypertensive rats. Another possible antihypertensive mechanism of hesperidin proposed in this study involved its anti-oxidant effect. Hesperidin reduced p47^{phox} and gp91^{phox} expression, $O_2^{\bullet -}$ production and plasma MDA levels in 2K-1C hypertensive rats which were associated with the improvement of vascular function and plasma NOx level. There are substantial data to indicate the anti-oxidant property of hesperidin (Homayouni *et al.*, 2017). Yamamoto and coworkers firstly reported the anti-hypertensive effect of hesperidin in spontaneously hypertensive rats associated with improved vascular function and a reduction of oxidative stress (Yamamoto *et al.*, 2008). In RAS-dependent hypertension in rats, this study indicated that the antihypertensive effect of hesperidin might involve the anti-oxidant property or a sequential suppression of RAS cascade or both.

Sympathetic activation is an important factor to stimulate and maintain high blood pressure in renovascular hypertensive rats (Bergamaschi *et al.*, 1995). Sympathoexcitation was confirmed in renovascular hypertension as indicated by a significant rise in low-frequency SP during the mid-developmental and maintenance phases of the hypertension in 2K-1C hypertensive rats (Oliveira-Sales *et al.*, 2014). A recent study by Nishi and coworkers showed sympathetic vasomotor hyperactivity and baroreflex dysfunction which contributed to the development and maintenance of renovascular arterial hypertension (Nishi *et al.*, 2010). The present results demonstrated that there was an enhancement of contractile responses to sympathetic nerve stimulation without affecting the response to exogenous NA in 2K-1C rats, suggesting the augmentation of NA release from pre-junctional sites. This result could be supported with high plasma NA in 2K-1C rats. In addition, there is evidence to support findings that an increase in the vasoconstrictive response to peri-arterial nerve stimulation was uncovered in isolated mesenteric vascular beds from 2K-1C rats (Zimmerman *et al.*, 1987). The authors suggested that this enhancement was partially mediated by Ang II-induced facilitation of NA release (Zimmerman *et al.*, 1987). In fact, Ang II increases sympathetic nerve activity via activation of the AT_1 receptor in the rostral RVLM, a pressor area of the medulla, and activates sympathetic outflow to enhance NA release from sympathetic nerve terminals (Oliveira-Sales *et al.*, 2009). Furthermore, treatment with hesperidin suppressed the nerve-mediated contractile response in 2K-1C hypertensive rats, which was relevant to a decreased plasma NA level. An intriguing possibility is that hesperidin inhibited the RAS cascade and subsequently reduced oxidative stress and sympathoexcitation.

In the present study, losartan was used as a positive control and was able to decrease blood pressure, RAS activation, oxidative stress, and improve vascular function in the 2K-1C hypertensive rats. The antihypertensive actions of losartan as an AT_1 receptor antagonist in 2K-1C hypertensive rats were strongly documented (Martins-Oliveira *et al.*, 2013). Losartan also caused a significant reduction of brain AT_1 receptors with attenuation of the brain and plasma oxidative stress in renovascular hypertensive rats (Boshra and Abbas, 2017).

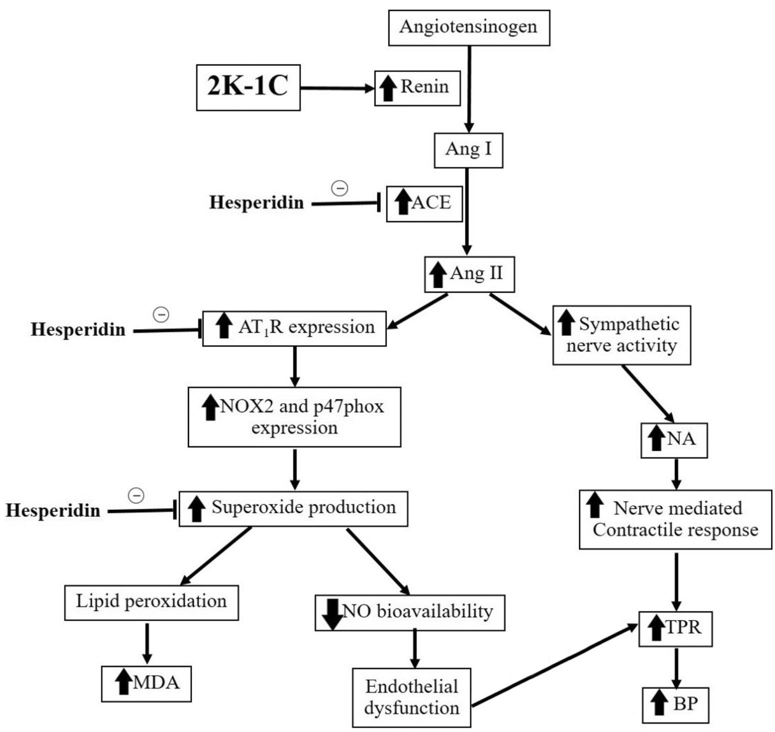


Figure 8. Proposed mechanisms of action of hesperidin. TPR represents total peripheral resistance.

In conclusion, the results of this present study indicate that hesperidin reduced blood pressure by suppressing RAS cascade mediated-NOX overexpression and sympathoexcitation or it directly reduced oxidative stress (Fig. 8). It could therefore be suggested that hesperidin might potentially be further developed as a complimentary agent in the treatment of hypertension.

Acknowledgments

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Hesperidin Prevents Nitric Oxide Deficiency-Induced Cardiovascular Remodeling in Rats via Suppressing TGF-β1 and MMPs Protein Expression

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Abstract: Hesperidin is a major flavonoid isolated from citrus fruits that exhibits several biological activities. This study aims to evaluate the effect of hesperidin on cardiovascular remodeling induced by N-nitro L-arginine methyl ester (L-NAME) in rats. Male Sprague-Dawley rats were treated with L-NAME (40 mg/kg), L-NAME plus hesperidin (15 mg/kg), hesperidin (30 mg/kg), or captopril (2.5 mg/kg) for five weeks (n = 8/group). Hesperidin or captopril significantly prevented the development of hypertension in L-NAME rats. L-NAME-induced cardiac remodeling, i.e., increases in wall thickness, cross-sectional area (CSA), and fibrosis in the left ventricular and vascular remodeling, i.e., increases in wall thickness, CSA, vascular smooth muscle cells, and collagen deposition in the aorta were attenuated by hesperidin or captopril. These were associated with reduced oxidative stress markers, tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta 1 (TGF-β1), and enhancing plasma nitric oxide metabolite (NOx) in L-NAME treated groups. Furthermore, up-regulation of tumor necrosis factor receptor type 1 (TNF-R1) and TGF- β1 protein expression and the overexpression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) was suppressed in L-NAME rats treated with hesperidin or captopril. These data suggested that hesperidin had cardioprotective effects in L-NAME hypertensive rats. The possible mechanism may involve antioxidant and anti-inflammatory effects.

Keywords: hesperidin; L-NAME; cardiovascular remodeling; oxidative stress; inflammation

1. Introduction

Nitric oxide (NO) is a crucial vasodilator derived from vascular endothelium to regulate vascular tone [1]. A reduction of NO production results in increased vascular resistance and high blood pressure. N^{ω}-nitro L-arginine methyl ester (L-NAME), an L-arginine analogue, is widely used as an inhibitor of nitric oxide synthase (NOS) activity to represent an animal model of hypertension. It has been reported that L-NAME-induced hypertension in rats is characterized by insufficient NO production, increased systemic oxidative stress, inflammation, and endothelial dysfunction [2]. Furthermore, L-NAME-induced hypertension and cardiovascular remodeling have also been reported in rats. For example, the administration of L-NAME (40 mg/kg) for four or five weeks causes high blood pressure and cardiovascular remodeling, including left ventricular hypertrophy, myocardial fibrosis, and thickening of the vascular wall [3–5]. It is generally known that the main sequel of cardiovascular remodeling is heart failure, which is the major cause of death worldwide [6].

The initial stage of cardiac remodeling is myocardial hypertrophy because of the adaptive response to a high-pressure load to preserve cardiac function and obtain normal cardiac work. In addition, the cardiac remodeling process in L-NAME-treated rats is involved in the production of myocardial fibrosis [7]. There are substantial data to show the molecular mechanism of extensive areas of cardiac fibrosis which is associated with the activation of various downstream inflammatory [8] and oxidative stress initiatives [9,10]. For example, a high level of tumor necrosis factor (TNF-α), a pro-inflammatory cytokine, developed in response to oxidative stress in L-NAME-induced hypertension has been reported [4,11]. These inflammatory responses subsequently activate the profibrotic mediator of the transforming growth factor β1 (TGF-β1) [11]. It is well-established that TGF-β1 plays a key role in fibrogenesis by activating apoptosis, collagen, and matrix protein synthesis [12–14]. For vascular structural changes in hypertension, it is known to be an adaptive response to an increase in wall tension [15]. This response is also related to the extracellular matrix degradation of elastic fibers since the up-regulation of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) expression in vessel tissue has been confirmed in animal models of hypertension. Several lines of evidence have indicated that the activation of MMP-2/9 protein expression found in the vascular remodeling process is mediated by the inflammatory cytokine, TNF-α [16–18]. Thus, it is noteworthy that natural products with high antioxidant and anti-inflammatory activities might be useful for alleviating cardiovascular alterations induced by nitric oxide deficiency.

Captopril is an angiotensin-converting enzyme (ACE) inhibitor and is commonly used as an anti-hypertensive drug [19]. Its mechanism of action has been well-documented to reduce angiotensin II production, which subsequently suppresses the renin-angiotensin-aldosterone system (RAAS) [19]. Other possible anti-hypertensive mechanisms include increased bradykinin and prostaglandins levels [20], the inhibition of superoxide production [21], and the free radical scavenging effect [22]. Many studies have already reported on the cardiovascular effects of captopril in nitric oxide-deficient hypertensive rats, i.e., lowering high blood pressure, improving vascular function [21], and preventing cardiovascular remodeling [23]. In L-NAME hypertensive rats, there is evidence showing the up-regulation of angiotensin II receptor type 1 (AT1R) which mediates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression and superoxide formation [10]. This study used captopril as a positive control agent because the L-NAME hypertension model is also involved in the activation of the RAAS, where captopril inhibits the RAAS.

Hesperidin is a flavanone glycoside, a subclass of flavonoids, abundantly found in citrus fruits such as lemon or orange peels or juices [24]. Numerous beneficial effects of hesperidin have been published. For example, the antioxidant effect of hesperidin has been reported to be able to sequester 1,1-diphenyl-2-picrylhydrazyl (DPPH) and protect cell injury-induced by paraquat and hydrogen peroxide [25], reduce plasma levels of lipid peroxidation markers, and increase antioxidant enzyme activities in heart tissue in experimentally ischemic myocardial rats [26]. Hesperidin has also exhibited an anti-inflammatory effect by reducing circulating inflammatory markers, i.e., TNF-α, interleukin 6 (IL-6), and a high-sensitivity C-reactive protein (hs-CRP), in patients with type 2 diabetes [27] and suppressed inflammatory responses in lipopolysaccharide-induced RAW 264.7 cells [28]. Subsequently, a clinical study revealed that a combination of hesperidin, diosmin, and troxerutin was effective in relieving the symptoms of acute hemorrhoidal disease [29]. Recently, the current authors have demonstrated an anti-hypertensive effect of hesperidin in renovascular hypertensive rats that involved the suppression of the renin-angiotensin system [30]. This study was intended to further explore whether hesperidin could prevent L-NAME-induced hypertension and cardiovascular remodeling in rats.

2. Materials and Methods

2.1. Drugs and Chemicals

Hesperidin (purity ≥ 98%) was purchased from Chem Faces Company (Wuhan, Hubei, China). N(G)-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and captopril were purchased from Sigma-Aldrich Corp (St. Louis, MO, USA). All the other chemicals used in this study were obtained from standard companies and were of analytical grade quality.

2.2. Animals and Experimental Protocols

Male Sprague-Dawley rats (body weight 220–250 g) were supplied by Nomura Siam International Co., Ltd., Bangkok, Thailand. The animals were housed in a Heating, Ventilation and Air-Conditioning (HVAC) System (25 ± 2 °C) facility and maintained on a 12 h light and 12 h dark cycle with free access to a standard rat diet and water at the Northeast Laboratory Animal Center, Khon Kaen University. All the experimental protocols in this study were in accordance with the standards for the care and use of experimental animals and approval for all the experiments was obtained from the Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (AEKKU-NELAC 37/2559).

After a seven-day acclimatization period, the rats were randomly assigned to 5 groups (8/group). The control group animals received tap water and were orally administrated propylene glycol (PG, 1.5 mL/Kg) as a vehicle. L-NAME treated rats received L-NAME (40 mg/kg/day) in their drinking water and were further divided into the following 4 groups; L-NAME plus PG, L-NAME plus hesperidin at a dose of 15 mg/kg (L-NAME + H15 group), L-NAME plus hesperidin 30 mg/kg (L-NMAE + H30 group), L-NAME group plus captopril at a dose of 2.5 mg/kg (L-NAME + Cap group). Additionally, normal rats (n = 5) were orally treated with hesperidin (30 mg/kg) for 5 weeks to test the hypotensive effect of hesperidin. Hesperidin and captopril were dissolved in vehicle and intragastrically administered once daily for five weeks. The doses of hesperidin and captopril used in this study were influenced by previous studies in this laboratory [10,30].

2.3. Blood Pressure Measurements

To monitor blood pressure changes throughout the experimental period, systolic blood pressure (SP) was obtained in awake rats once a week for 5 weeks using tail-cuff plethysmography (IITC/Life Science Instrument model 229 and model 179 amplifier; Woodland Hills, CA, USA). At the end of the final experimental day, the rats were anesthetized with pentobarbital sodium (60 mg/kg, ip.). Then, the femoral artery was cannulated and connected to a pressure transducer for monitoring the baseline values of SP, diastolic blood pressure (DP), mean arterial pressure (MAP), and heart rate (HR) using the Acknowledge Data Acquisition software (Biopac Systems Inc., Santa Barbara, CA, USA).

2.4. Collection of Blood and Organs

After the blood pressure measurement, the rats were sacrificed by exsanguination and blood samples were collected from abdominal aortas into Ethylenediaminetetraacetic acid (EDTA) or heparin tubes for assays of oxidative stress and inflammatory markers. The carotid arteries were rapidly excised for analysis of superoxide ($O_2^{\bullet-}$) production. The thoracic aortas and heart tissues were collected for western blotting and morphometric analysis.

2.5. Assays of Vascular $O_2^{\bullet-}$ Production, Plasma Malondialdehyde (MDA), Plasma Nitric Oxide Metabolite (Nitrate/Nitrite, NOx), Plasma TNF-α and Plasma TGF- β1 Levels

The carotid arteries were cleaned of connective tissues, cut into 0.5 cm lengths, and incubated with 1 mL oxygenated Krebs-KCl solution at pH 7.4, 37 °C for 30 min. The production of $O_2^{\bullet-}$ in the carotid arteries was determined by lucigenin-enhanced chemiluminescence, as previously described [31], with some modifications [32]. Plasma NOx was assayed using an enzymatic conversion method [33], with some modifications [32]. The concentrations of plasma TNF-α and TGF-β1 were measured using enzyme-immunoassay assay (ELISA) kits (eBioscienc, Inc., San Diego, CA, USA and ab119557, Abcam Plc, Cambridge, UK).

2.6. Morphometric Analysis of Thoracic Aorta and Heart Tissue

Heart weight (HW) and left ventricular weight (LVW) were measured, and calculated as an LVW/BW ratio. Thereafter, the left ventricles and thoracic aortas were fixed with 4% paraformaldehyde and then embedded in paraffin and cut into serial 5-μm-thick sections. Each section was stained with hematoxylin and eosin (H&E) and/or Picrosirius Red. Sections were captured with a Digital sight DS-2MV light microscope (Nikon, Tokyo, Japan) or a stereoscope (Nikon SMZ745T with NIS-elements D 3.2, Tokyo, Japan). Morphometric evaluations of the sections were performed with Image J software (National Institutes of Health, Bethesda, MD, USA).

2.7. Western Blot Analysis of Tumor Necrosis Factor Receptor 1 (TNF-R1), TGF- β1, MMP-2 and MMP-9 Protein Expressions in Cardiac and Aortic Tissues

Protein samples were prepared through the homogenization of cardiac and aortic tissues in a lysis buffer (Cell Signaling Technology Inc., Danvers, MA, USA). The proteins were then electrophoresed on a sodium dodecylsulfate polyacrylamide gel electrophoresis system and transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) with 0.1% Tween 20 for 2 h at room temperature before overnight incubation at 4 °C with primary antibodies against TNF-R1, TGF-β1, MMP-2, MMP-9, or β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Thereafter, the membranes were washed three times with TBS and then incubated for 2 h at room temperature with a horseradish peroxidase conjugated secondary antibody. The protein bands were detected using Luminata™ Forte horseradish peroxidase (HRP) detection reagent (Merck KGaA, Darmstadt, Germany) and the densitometric analysis was performed using ImageQuantTM 400 (GE Healthcare Life Sciences, Piscataway, NJ, USA). The intensity of each band was normalized to that of β-actin, and data were expressed as a percentage of the values determined in the control group from the same gel.

2.8. Statistical Analysis

Data are expressed as mean ± S.E.M. The differences among the treatment groups were analyzed through a one-way analysis of variance (ANOVA) followed by Bonferini's post-hoc test. A *p*-value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Effects of Hesperidin and Captopril on Blood Pressure in Conscious Rats

There were no significant differences in the systolic blood pressure of all the rats at the beginning of the study. The administration of L-NAME caused a gradual increase in the SP of all the rats compared to the control rats (SP at 5th week, 200.21 ± 6.52 vs. 122.14 ± 1.75 mmHg, $p < 0.01$, Figure 1). The co-administration of L-NAME and hesperidin at doses of 15 or 30 mg/kg (2.5 mg/kg) partially prevented L-NAME-induced high blood pressure in a dose-dependent manner compared to that of untreated rats (SP at 5th week, 177.50 ± 3.91 and 162.74 ± 2.82 mmHg, $p < 0.05$). Captopril also partially alleviated L-NAME-induced hypertension (152.19 ± 5.01 mmHg) compared to untreated rats ($p < 0.05$). In addition, captopril produced a greater preventive effect on SP than hesperidin (15 and 30 mg/kg).

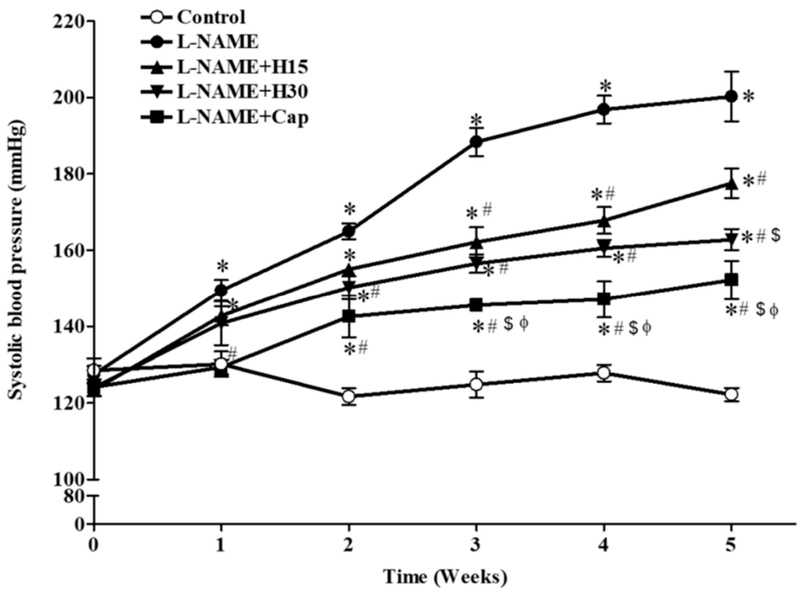

Figure 1. Time-course changes in systolic blood pressures of all experimental groups. Data are expressed as mean ± S.E.M (n = 7–8/group), * $p < 0.05$ vs. control, # $p < 0.05$ vs. L-NAME, $ $p < 0.05$ vs. L-NAME + hesperidin (15 mg/kg), $^{\Phi}$ $p < 0.05$ vs. L-NAME + hesperidin (30 mg/kg) group.

3.2. Effects of Hesperidin and Captopril on SP, DP, MAP, and HR in Anesthetized Rats

The blood pressure data obtained using the indirect blood pressure measurement method were consistent with the values from the direct method since L-NAME treated rats exhibited high blood pressure, including high SP, DP, MAP, and high HR compared to those of control rats ($p < 0.05$, Table 1). Hesperidin at doses of 15 and 30 mg/kg significantly decreased SP, DP, and MAP in a dose-dependent manner compared to the untreated group ($p < 0.05$). Similarly, captopril reduced the development of hypertension induced by L-NAME compared to untreated rats ($p < 0.05$). Hesperidin at a dose 30 mg/kg, however, also affected the elevation of HR compared to untreated rats ($p < 0.05$, Table 1). Furthermore, hesperidin had no effect on blood pressure in normotensive rats (SP = 122.29 ± 4.05 mmHg, n =4).

Table 1. Effects of hesperidin and captopril on blood pressure and heart rate in anesthetized rats.

Parameters	Control	L-NAME	L-NAME + H15	L-NAME + H30	L-NAME + Cap
SP (mmHg)	120.92 ± 2.27	205.88 ± 3.19 *	179.38 ± 16.51 *,#	154.07 ± 4.88 *,#,$	140.14 ± 7.06 #,$
DP (mmHg)	72.68 ± 3.31	141.65 ± 5.73 *	114.13 ± 16.57 *,#	86.89 ± 5.74 *,#,$	91.48 ± 7.36 #,$
MAP (mmHg)	88.76 ± 2.47	161.41 ± 4.01 *	135.88 ± 16.00 *,#	109.28 ± 5.39 *,#,$	107.70 ± 6.27 #,$
HR (beat/min)	367.86 ± 11.90	419.30 ± 11.96 *	391.93 ± 14.35	351.44 ± 13.47 #,$	384.28 ± 17.31

SP: systolic blood pressure; DP: diastolic blood pressure; MAP: mean arterial pressure; HR: heart rate. Values are mean ± S.E.M (n = 7–8/group), * $p < 0.05$ vs. control, # $p < 0.05$ vs. L-NAME, $ $p < 0.05$ vs. L-NAME + H15.

3.3. Effects of Hesperidin and Captopril on Left Ventricular (LV) Morphometry and Fibrosis

Rat body weights did not differ among all experimental groups. After 5 weeks of L-NAME administration, the HW, LVW, and LVW/BW ratios were significantly increased compared to those of control rats. The co-administration of L-NAME and hesperidin or captopril significantly decreased those values when compared to the untreated group (Table 2). Morphometric analysis of hearts showed that the chronic administration of L-NAME significantly increased LV wall thickness and LV muscle fiber cross-sectional area (CSA) compared to the normal control group ($p < 0.05$, Table 2). Hypertensive rats that received hesperidin or captopril had significantly reduced wall thicknesses and CSA of the LV compared to untreated rats ($p < 0.05$) (Table 2, Figure 2A). LV fibrosis was significantly increased in the L-NAME-treated rats compared to the normal control rats ($p < 0.05$). Hesperidin or captopril treatment significantly prevented L-NAME-induced LV fibrosis compared to the untreated rats ($p < 0.05$) (Figure 2B).

Table 2. Effect of hesperidin and captopril on the cardiac mass indices and cardiovascular structural modifications in left ventricle and thoracic aorta.

Cardiac Mass Indices				
Groups	**Body Weight (g)**	**Heart Weight/BW (mg/g)**	**LVW/BW (mg/g)**	
Control	434 ± 6.8	3.14 ± 0.17	2.06 ± 0.10	
L-NAME	413 ± 16.9	4.21 ± 0.26 *	3.04 ± 0.18 *	
L-NAME + H30	406 ± 9.7	3.11 ± 0.23 #	2.23 ± 0.17 #	
L-NAME + Cap	401 ± 9.7	3.12 ± 0.18 #	2.07 ± 0.12 #	
Left Ventricle				
Groups	**LV Wall Thickness (mm)**	**LV CSA (mm²)**	**LV Fibrosis (%)**	
Control	2.72 ± 0.05	57.58 ± 1.05	0.69 ± 0.04	
L-NAME	3.28 ± 0.04 *	72.42 ± 0.51 *	2.72 ± 0.15 *	
L-NAME + H30	2.90 ± 0.06 #	61.12 ± 1.75 #	0.92 ± 0.09 #	
L-NAME + Cap	2.79 ± 0.09 #	59.87 ± 1.63 #	1.00 ± 0.06 #	
Thoracic Aorta Structural Modifications				
Groups	**Wall Thickness (μm)**	**CSA (×10³ μm²)**	**VSMCs (cells/CSA)**	**Collagen Deposition (% Area Fraction)**
Control	106.39 ± 1.02	579.00 ± 15.16	1298.00 ± 73.64	15.78 ± 0.70
L-NAME	150.58 ± 2.09 *	810.50 ± 18.64 *	2013.71 ± 51.62 *	31.32 ± 1.00 *
L-NAME + H30	127.11 ± 2.90 *,#	617.95 ± 18.65 #	1540.16 ± 46.88 *,#	24.84 ± 0.69 *,#
L-NAME + Cap	129.91 ± 6.50 *,#	658.38 ± 40.22 #	1671.78 ± 24.90 *,#	23.68 ± 0.63 *,#

LV: left ventricular, LVW: left ventricular weight, BW: body weight, CSA: cross-sectional area, VSMCs: vascular smooth muscle cells. Values are expressed as mean ± S.E.M, (n = 6/group). * $p < 0.05$ when compared to the control group, and # $p < 0.05$ when compared to the L-NAME group.

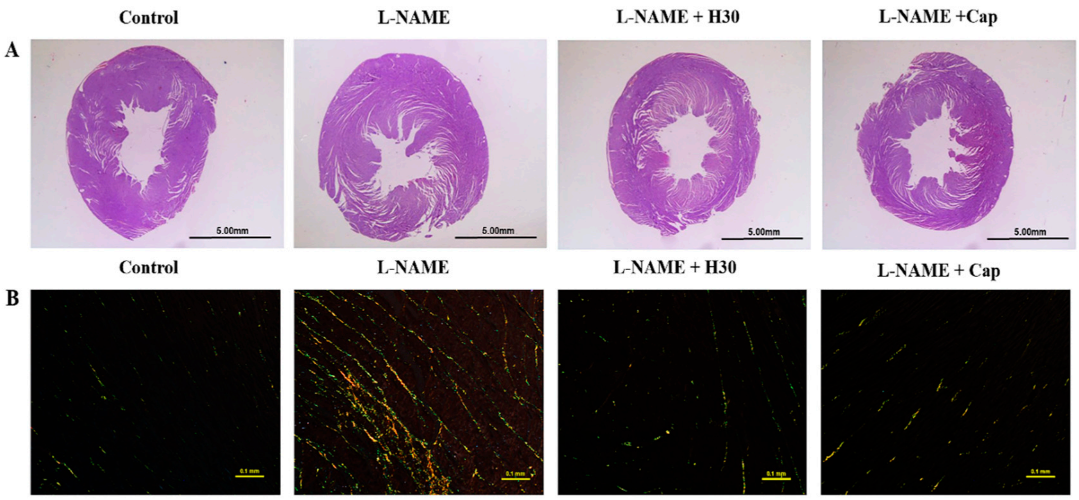

Figure 2. The histology and morphology of LV from control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. Representative images of LV sections, (**A**) stained with hematoxylin and eosin under stereomicroscopes, and (**B**) stained with picrosirius red under a polarized light microscope using a 20× objective lens.

3.4. Effect of Hesperidin and Captopril on Vascular Morphology

Vascular wall hypertrophy was observed in thoracic aortas collected from L-NAME hypertensive rats (Figure 3A) with significant increases in vascular wall thickness, CSA, and smooth muscle cells numbers compared to those of the control rats ($p < 0.05$; Table 2, Figure 3A). Moreover, the relative amounts of collagen depositions (Figure 3B) in the aortic walls of L-NAME hypertensive rats were also clearly observed ($p < 0.05$; Table 2, Figure 3B). Hesperidin or captopril treatment partially prevented the vascular structural abnormalities in aortas induced by L-NAME ($p < 0.05$).

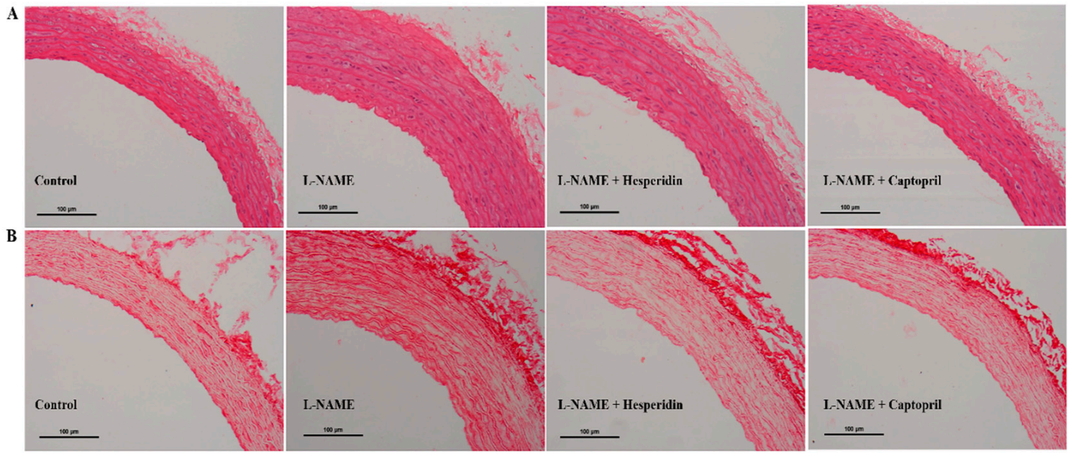

Figure 3. The histology and morphology of thoracic aorta from control, L-NAME, L-NAME + hesperidin (30 mg/kg), and L-NAME + captopril (2.5 mg/kg) groups. Representative images of aortic sections, (**A**) stained with hematoxylin and eosin and (**B**) stained with picrosirius red under a light microscope using a 20× objective lens.

3.5. Effects of Hesperidin and Captopril Supplementation on Oxidative Stress Markers, Plasma Nitric Oxide Metabolites (NOx) Levels in L-NAME Treated Rats

L-NAME treated rats showed a significant increase in the production of vascular $O_2^{\bullet -}$ (263.26 ± 11.20 vs. 71.42 ± 15.97 count/mg dry wt/min, $p < 0.001$) and plasma MDA levels compared to the control groups (10.24 ± 0.4 vs. 3.11 ± 0.27 µM, $p < 0.05$). When treated with hesperidin or captopril, the elevations of vascular $O_2^{\bullet -}$ and plasma MDA were mitigated compared to those of untreated rats (7.91 ± 0.92, 4.83 ± 0.74 and 3.88 ± 0.25 count/mg dry wt/min and 138.86 ± 28.75, 97.28 ± 16.67 and 92.14 ± 12.90 µM, $p < 0.05$) (Figure 4A,B). In addition, low levels of plasma NOx were found in L-NAME hypertensive rats compared to control rats (3.49 ± 1.0 vs. 10.17 ± 0.95 µM, $p < 0.05$). These low levels of plasma NOx were improved by hesperidin or captopril supplementation (4.38 ± 1.15, 7.48 ± 1.03 and 8.48 ± 1.21 µM, $p < 0.05$) (Figure 4C).

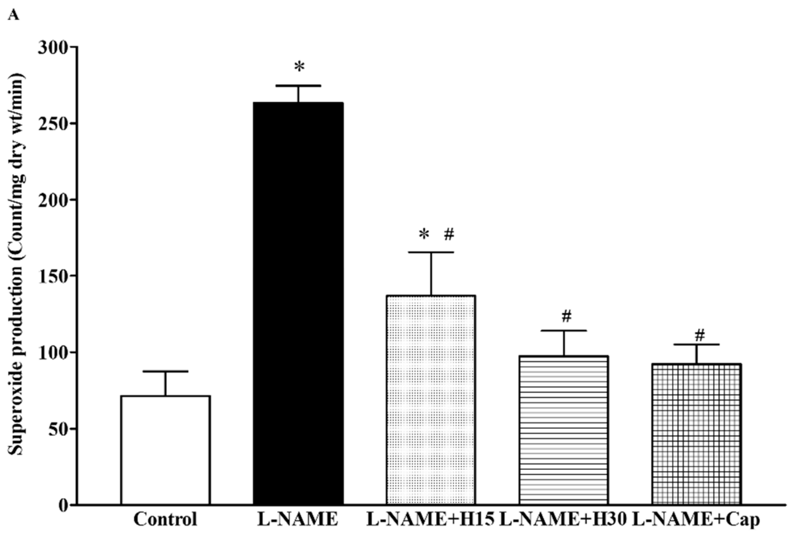

Figure 4. *Cont.*

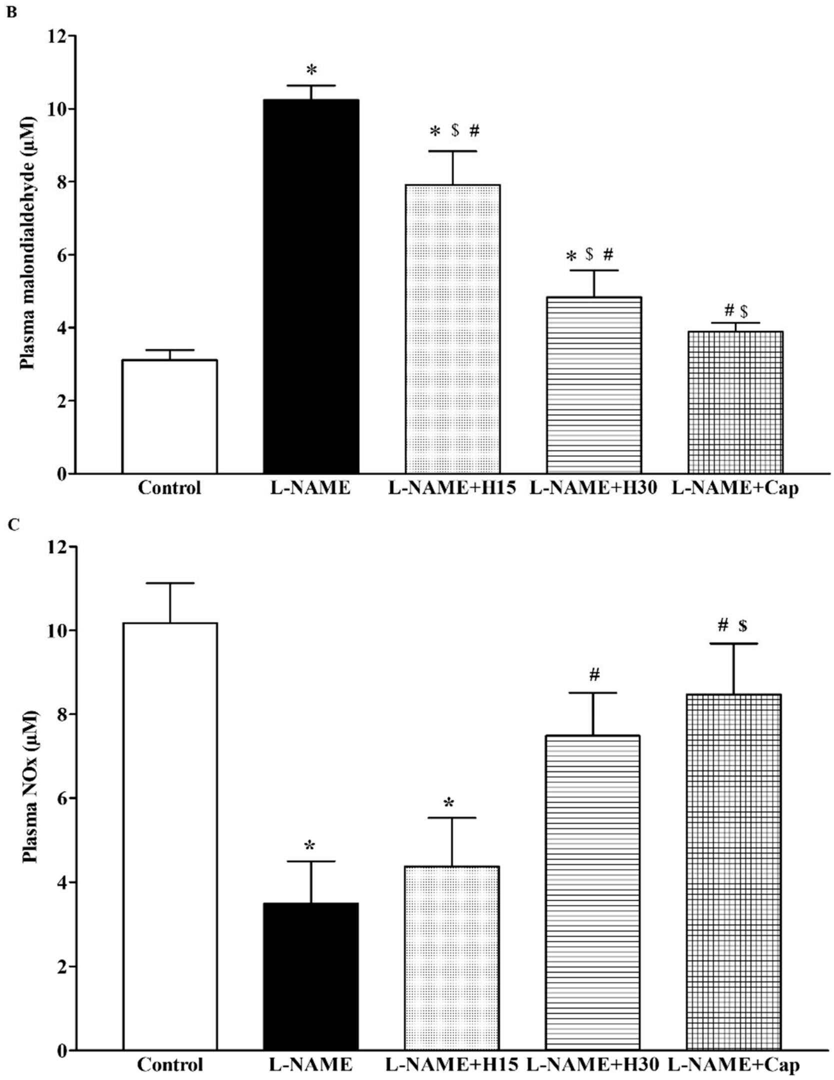

Figure 4. Effects of hesperidin and captopril supplementation on vascular $O_2^{\bullet-}$ production, (**A**) plasma MDA (**B**) and plasma NOx (**C**) levels in control, L-NAME, L-NAME + hesperidin (15 mg/kg), L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (5 mg/kg) groups. Data are expressed as mean ± S.E.M (n = 7–8/group), * $p < 0.05$ vs. control, $^{\#}$ $p < 0.05$ vs. L-NAME group, $^{\$}$ $p < 0.05$ vs. L-NAME + H15.

3.6. Effects of Hesperidin and Captopril on Protein Expression of TNF-R1 and TGF-β1 in Heart Tissues and Concentrations of TNF-α and TGF-β1 in Plasma

Over-expressions of TNF-R1 and TGF-β1 proteins were found in heart tissues collected from the hypertensive group compared to the control group ($p < 0.001$). Interestingly, supplementation with hesperidin and captopril partially reversed these protein up-regulations ($p < 0.01$; Figure 5A,B). These results were consistent with the results in that high levels of plasma TNF-α and TGF-β1 were observed in L-NAME hypertensive rats compared to those of control rats (168.49 ± 13.05 vs. 24.21 ± 8.51 pg/mL and 23.54 ± 3.91 vs. 4.90 ± 0.50 ng/mL, $p < 0.01$). The administration of hesperidin or captopril attenuated these high levels of plasma TNF-α (58.23 ± 14.71 or 20.97 ± 6.97 pg/mL) and TGF-β1 (5.23 ± 0.32 or 4.79 ± 0.55 ng/mL, $p < 0.05$) in hypertensive rats (Figure 5C,D).

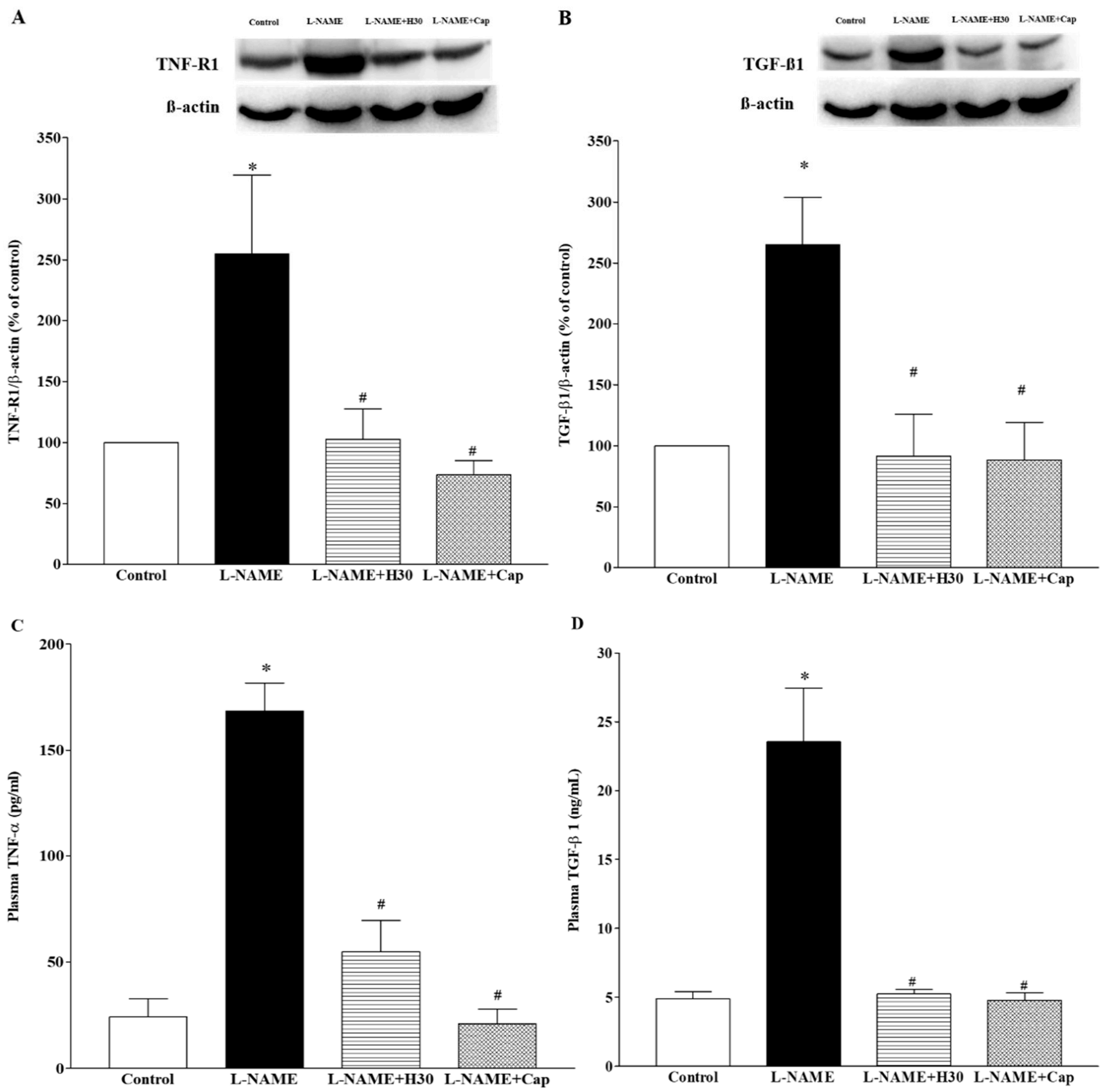

Figure 5. Effects of hesperidin and captopril on protein expression of TNF-R1, (**A**) and TGF-β1, (**B**) in heart tissue and on concentrations of plasma TNF-α, (**C**) and TGF-β1, (**D**) collected from control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. The top panel shows the representative bands of TNF-R1, (**A**) and TGF-β1, (**B**) protein expression in heart tissues. Values are mean ± S.E.M ($n = 4$ for each group), * $p < 0.05$ vs. control, # $p < 0.05$ vs. L-NAME group.

3.7. Effects of Hesperidin and Captopril on Protein Expression of MMP-2 and MMP-9 in Aortic Tissue

A significant increase in MMP-2 and MMP-9 protein expression was observed in thoracic aortic tissues collected from the hypertensive group compared to the control group (Figure 6A,B, $p < 0.05$). Hesperidin or captopril treatment significantly suppressed the level of MMP-2 and MMP-9 protein expression compared to untreated rats, ($p < 0.05$).

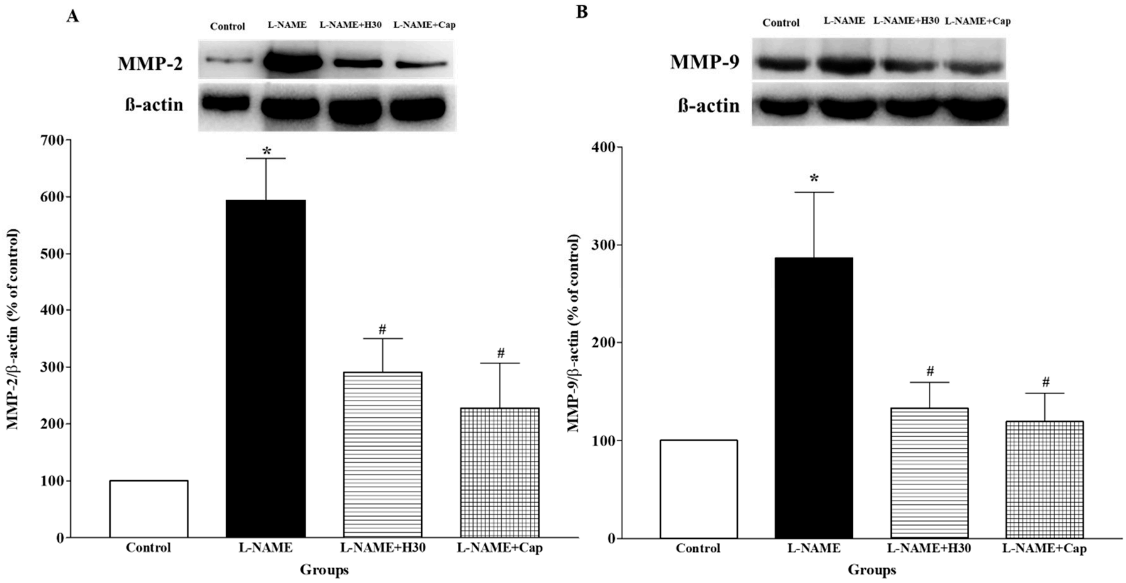

Figure 6. Effects of hesperidin and captopril on protein expression of MMP-2, (**A**) and MMP-9, (**B**) in aortic tissue collected from control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. The top panels show the representative bands of MMP-2, (**A**) and MMP-9, (**B**) protein expression in thoracic aortas. Values are mean ± S.E.M (n = 4 for each group), * $p < 0.05$ vs. control, # $p < 0.05$ vs. L-NAME group.

4. Discussion

This study demonstrates that rats that received L-NAME developed hypertension and cardiovascular remodeling. Hesperidin mitigated high blood pressure and cardiac remodeling by reducing the left ventricular hypertrophy and fibrosis associated with down-regulations of TGF-β1 and TNF-R1 protein expression and a reduction of plasma TGF-β1 levels in L-NAME-induced hypertension in rats. Vascular remodeling, including vascular hypertrophy and increased collagen deposition, induced by L-NAME in rats was inhibited by hesperidin supplementation. This was consistent with the decreased protein expression of MMP-2 and MMP-9 in aortic tissue. Furthermore, hesperidin preventing cardiovascular remodeling induced by L-NAME in the present study was linked to the reduction of an inflammatory cytokine, oxidative stress markers, and enhanced NO availability.

It was found that chronic treatment of L-NAME led to the development of NO-deficient hypertension as well as cardiovascular remodeling. These remodelings included increases in LVW/HW ratio, LV wall thickness, LV CSA, LV fibrosis, aortic wall thickness, aortic cross-sectional areas, aortic smooth muscle cell numbers, and collagen deposition. It is well-accepted that the chronic inhibition of NO synthase using L-NAME results in NO depletion, increased vascular tone, and high blood pressure [34]. Several studies have demonstrated that cardiovascular remodeling occurs after chronic treatment with L-NAME (40 mg/kg) for five weeks [4,10,35]. The mechanisms involved in cardiac remodeling in an animal model of nitric oxide-deficient hypertension are still unclear; however, two possible mechanisms related to hemodynamics and non-hemodynamic aspects have been described [36]. Hemodynamic overload in hypertension provoked left ventricular hypertrophy because of the adaptive response to conserve cardiac output [37]. A reduction in NO is one of several non-hemodynamic factors that participate in cardiac remodeling because when NO is suppressed, hypertensive cardiac remodeling through the cyclic guanosine monophosphate/protein kinase G (cGMP/PKG) pathway is initiated to inhibit fibrotic synthesis [38]. It is well-documented that vascular remodeling in hypertension occurs in response to long-term modifications of hemodynamic conditions [39,40]. Furthermore, numerous studies have reported that vascular remodeling is characterized by increases in wall thickness, CSA, and smooth muscle cell numbers in L-NAME hypertensive rats [3,4,41]. In this study, hesperidin partially inhibited the development of hypertension as well as cardiovascular remodeling induced by chronic L-NAME treatment. These effects may have involved an increase in NO bioavailability, reductions of oxidative stress, and inflammation as further possibilities.

Oxidative stress is one of the important mechanisms of L-NAME-induced hypertension since L-arginine analogues activate eNOS uncoupling, leading to an overwhelming vascular superoxide generation [42]. Then, superoxide can rapidly react with nitric oxide to form peroxynitrite [43]. This reaction results in reducing nitric oxide bioavailability [44]. In the present study, increases in plasma MDA levels and vascular superoxide production were accompanied by decreased plasma NOx levels observed in the L-NAME hypertensive rats. Hesperidin alleviated L-NAME-induced oxidative stress and thus increased NO bioavailability with an increase in the plasma NOx level. Many studies have confirmed that hesperidin has a strong antioxidant activity [26,45]. Hesperidin exhibits its antioxidant properties with two main mechanisms, including directly scavenging reactive oxygen species [46], and boosting cellular antioxidant defense [25]. Thus, this is one of the possible mechanisms of the cardiovascular protective effects of hesperidin in this study that might have involved its antioxidant capability, resulting in increased NO bioavailability, which reduced vascular resistance.

There is substantial evidence to suggest that inflammation is one of pathologies that occurs in L-NAME hypertensive rats [47,48]. The results of this study proved that, as in the previous studies, there were increases in the levels of pro-inflammatory cytokine, TNF-α, in plasma and expression of TNF-α protein in the heart tissue of L-NAME hypertensive rats. Myocardial TGF-β protein expression was also observed in L-NAME hypertensive rats. It is well-established that TGF-β plays an important role in responses to inflammation to activate fibrogenesis, which is an important pathological process for cardiac remodeling [49,50]. The present study has also shown that hesperidin attenuated cardiac remodeling, accompanied by decreased systemic and heart inflammation in L-NAME hypertensive rats. The protein expression of TGF-β in cardiac tissue was also down-regulated in the hesperidin supplemented group. The anti-inflammatory effect of hesperidin has been clearly revealed in both cellular and animal models. In human umbilical vein endothelial cells, hesperidin significantly suppressed TNF-α [51]. Li and coworkers demonstrated that hesperidin decreased the production of IL-1β, IL-6, and TNF-α in a rat model of rheumatoid arthritis [52]. Thus, the current results confirmed that the cardiprotective effect of hesperidin was associated with its great anti-inflammatory effect.

Additionally, vascular remodeling with collagen deposition was associated with the overexpression of MMP-2 and MMP-9 in aortic tissue in L-NAME hypertensive rats, as shown in this study. Several studies report that MMPs play an important role in physiological processes that contribute to hypertension-induced maladaptive arterial changes and sustained hypertension [53,54]. The overexpression of MMP-mediated vascular remodeling was stimulated by oxidative stress and inflammatory cytokines [54]. Del Mauro and coworkers demonstrated that MMP-2 and MMP-9 activity was a pathologic process in L-NAME-induced morphometric alterations in the aorta [55]. Interestingly, the authors of the present study first reported L-NAME-induced hypertension and vascular remodeling in rats in which there was an up-regulation of MMP-2 and MMP-9 protein expression in response to oxidative stress. Hesperidin prevented vascular remodeling induced by L-NAME associated with the down-regulation of MMP-2 and MMP-9. This effect might be involved in its antioxidant and anti-inflammatory effects, which further inhibited MMP activation and collagen degradation.

Captopril was used as a positive control to prevent the development of hypertension and cardiovascular remodeling. These findings are supported by previous studies that found that captopril prevented high blood pressure, left ventricular hypertrophy, and vascular remodeling induced by L-NAME in rats [56,57]. Captopril also reduced oxidative stress and inflammatory markers and suppressed protein expressions of TNF-R1, TGF-β1, and MMPs. An antioxidative effect of captopril in the present study might be associated with two main mechanisms, direct and indirect effects. Captopril contains free sulfhydryl groups that directly scavenge oxygen free radicals [58], or it suppresses AT1R-mediated NADPH oxidase expression and superoxide production [10]. It has been demonstrated that captopril improved ventricular hypertrophy in rats by suppressing MMP-2 and MMP-9 expression [59]. In addition, an anti-inflammatory effect of captopril in the animal model of hypertension has been reported [60].

In conclusion, the findings of this study indicated that hesperidin had cardiovascular protective effects by preventing the L-NAME-induced development of hypertension and cardiovascular remodeling in rats. These effects were affirmed by reducing oxidative stress and inflammation.

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Nobiletin, a citrus flavonoid, exerts a wide range of biological activities. This study investigated the effect of nobiletin on vascular dysfunction and remodeling in L-NAME-induced hypertensive rats. Male Sprague-Dawley rats were given L-NAME (40 mg/kg) for five weeks to induce hypertension and treated with nobiletin (20 or 40 mg/kg) or captopril (5 mg/kg) for the last two weeks. Nobiletin or captopril significantly reduced blood pressure and the enhancement of the contractile response to sympathetic nerve stimulation in the mesenteric vascular beds of L-NAME rats ($p<0.05$). Both agents improved the impairment of vasorelaxation responses to acetylcholine in mesenteric vascular beds and aortic rings in L-NAME rats ($p<0.05$). Moreover, nobiletin and captopril decreased oxidative stress markers, restored the abnormality of plasma NOx and protein expression of eNOS, Nrf-2 and HO-1 observed in L-NAME rats ($p<0.05$). Increases in aortic wall thickness, cross sectional area, vascular smooth muscle cells and collagen deposition that occurred in L-NAME rats were reduced by nobiletin or captopril ($p<0.05$). These reductions were associated with suppression of matrix metalloproteinase (MMP)-2 and MMP-9 protein expression ($p<0.05$). These findings indicated that nobiletin had antihypertensive effects with amelioration of vascular alterations. The molecular mechanism is likely to involve the restoration of Nrf-2/HO-1/MMPs signaling pathways.

1. Introduction

Flavonoid is found in vegetables and fruits and is reported to reduce risks of hypertension by its beneficial effects such as vasorelaxation, anti-inflammation and antioxidation.[1,2] Nobiletin, a polymethoxylated flavone, is a unique flavonoid exclusively found in citrus peels which possess extensive bioactivities.[3,4] A growing body of evidence suggests that nobiletin has strong anti-inflammatory and antioxidant effects. For example, 4′-demethylnobiletin, a major metabolite of nobiletin, significantly reduced expression of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and inducible nitric oxide synthase (iNOS) in lipopolysaccharide-treated RAW 264.7 macrophages. It also activated antioxidant transcription factor nuclear factor erythroid 2–related factor 2 (Nrf-2) and its dependent genes, heme oxygenase-1 (HO-1), and NAD(P)H dehydrogenase (quinone 1) (NQO1).[5] Furthermore, it has been reported that nobiletin prevented cardiac hypertrophy via inhibition of nicotinamide adenine dinucleotide phosphate (NAPDH) oxidases and alleviated endoplasmic reticulum stress in mice.[6] Supplementation of nobiletin ameliorated cardiac dysfunction, oxidative stress and inflammation in streptozotocin-induced diabetic cardiomyopathy mice.[7] Ikemura and co-workers demonstrated the protective effect of nobiletin on hypertension and thrombogenicity in cerebral vessels of stroke-prone spontaneously hypertensive rats. They suggested the mechanisms underlying these beneficial effects of nobiletin are mediated by strong antioxidant properties and, therefore increasing NO bioavailability.[8] Additional studies indicated that nobiletin reduced dyslipidemia, increased insulin sensitivity and attenuated atherosclerosis in mice with diet-induced insulin resistance.[9]

N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) inhibits nitric oxide (NO) synthesis by competitive binding with L-arginine to the NO synthase.[10] It is generally accepted that NO is a potent vasodilator and plays an important role in vascular tone regulation.[11] Chronic inhibition of NO production by L-NAME promotes persistent peripheral vasoconstriction and contributes to hypertension.[12] Previous studies indicated that L-NAME induced high blood pressure which contributed to endothelial dysfunction and vascular remodeling.[13,14] L-NAME hypertensive rats had an impairment of endothelium-dependent vasorelaxation in both conduit and resistance vessels.[15,16] Moreover, sympathetic overactivity was found in L-NAME treated rats since NO depletion enhanced endogenous noradrenaline release from postganglionic adrenergic sites.[17,18] This vascular dysfunction was supported

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by the reduction of eNOS protein expression in vessels and systemic nitric oxide metabolites (NOx) levels in L-NAME hypertensive rats.[19,20] Subsequently, it was shown that matrix metalloproteinases (MMPs) are involved in vascular remodeling processes since MMP-2 and MMP-9 are mainly expressed in arteries in animal models of hypertension.[21,22] Recently, Lee and Griendling provided evidence to support that the alterations of vascular morphology and function are mediated by overproduction of reactive oxygen species (ROS) in vasculature.[23]

Oxidative stress has been addressed to play an important role in pathogenesis of L-NAME induced hypertension in rats[24] because increases in tissue and systemic oxidative stress markers were observed in this animal model.[25] In fact, oxidative stress can reduce NO bioavailability as superoxide ($O_2^{\bullet -}$) rapidly binds to NO to form peroxynitrite ($ONOO^-$).[26] In addition, an imbalance between ROS production and the endogenous antioxidant mechanism in rats treated with L-NAME has been revealed.[27,28] These conditions may involve the suppression of the Nrf-2 signaling pathway. Nrf-2 is a cytoprotective transcription factor which exists to protect cells under stress conditions by influencing the transcription of antioxidative genes including HO-1.[29,30] Recently, Omobowale and coworkers reported that there was a reduction of Nrf-2 expression in L-NAME-induced hypertensive rats.[31]

Captopril is a standard anti-hypertensive drug with its angiotensin converting enzyme (ACE) inhibitor effect.[32] It also has a vasodilator property in hypertensive patients.[33] Moreover, other possible mechanisms, the increase of NO generation and decrease of reactive oxygen species, which may respond for beneficial effects of an ACE inhibitor in addition to direct reduction of angiotensin II and elevation of bradykinin production have been clearly demonstrated in animal models of hypertension.[34,35] For example, captopril decreases blood pressure associated with improving vascular function and structure and reducing oxidative stress in spontaneously hypertensive rats.[36] In L-NAME hypertensive rats, captopril has cardioprotective effects resulting from its antioxidant effect and enhancing NO bioavailbility.[37] There is, however, no evidence regarding the effect of nobiletin on high blood pressure, vascular alterations in NO-deficient rats. This study aimed to investigate the effects of nobiletin on blood pressure, vascular dysfunction and vascular remodeling as well as the underlying mechanisms involved in L-NAME-induced hypertensive rats.

2. Materials and methods

2.1. Drugs

Nobiletin (99%) was purchased from INDOFINE Chemical Company, Inc. (NJ, USA). L-NAME and captopril were purchased from Sigma-Aldrich Corp (St Louis, MO, USA). All other chemicals used in this study were obtained from standard companies and were of analytical grade quality.

2.2. Animals and Experimental Protocols

Male Sprague-Dawley rats weighing 220-250 g were purchased from Nomura Siam International Co, Ltd., Bangkok, Thailand. Rats were housed in the HVAC (Heating, Ventilation and Air-Conditioning) System (25±2°C) with a 12 h dark-light cycle at Northeast Laboratory Animal Center. All procedures were complied with the standards for the care and use of experimental animals and approved by Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (ACUC-KKU-29/60). After seven days of adaptation, the animals were divided into a control group that received drinking water and an L-NAME treated group that received L-NAME (40 mg/kg) in drinking water for five weeks to induce hypertension. Hypertensive rats were subdivided into 4 groups and they were intragastrically administered propylene glycol (vehicle) or nobiletin (20 or 40 mg/kg) or captopril (5 mg/kg) for the last two weeks (n=8/each group).

2.3. Indirect measurement of blood pressure in conscious rats

Systolic blood pressure (SP) was measured in conscious rats by the tail-cuff plethysmography (IITC/Life Science Instrument model 229 and model 179 amplifier; Woodland Hills, CA, USA) method for recording SP weekly throughout five weeks of the experimental period.

2.4. Direct measurement of blood pressure in unconscious rats

At the end of the experimental period, rats were anesthetized with pentobarbital sodium (60 mg/kg, ip.). The left femoral artery was identified, cleaned of connective tissue and cannulated by a polyethylene tube. Baseline values of SP, diastolic blood pressure (DP), mean arterial pressure (MAP), and heart rate (HR) were continuously monitored for 20 min and recorded using Acknowledge Data Acquisition software (Biopac Systems Inc., Santa Barbara, CA, USA).

2.5. Vascular Function Study

2.5.1. Experimental protocols in isolated mesenteric vascular beds

The animals were killed by exsanguinations after hemodynamic assessment. The superior mesenteric artery was cannulated the hypodermic needle and gently separated. The isolated mesenteric vascular beds were placed on a stainless-steel grid (7x5 cm) in a humid chamber. The preparations were perfused with physiological Krebs' solution at a constant flow rate of 5 ml/min, using a peristaltic pump (07534-04, Cole-Palmer Instrument, Illinois, USA). Kreb's solution was composed of the following (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.8, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 1.25 and glucose 11.1.[38] The solution was maintained at 37°C and continually gassed with a 95% O_2 and 5% CO_2 gas mixture. The mesenteric vascular beds were pretreated with capsaicin (0.1 µM) for 20 min followed by 30 min washout period to facilitate a desensitization of vanilloid receptors and to cause a diminution of sensory neurotransmitters.[39] After the washout period, electrical field stimulation (EFS) (5-40 Hz, 90 V, 1 ms,

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for 30 s at 5-min intervals) was performed with a Grass SD9 stimulator (Grass SD9 B Square Pulse Stimulator, Rhode Island, USA). This stimulator passes a current between the needle and the wire grid on which the preparation placed. The preparation was allowed to achieve a stable baseline, then the mesenteric vascular beds were evaluated for contractile responses to exogenous noradrenaline (NA) (0.15-15 nmol). The preparations were allowed to equilibrate for 30 min before the next trial. To determine vasoactive performance of small resistance arteries, methoxamine (5-7 µM) was added to the Kreb's solution to raise tone (70-90 mmHg above baseline). Subsequently, different doses of vasoactive agents, acetylcholine, endothelium-dependent vasorelaxation, (ACh, 1 nM-0.01 µM) and sodium nitroprusside, a nitric oxide donor, (SNP, 1 nM-0.01 µM) were injected. Contractile responses and relaxation responses to vasoactive agents of the preparations were detected as changes in mean perfusion pressure (mmHg) using a pressure transducer and recorded via the BIOPAC System (BIOPAC Systems Inc., California, USA).

2.5.2. Experimental protocols in isolated aortic rings

To assess vasoactive performance of the conduit arteries, the thoracic aorta was rapidly isolated, carefully cleaned of adhering connective tissues and cut into rings 2-3 mm in length for tension measurement. The rings were suspended in baths containing Krebs' solution at 37°C and gassed with a 95% O_2 and 5% CO_2 gas mixture. Isometric contractions were recorded with a resting tension of 1g using a transducer connected to a 4-channel bridge amplifier and a PowerLab A/D converter and a PC running Chart v5 (PowerLab System, ADInstruments, Australia). Vascular responses to ACh (0.01-3 µM) and SNP (0.01-3 µM) were assessed under raised tone conditions with phenylephrine, α1-adrenoceptor agonist, (10 µM) and relaxation expressed as percent of the phenylephrine-induced contraction.

2.6. Measurement of vascular $O_2^{\bullet-}$ production and plasma malondialdehyde (MDA)

$O_2^{\bullet-}$ production in carotid arteries was determined by lucigenin-enhanced chemiluminescence as previously described[40] with some modifications.[41] Both sides of carotid artery were rapidly removed and placed in ice-cold saline and clean off connective tissues. The vessel was cut into 1 cm in length and incubated with 1 mL oxygenated Krebs-KCl solution at pH 7.4, 37 °C for 30 minutes. Thereafter, lucigenin 100 mM was added in sample tube and placed in a luminometer (Turner Biosystems, CA, USA). Luminometer counts were integrated every 30 second for 5 minutes and averaged. Vascular tissue $O_2^{\bullet-}$ production was expressed as relative light unit counts per minute per dried weight of vascular tissues.

Blood samples were collected from the abdominal aorta in an EDTA tube and placed on ice for plasma MDA measurement. The concentration of plasma MDA was measured as thiobarbituric acid reactive substances by a spectrophotometric method as previously described.[19] The absorbance of the supernatant was measured at 532 nm by a spectrophotometer.

2.7. Assay of plasma nitric oxide metabolites (nitrate/nitrite)

Accumulation of nitrate/nitrite, the end products of NO metabolism was used as index of NOS activity by using Griess reagents, and the resulting reaction product, an azoic compound, was measured spectrophotometrically at 540 nm. Plasma samples were deprotenized by ultrafiltration using centrifugal concentrations (NANOSEPTM, PI Filtration, USA). The supernatant was mixed with 1.2 µM NADPH, 4 mM G-6-P, 1.28 unit/ml G-6-PD and 0.8 unit nitrate reductase, and then incubated at 30°C for 30 minutes. After that, the mixer was reacted with a Griess solution of 4% sulfanilamide in 0.3% N-naphthyl-ethylenediamine for 15 min. The absorbance of samples was measured on a plate reader with a filter wavelength of 540 nm (Tecan GmbH., Groding Australia). A standard curve was established with a set of serial dilution of $NaNO_2$.[41]

2.8. Histology and morphometry

The thoracic aorta was fixed for 24 h in 4% paraformaldehyde then embedded in paraffin and cut into serial 5-µm thick sections. Sections were stained with hematoxylin and eosin (Bio-Optica Milano SpA., Milano, Italy) and by picro-sirius red (Polysciences, Warrington, PA, USA). Images were obtained under DS-2Mv light microscope and Eclipse LV100 POL polarized light microscope (Nikon, Tokyo, Japan). Morphometric evaluations were analyzed with the ImageJ morphometric software (National Institutes of Health, Bethesda, MD, USA). Aortic collagen deposition was expressed as a percentage of positively stained area to medial area.

2.9. Western blot analysis

The aortic tissues were homogenized and the proteins were electrophoresed on a sodium dodecylsulfate polyacrylamide gel electrophoresis system. Thereafter, the proteins were electrotransfered onto a polyvinylidenedifluoride membrane and blocked with 5 % skimmed milk in Tris-buffered saline with 0.1 % Tween 20 (TBST) for 2 h at room temperature followed by incubation overnight at 4 °C with mouse monoclonal antibodies to eNOS (BD Biosciences, CA, USA), mouse monoclonal antibodies to Nrf-2, HO-1, MMP-2 and MMP-9 and goat polyclonal IgG to β-actin (Santa Cruz Biotechnology, Indian Gulch, CA, USA). After the incubation period, the membranes were washed with TBST and then incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibody. The blots were developed in Luminata™ Forte Western HRP Substrate (Millipore Corp., Billerica, MA, USA), and densitometric analysis was performed using an Amersham Imager 600 (GE Healthcare Life Sciences, Uppsala, Sweden). The intensity of bands was normalized to that of β-actin, and data were expressed as a percentage of the values determined in control group from the same gel.

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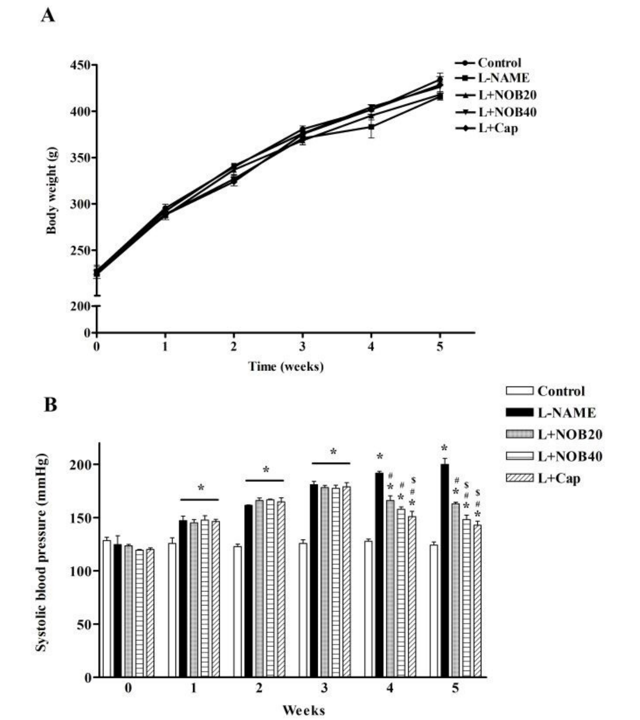

Fig.1 Effects of nobiletin on body weight **(A)** and systolic blood pressure **(B)** in conscious rats. Data are expressed as mean ± SEM (n=8/group), *p<0.05 vs. control, #p<0.05 vs. L-NAME, $p<0.05 vs. L+NOB20, L+NOB20, L-NAME + nobiletin 20 mg/kg; L+NOB40, L-NAME + nobiletin 40 mg/kg; L+Cap, L-NAME + captopril 5 mg/kg

2.10. Statistical analysis

Data were expressed as mean ± S.E.M. The differences among treatment groups were analyzed by one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of nobiletin on blood pressure in L-NAME-induced hypertensive rats

3.1.1. Effects of nobiletin on blood pressure in conscious rats

There are no significant differences of body weight in all groups of rats as shown in figure 1A. At baseline, SP was not significant different among experimental groups. Daily administration of L-NAME for five weeks caused significant increase in SP (200.00 ± 5.66 mmHg) compared to the control group (124.29 ± 2.68 mmHg) (p<0.05). Treatment with nobiletin at 20 or 40 mg/kg significantly reduced SP in a dose-response dependent manner (163.00 ± 1.59 and 148.20 ± 4.22 mmHg, p<0.05) compared to untreated group (p<0.05). Thus, this present study used nobiletin at dose 40 mg/kg as an effective dose. Moreover, captopril significantly reduced SP 142.95 ± 3.57 mmHg in L-NAME rats compared to L-NAME-untreated rats (p<0.05, Fig. 1B).

3.1.2. Effects of nobiletin on blood pressure and heart rate in anesthetized rats

The value of blood pressure obtained for direct method was consistent with those of indirect method since after 5 weeks of experimental period, SP, DP, MAP and HR in L-NAME group were significantly increased compared to those of control group (p<0.05). Nobiletin significantly decreased SP, DP, MAP and HR in L-NAME rats comparing to L-NAME-untreated rats (p<0.05). Moreover, captopril significantly reduced hypertension induced by L-NAME as there were reductions of SP, DP and MAP in L-NAME treated rats that received captopril (p<0.05, Table 1).

3.2. Effects of nobiletin on vascular function in L-NAME-induced hypertensive rats

3.2.1. Effects of nobiletin on contractile responses to electrical field stimulation (EFS) and exogenous NA in mesenteric vascular beds

To investigate the role of sympathetic nerve activity, EFS was performed to produce an increase in perfusion pressure that was frequency-dependent vasoconstriction in all preparations. The contractile responses evoked by sympathetic nerve stimulation was larger in the mesenteric vascular bed isolated from L-NAME hypertensive rats than those found in control rats (at 40 Hz, 76.24 ± 1.67 vs. 44.72 ± 7.95 mmHg, p<0.05). Contractile response to EFS in L-NAME hypertensive rats-treated with nobiletin at dose 40 mg/kg and rats treated with captopril were markedly reduced (at 40 Hz, 44.97 ± 4.46 and 43.66 ± 5.02 mmHg, respectively, p<0.05) compared to the response from L-NAME hypertensive rats (Fig. 2A). The contractile response to exogenous NA (0.15-15 nmol), however, was not different among groups (Fig. 2B).

3.2.2. Effects of nobiletin on vascular responses to vasoactive agents in perfused mesenteric vascular beds of the rats

Vasorelaxation responses to ACh and SNP was expressed as a reduction of perfusion pressure. Vasorelaxation response to

Table 1 Effects of nobiletin on blood pressure and heart rate in anesthetized rats

Parameters	Control	L-NAME	L+NOB20	L+NOB40	L+Cap
SP (mmHg)	119.76± 3.10	185.81±5.77 *	152.09±4.62 $^{*\#}$	137.68± 3.90$^{*\#\$}$	134.40± 2.81 $^{*\#\$}$
DP (mmHg)	74.47± 3.81	132.46±3.92 *	109.40± 2.63 $^{*\#}$	101.06± 4.46 $^{*\#}$	95.42± 3.63$^{*\#\$}$
MAP (mmHg)	89.56 ± 3.06	150.24 ±3. 11 *	123.63±2.84 $^{*\#}$	113.26± 4.06 $^{*\#\$}$	108.41± 3.28 $^{*\#\$}$
HR (beat/min)	354.70± 11.88	417.57±10.58 *	384.08±8.81 $^{*\#}$	365.35± 5.29$^{\#}$	369.11± 8.17 $^{\#}$

SP: systolic blood pressure; DP: diastolic blood pressure; MAP: mean arterial pressure; HR: heart rate; NOB: nobiletin; Cap: captopril. Data are expressed as mean ± SEM (n=8/group). *p<0.05 vs. control; #p<0.05 vs. L-NAME; $p<0.05 vs. L+NOB20

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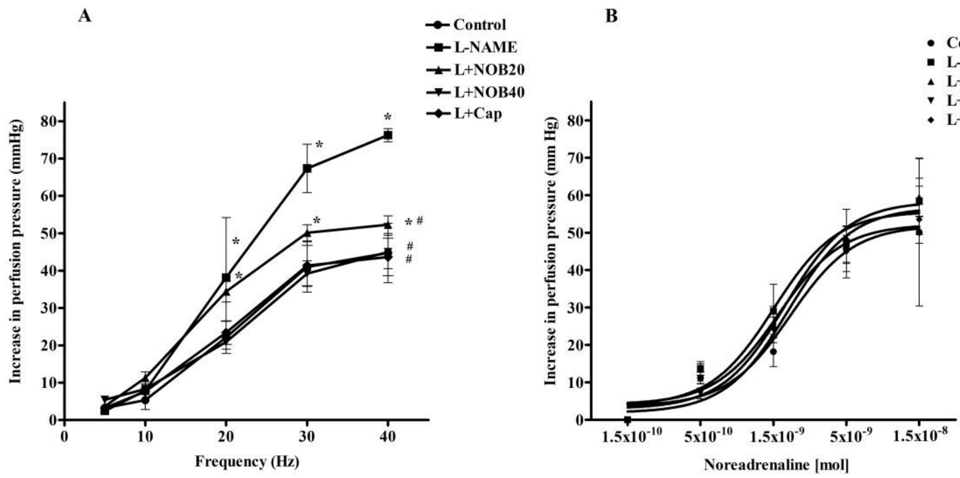

Fig.2 Effects of nobiletin on contractile responses to EFS (5-40 Hz, 90 V, 1 ms, for 30 s at 5-min intervals) **(A)** and exogenous NA **(B)** in mesenteric vascular beds. Data are expressed as mean ± SEM (n=5/group), *p<0.05 vs. control; #p<0.05 vs. L-NAME

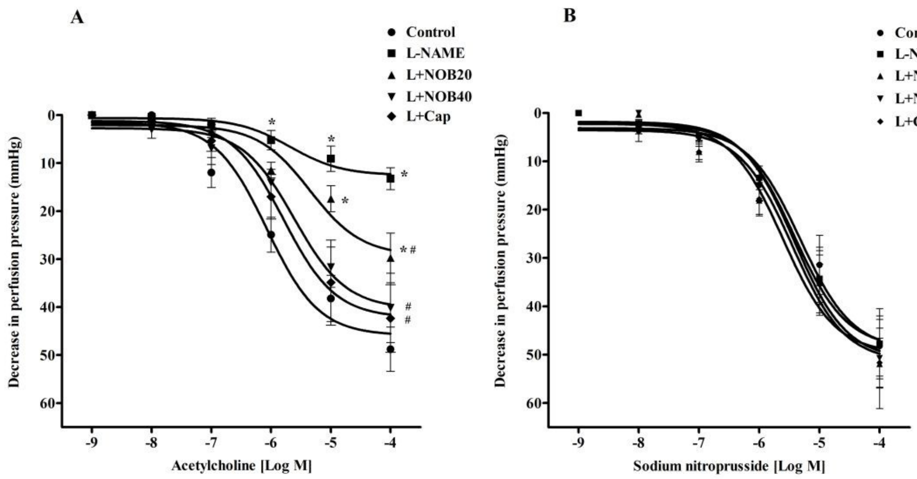

Fig.3 Effects of nobiletin on vascular responses to exogenous ACh **(A)** and SNP **(B)** in mesenteric vascular beds. Data are expressed as mean ± SEM (n=5/group), *p<0.05 vs. control; #p<0.05 vs. L-NAME.

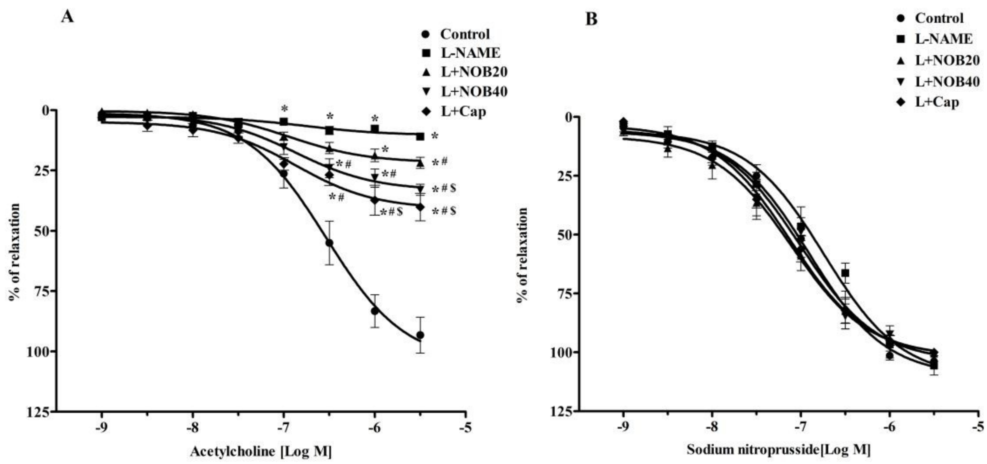

Fig.4 Effects of nobiletin on vascular function in thoracic aorta. Vasorelaxation responses to exogenous ACh **(A)** and SNP **(B)** in thoracic aorta. Data are expressed as mean ± SEM (n=5/group), *p<0.05 vs. control; #p<0.05 vs. L-NAME; $p<0.05 vs. L+NOB20.

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ACh (0.1 µM–0.1 mM) in the mesenteric vascular bed were significantly blunted in L-NAME group compared to control group (0.1 mM ACh, 13.23 ± 2.27 vs. 48.78 ± 4.62 mmHg) ($p<0.05$). Treatment with nobiletin at dose 40 mg/kg improved the response to ACh in L-NAME treated rats compared to untreated rats (0.1 mM ACh, 40.18 ± 7.22 mmHg) ($p<0.05$). Moreover, L-NAME rats treated with captopril significantly improved the response to ACh compared to L-NAME hypertensive rats (0.1 mM ACh, 42.37 ± 7.04 mmHg) ($p<0.05$; Fig. 3A). There were no significant differences in the vasorelaxation responses to SNP among groups, indicating normal vascular smooth muscle cell function (Fig. 3B).

3.2.3. Effects of nobiletin on vascular responses to vasoactive agents in the thoracic aorta

Endothelium-dependent vasorelaxation responses to ACh (0.01–3 µM) were significantly blunted in aortic rings from L-NAME hypertensive rats compared to control rats (3 µM ACh, 10.96 ± 0.74 vs. 93.23 ± 7.41% of relaxation) ($p<0.01$). Nobiletin at a dose of 40 mg/kg improved vascular response to ACh compared to untreated group (3 µM ACh, 33.04 ± 2.26% of relaxation; $p<0.05$). Moreover, rats treated with captopril had significant improvement of the response to ACh compared to L-NAME hypertensive rats (3 µM ACh, 40.17 ± 5.70% of relaxation; $p<0.05$) (Fig. 4A). In addition, the vasorelaxation response to SNP, a NO donor, did not differ significantly among groups (Fig. 4B).

3.3. Effects of nobiletin on vascular $O_2^{\bullet -}$ production and plasma MDA in L-NAME-induced hypertensive rats

There were a significant increase in vascular $O_2^{\bullet -}$ production (167.63 ± 29.31 vs. 43.62 ± 6.57 count/mg dry wt/min, $p<0.01$) and plasma MDA (8.96 ± 0.80 vs. 2.85 ± 0.25 µM, $p<0.05$) in L-NAME hypertensive group compared to control group. The rise of $O_2^{\bullet -}$ production and plasma MDA were significantly reduced in L-NAME rats treated with nobiletin (20 and 40 mg/kg) or captopril compared to L-NAME untreated rats (91.16 ± 5.97, 52.31 ± 6.40 and 39.60 ± 8.17 count/mg dry wt/min and 5.19 ± 0.52, 4.83 ± 0.30 and 3.39 ± 0.43 µM, respectively, $p<0.05$) (Fig. 5A and B). This study, we measured MDA in plasma to indicate systemic oxidative stress, which might be fair representative for ROS source from vascular and other tissues.

3.4. Effects of nobiletin on plasma NOx and eNOS protein expression in L-NAME-induced hypertensive rats

Plasma NOx concentration was decreased in L-NAME-induced hypertensive rats compared to those of control rats (3.42 ± 0.57 vs. 9.86 ± 0.43 µM, $p<0.01$). The level of plasma NOx was significantly restored in L-NAME rats treated with nobiletin at 20 mg/kg, 6.12 ± 0.16 and at 40 mg/kg 6.96 ± 0.53 or captopril at 7.83 ± 0.75 µM, $p<0.05$, compared with untreated rats (Fig. 6A). This was consistent with the downregulation of eNOS protein expression in aortic tissue of L-NAME hypertensive

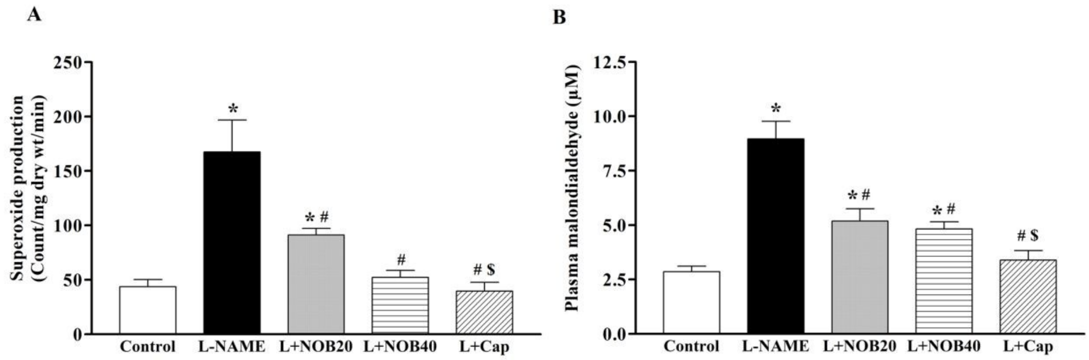

Fig. 5 Effects of nobiletin and captopril supplementation on vascular $O_2^{\bullet -}$ production **(A)** and plasma MDA **(B)** in all experimental groups. Data are expressed as mean ± SEM (n=7-8 /group), $^*p<0.05$ vs. control; #$p<0.05$ vs. L-NAME; \$$p<0.05$ vs. L+NOB20.

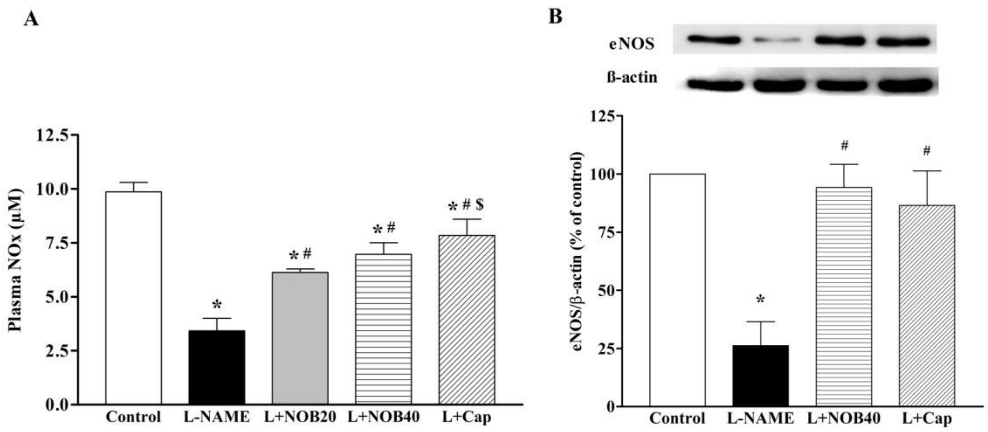

Fig. 6 Effects of nobiletin on plasma NOx **(A)** in all experimental groups and eNOS protein expression **(B)** of control, L-NAME, L-NAME + nobiletin (40 mg/kg) and L-NAME + captopril (5 mg/kg) groups. Data are expressed as mean ± SEM (n=7-8/group), $^*p<0.05$ vs. control; $^{\#}p<0.05$ vs. L-NAME; $^{\$}p<0.05$ vs. L+NOB20.

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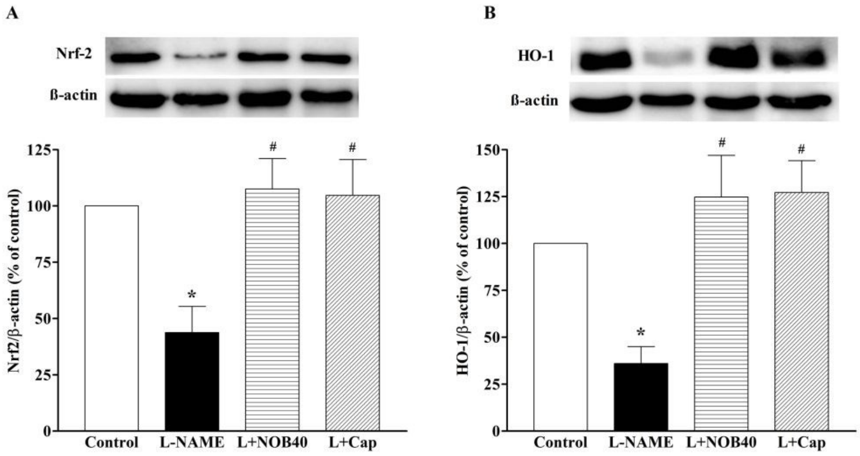

Fig.7 Effects of nobiletin and captopril on protein expression of Nrf-2 **(A)** and HO-1 **(B)** in aortic tissue of control, L-NAME, L-NAME + nobiletin (40 mg/kg) and L-NAME + captopril (5 mg/kg) groups. The top panel shows representative bands of Nrf-2 (A) and HO-1 (B) protein expression in thoracic aortas. Values are mean ± S.E.M (n=4/group), *p<0.05 vs. control; #p<0.05 vs. L-NAME

rats. Nobiletin or captopril treatment significantly restored eNOS protein expression compared with untreated group (Fig. 6B, $p<0.05$).

3.5. Effects of nobiletin on Nrf-2 and HO-1 protein expression in aortic tissue

L-NAME hypertensive group had low level of Nrf-2 and HO-1 protein expression compared with the control group (Fig. 7A and B, respectively, $p<0.05$). Nobiletin treatment significantly upregulated Nrf-2 and HO-1 protein expression compared with untreated rats ($p<0.05$).

3.6. Effects of nobiletin on vascular morphology and collagen deposition in L-NAME-induced hypertensive rats

Increases in vascular wall thickness, cross sectional area, wall/lumen ratio and vascular smooth muscle cell numbers were observed in thoracic aortas of L-NAME treated group compared with those of controls. Treatment with nobiletin significantly attenuated abnormalities of vascular morphology in L-NAME-induced hypertensive rats (Fig. 8A, C, D, F and G, $p<0.05$). There were no significant differences in luminal diameters among groups (Fig. 8E). In addition, collagen deposition was significantly increased in the L-NAME group and this was attenuated in nobiletin and captopril treated groups (Fig. 8B and H, $p<0.05$).

3.7. Effects of nobiletin on MMP-2 and MMP-9 protein expression in aortic tissue

Protein expressions of MMP-2 and MMP-9 were upregulated in hypertensive rats compared with the control group (Fig. 9A and B, $p<0.05$). Nobiletin and captopril treatment significantly decreased the level of MMP-2 and MMP-9 protein expressions compared with untreated rats ($p<0.05$).

4. Discussion

The present study demonstrated that rats treated with L-NAME for five weeks had high blood pressure, vascular dysfunction and vascular remodeling. Nobiletin improved endothelial-dependent vasorelaxation in both aortic rings and mesenteric vascular beds. The augmentation of nerve-mediated contractile responses in mesenteric vascular beds collected from L-NAME treated rats was found and this was diminished by nobiletin and captopril treatment. L-NAME-induced hypertensive rats showed increases in oxidative stress markers and a low level of NO metabolites, eNOS, Nrf-2 and HO-1 protein expression. Moreover, nobiletin and captopril alleviated L-NAME induced vascular morphology abnormalities and collagen deposition. These results were associated with increases in MMP-2 and MMP-9 protein expression.

Hypertension induced by L-NAME in rats in the present study was alleviated by nobiletin. This antihypertensive effect of nobiletin was possibly linked to improving vascular function. It is well established that blood pressure is determined by cardiac output and total peripheral resistance. Vascular diameter is partially regulated by adrenergic, cholinergic, nitrergic and sensory innervations.[42] EFS produces a vasoconstrictor response of mesenteric arteries by releasing NA from adrenergic nerves.[38,43] It has been reported that NO can modulate sympathetic activity by decreasing biological activity of NA released from sympathetic nerves.[44] This was supported by the study that L-NAME enhanced a nerve-mediated contractile response and NA overflow in mesenteric vascular beds.[45,46] In the present study, chronic inhibition of NO production augmented sympathetic vasoconstriction mediated by enhancement of prejunctional sites indicated by the fact that vasoconstrictive responses to exogenous NA did not differ among groups. NO deficiencies have been characterized by endothelial dysfunction and an increase in total peripheral resistance contributing to hypertension.[47] The present study is consistent with the previous study when the vasorelaxation responses to ACh in both aortic rings and mesenteric vascular beds were blunted in L-NAME hypertensive rats. In contrast, vascular responses to SNP, a NO

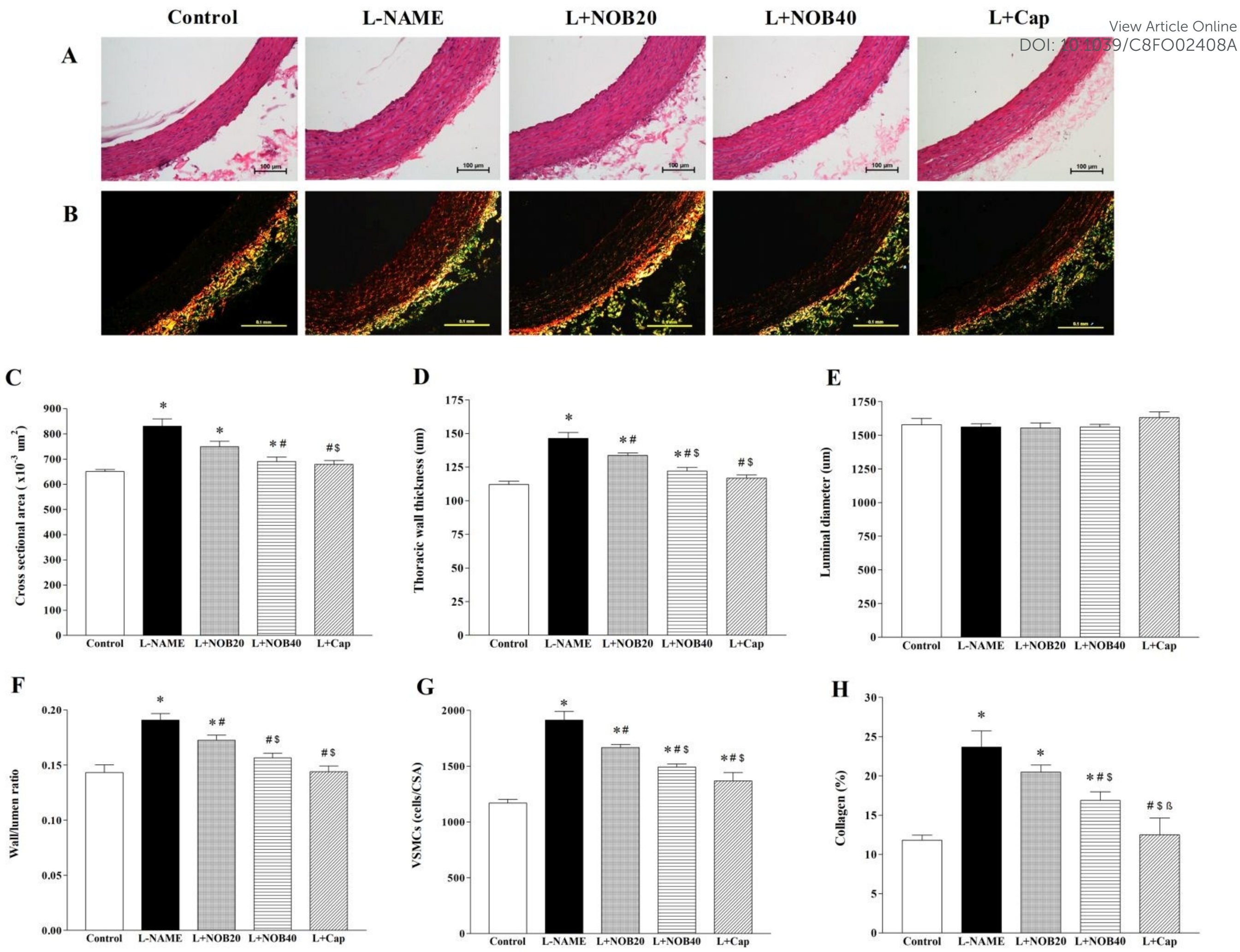

Fig.8 Effects of nobiletin on histology and morphology of thoracic aortas from each experimental group; Hematoxylin and eosin staining under light microscopy **(A)** and Picro-sirius red staining under polarized light microscopy **(B)**. Results of the quantitative analyses of vascular remodeling are represented by wall thicknesses **(C)**, cross sectional areas **(D)**, luminal diameters **(E)**, the ratios of the wall to the lumen **(F)** and vascular smooth muscle cell numbers **(G)**. Results of the quantitative analyses of collagen deposition are represented by the percentages of collagen **(H)**. Data are expressed as mean ± S.E.M, (n=8/group). *p<0.05 vs. control, #p<0.05 vs. L-NAME, $p<0.05 vs. L+NOB20, ßp<0.05 vs. L+NOB40.

donor, did not differ indicating normal smooth muscle cell functions. The impairment of endothelium-dependent vasorelaxation in L-NAME-induced hypertension is associated with oxidative stress and decreased eNOS expression and NO metabolite levels.[15,48,49] Nobiletin reduced the contractile responses to EFS as well as improved endothelium-dependent vasorelaxation in L-NAME hypertensive rats. Yang and coworkers (2016) demonstrated that nobiletin had an endothelium-dependent vasodilatory effect in rats.[50] The effect of nobiletin on vascular dysfunction in this study was associated with its antioxidant capacity since oxidative stress markers were decreased in the nobiletin treated group. This was consistent with the restoration of eNOS protein expression and plasma NOx after nobiletin treatment in L-NAME hypertensive rats.

One of the principal mechanisms in L-NAME-induced hypertension is oxidative stress. This study found the elevation of oxidative stress markers in hypertensive rats associated with downregulation of Nrf-2 and HO-1 protein expression in L-NAME-induced hypertensive rats. It is possible that L-NAME competitively bound at the L-arginine binding site led to eNOS uncoupling, which stimulated the production of $O_2^{\bullet -}$ in vasculature. $O_2^{\bullet -}$ then quickly reacted with NO to form $ONOO^-$ which can damage blood vessels extensively.[51,52] When excessive production of ROS is determined by $O_2^{\bullet -}$ production, MDA in L-NAME-induced hypertension in animals has been reported.[53,54] Moreover, impairment of endogenous antioxidant system in NO deficient rats has been introduced.[27] Nrf-2 is a crucial transcription factor that regulates various antioxidant genes. Under stress conditions, Nrf-2 is activated and translocated to the nucleus and subsequently bonded to antioxidant response elements. This signaling pathway causes transcription of many antioxidant genes especially HO-1. HO-1 degrades heme to biliverdin, ferrous iron, and carbon monoxide that mitigates oxidative stress induced cell injury.[55] It was found that Nrf-2 downregulation resulted in an imbalance between antioxidant capacity and oxidative stress and vascular dysfunction.[56] In L-NAME-induced hypertensive rats there was suppression of Nrf-2 expression.[31] In the present study, nobiletin normalized the Nrf-2/HO-1 cascade as

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well as oxidative stress markers, which raised NO bioavailability and then improved vascular function. The antioxidant capacity of nobiletin reducing oxidative stress and increasing antioxidant levels in various models of cardiovascular diseases has been supported by several studies.[6,57]

Vascular remodeling associated with upregulation of MMP-2/9 expression was observed in L-NAME hypertensive rats in the present study. There were two main factors that affected the vascular remodeling process.[58] The vascular morphology changes in response to long-term exposure to hemodynamic alterations has been revealed.[59] Besides the role of NO, a non-hemodynamic factor involved in vascular remodeling has been proposed since loss of NO bioavailability with increased oxidative stress caused by L-NAME led to remodeling of aortas.[20,60] NO is well accepted to have an antiproliferative effect.[61] This effect was confirmed by the study that NO showed inhibitory effect on VSMC proliferation, collagen levels and a production of extracellular matrix components.[62,63] The possible underlying induction mechanism of L-NAME hypertensive rats in the present study may have involved two main factors as mentioned above since there were both pressure overload and NO deficiency in this animal model. Furthermore, the vascular remodeling process in hypertension is involved in the upregulation of MMPs.[64] It is well established that excessive ROS and cytokines generation can modulate MMPs activity resulting in a synthesis or degradation of the matrix and collagen deposition.[65,66] Interestingly, there is evidence showing that Nrf2/HO-1 axis mediated the expression of MMP-2 and MMP-9.[67,68] The inhibitory effect of NO on MMP-induced fibrosis has been suggested.[69] This was associated with the role of NO that suppressed NADPH oxidase-mediated $O_2^{\bullet-}$ production via activation of HO-1.[70] Recently, Maneesai and coworkers reported an increase in protein expression of MMP-2 and MMP-9 associated with vascular remodeling in L-NAME hypertensive rats.[37] Results of this current study firstly showed the association of Nrf2/HO-1 axis and MMPs expression in L-NAME induced vascular remodeling. Subsequently, nobiletin alleviated aortic remodeling with downregulation of MMP-2 and MMP-9 protein expressions in aortic tissues. It is likely to be associated with increased Nrf-2/HO-1 expression and increased NO bioavailability observed in the present study.

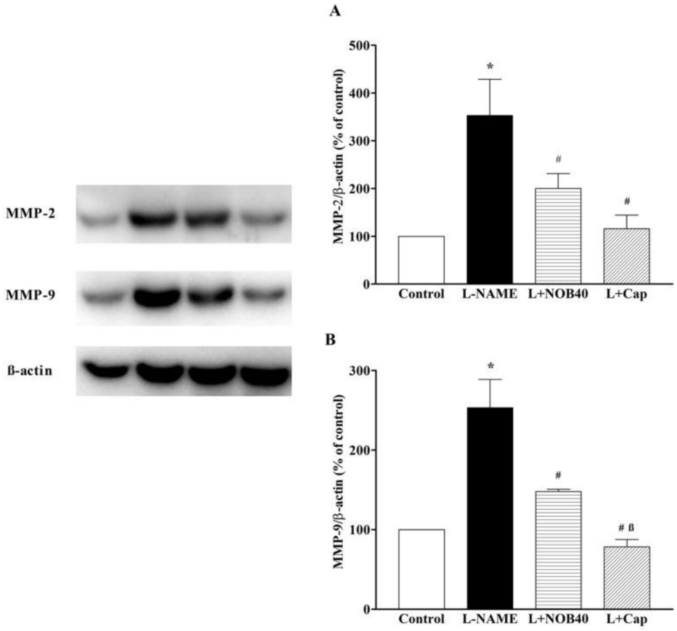

Fig. 9 Effects of nobiletin and captopril on protein expressions of MMP-2 **(A)** and MMP-9 **(B)** in aortic tissues of control, L-NAME, L-NAME + nobiletin (40 mg/kg) and L-NAME + captopril (5 mg/kg) groups. The panel shows representative bands of MMP-2 and MMP-9 protein expressions in thoracic aortas. Values are mean ± S.E.M (n=4/group), $^{*}p<0.05$ vs. control, $^{\#}p<0.05$ vs. L-NAME, $^{ß}p<0.05$ vs. L+NOB40.

Captopril was used as a positive control agent. The results showed that captopril is efficient in treatment of hypertension and has beneficial effects on vascular function and remodeling. These results are congruent with many studies in that in NO-deficient models, captopril decreased high blood pressure, improved endothelial-dependent vasorelaxation and vascular abnormalities associated with reducing oxidative stress and increasing NO.[49,71] Moreover, captopril was capable to increase the expression of Nrf-2 in L-NAME hypertension [31]. The beneficial effects of captopril on hypertension treatment are not only by ACE inhibition, it also has a potent antioxidant effect related to the thiol-containing molecule, which directly scavenges free radical molecules.[34] The effect of captopril (5 mg/kg) in comparison to nobiletin (40 mg/kg) on blood pressure is comparable in the present study. Moreover, nobiletin is as effective as captopril in suppression of oxidative stress and increased nitric oxide bioavailability. However, captopril was more effective on reducing collagen deposition and MMP-9 protein expression than nobiletin group. The long term efficacy in prevention of cardiovascular disease of nobiletin is yet to be studied. The dose of nobiletin used in this study followed a previous study.[8] These doses can be translated for appropriate doses in human using the formula for dose translation.[72] The doses in rats of 20 and 40 mg/kg were translated to human, being 3.24 or 6.48 mg/kg, respectively. Similarly, the dose of captopril (5mg/kg BW) used in rats was calculated based on the dose in human of 40 mg/day in human weighing 50 kg. which is consistent with the recommended dose in clinical practice in hypertensive patients (25-50 mg/day).[73]

5. Conclusion

In conclusion, the results of present study showed that nobiletin had an antihypertensive effect, alleviated vascular dysfunction and vascular remodeling and decreased oxidative stress in L-NAME-induced hypertensive rats. The possible molecular mechanisms were associated with the ability of nobiletin to up-regulate the Nrf-2/HO-1 axis and suppressed MMP expression in hypertensive rats.

Abbreviations

ACh, acetylcholine; DBP, diastolic blood pressure; EFS, electrical field stimulation; eNOS, endothelial nitric oxide synthase; HO-1, heme oxygenase-1; HR, heart rate; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MAP, mean arterial pressure; MDA, malondialdehyde; MMP, matrix metalloproteinase; NO, nitric oxide; Nrf-2, nuclear factor erythroid 2–related factor 2; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species; SBP, systolic blood pressure; SNP, sodium nitroprusside

Conflict of interest

There is no conflict of interest.

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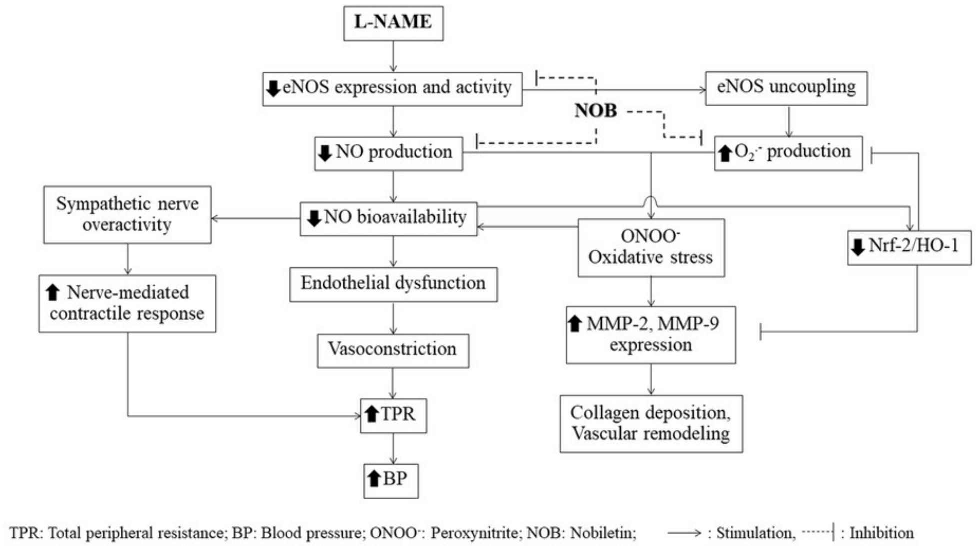

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Tangeretin mitigates L-NAME-induced ventricular dysfunction and remodeling through AT_1R/pERK1/2/pJNK signaling pathway in rats

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Tangeretin is a citrus flavonoid that exerts several beneficial effects, including anti-inflammation, anti-oxidation and neuroprotection. For this study, the aim was to test the effect of tangeretin on N^{ω}-Nitro-L-arginine methyl ester (L-NAME)-induced high blood pressure, left ventricular dysfunction and remodeling in rats. Rats were divided into five groups (n=8/each group): a control group, an L-NAME group and three L-NAME groups treated with tangeretin (15mg/kg) or tangeretin (30 mg/kg) or captopril (5 mg/kg) for the final two weeks. After five weeks of experiment, L-NAME groups had high systolic blood pressures, ventricular dysfunction and remodeling. Overexpression of angiotensin II type 1 receptor, phosphorylated-extracellular-regulated kinase 1/2 (pERK1/2), phosphorylated-c-Jun N-terminal kinases (pJNK) protein but downregulation of endothelial nitric oxide synthase (eNOS) protein expression in ventricular tissues were shown in hypertensive rats while protein expression of phosphorylated-mitogen activated protein kinase p38 did not differ among groups. Decrease in plasma NOx and increases in vascular superoxide generation, plasma malondialdehyde, angiotensin-converting enzyme activity and angiotensin II levels were found in hypertensive rats. These alterations were suppressed in hypertensive rats treated with tangeretin or captopril. In conclusion, tangeretin exhibits antihypertensive effects and alleviates ventricular dysfunction and remodeling in hypertensive rats. These effects are associated with inhibition of renin angiotensin system activation and restoration of pERK1/2, pJNK, eNOS protein expressions along with reducing oxidative stress and raising NO bioavailability

1. Introduction

Chronic administration of N^{ω}-Nitro-L-arginine methyl ester (L-NAME) causes nitric oxide (NO) deficiency resulting in hypertension in rats.[1, 2] Subsequently, L-NAME-induced deteriorations of cardiovascular morphology and function have been reported.[3-5] Previous studies found that left ventricular (LV) hypertrophy and fibrosis occurred in rats treated with L-NAME for 5 weeks.[6-8] In addition to pathological conditions of myocardial damage, ventricular pressure and volume overload that causes LV remodelling, the renin angiotensin system (RAS) has been proposed to involve the mechanism of L-NAME induced cardiac remodelling.[9] Gao and co-workers reported that administration of L-NAME increases angiotensin II (Ang II) levels and cardiac hypertrophy.[10] Recently, overexpression of Ang II Type I Receptor (AT_1R) has been proposed to be associated with L-NAME-induced LV wall hypertrophy and fibrosis in rats.[11] Once activated, AT_1R also transactivates serine/threonine kinases such as mitogen-activated protein kinases (MAPKs) to mediate cell growth and hypertrophy. Three MAPK families have been clearly described and called for their terminal components: extracellular signal-regulated kinases (ERK) 1/2, p38MAPK, and c-Jun N-terminal kinases (JNKs) that are implicated in cell growth, hypertrophy, and cell proliferation.[12] Activation of ERK1/2 is a key element to produce cardiac hypertrophy while activation of the p38MAPK cascades is predominantly involved in the inflammatory response and cardiac remodelling. Gómez-Guzmán and co-workers showed that the administration of L-NAME could produce pERK1/2 up-regulation and cardiac hypertrophy in rats.[13]

In an animal model of L-NAME-induced hypertension, the main consequence of ventricular remodelling is ventricular dysfunction, which is an important cause of heart failure. Under physiological conditions, NO is derived from endocardial endothelial cells, coronary microvascular and cardiac myocytes by eNOS to promote myocardial relaxation and diastolic properties.[14-16] Under pathological conditions, such as ischemia–reperfusion, LV hypertrophy, heart failure and myocarditis, NO is synthesized by eNOS to improve myocardial contractility.[17] That depletion of NO undoubtedly impairs cardiac function is supported by a previous study where cardiac dysfunction was observed in the NO-deficient model of hypertension.[18] In addition, Sheng and co-workers found that cardiac remodelling and dysfunction were associated with decreased eNOS phosphorylation, NOS activity, and NO quantity in L-NAME rats.[19] There is evidence that L-NAME induces eNOS uncoupling resulting in increased reactive oxygen species, especially superoxide, which in turn reduces NO bioavailability.[20] Increases in vascular superoxide production [21] and lipid peroxidation product[22, 23] in L-NAME-induced hypertension in rats have been documented.

Tangeretin is a polymethoxylated flavonoid; one of the most abundant compounds in citrus fruit peel.[24] Numerous pharmacological activities of tangeretin have been reported including, anti-neurodegeneration, anti-inflammation, and anti-oxidation.[25, 26] Tangeretin can reduce inflammation, oxidative stress, and modulate MAPKs and apoptotic pathways in cisplatin-induced acute hepatic injury.[27] It also decreases inflammatory markers and improves glucose uptake in adipocytes.[28] Eun and coworkers suggested that daily intake of

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tangeretin attenuates colitis in mice through suppression of interleukin 12 and tumor necrosis factor-α expression and nuclear factor kappa B activation.[29] Furthermore, tangeretin improves renal function and reduces the cognitive and memory impairments in chronic kidney disease related to its anti-oxidant and anti-inflammatory properties.[30] The effect of tangeretin on cardiac alterations induced by NO deficiency, however, is unknown.

Captopril inhibits angiotensin-converting enzyme (ACE) to convert angiotensin I to Ang II and is a common anti-hypertensive agent. It reduces blood pressure (BP) by suppressing the RAS and increasing bradykinin and prostaglandin levels.[31] Captopril contains sulfhydryl (SH) groups that can exhibit the free radical scavenging effects or antioxidant properties.[32] Several lines of evidence have reported other beneficial effects of captopril on the cardiovascular organ since it alleviates cardiac and vascular remodelling induced by L-NAME.[8, 11] A previous study demonstrated that captopril upregulated antioxidant enzyme and eNOS protein expression in the heart, but suppressed the RAS components.[33]

The previous study by the present authors demonstrated the overactivation of RAS system, rises in Ang II levels and vascular AT_1R protein expression, induced by NO deficiency.[34] In this study, the signalling downstream of AT_1R activation related to ventricular remodelling and dysfunction are further explored. Thus, the objective of this study was to evaluate whether tangeretin could decrease BP, alleviate cardiac alterations, suppress the activation of RAS and AT_1R signalling pathways and increase NO bioavailability in NO-deficient hypertensive rats.

2. Materials and methods

2.1 Drugs and chemicals

Tangeretin (purity ≥ 98%) was obtained from ChemFaces (Hubei, China). Propylene glycol (PG) was obtained from Ajax Finechem Pty Ltd. (NSW, Australia). L-NAME, captopril and ethylenediaminetetraacetic acid were purchased from Sigma-Aldrich Corp (St Louis, MO, USA). Lucigenin were obtained from Fluka Chemika Co. Ltd. (Buchs, Switzerland). Paraformaldehyde was obtained from Electron Microscopy Sciences (Hatfield, PA, USA). Xylene was purchased from Panreac Quimica S.A.U., E.U. Alcohol was purchased from ACI Lanscan., Thailand. Paraffin wax was purchased from Scientific, UK. Hematoxylin and Eosin (H&E) were purchased from Bio-Optica Milano SpA, Italy. Picrosirius Red Stain Kit was purchased from Polysciences, Inc., USA.

2.2 Animals and experimental protocols

Male Sprague-Dawley rats (220-240 g body weight) were purchased from Nomura Siam International Co., Ltd., Bangkok, Thailand. They were housed in plastic cages in a room with a regular 12-h dark–light cycle at a controlled temperature (22-24 °C) at the Northeast Laboratory Animal Center, Khon Kaen University, Khon Kaen, Thailand. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Khon Kaen University and experiments were approved by the Animal Ethics Committee of Khon Kaen University, Thailand (IACUC-KKU-98/60). Rats were allowed a week to acclimatize, then, hypertension was induced by addition of L-NAME (40 mg/kg/day) in their drinking water whereas control rats were given tap water. After three weeks of L-NAME treatment, high blood pressure was observed in rats. Thereafter, the rats were randomly divided into 5 groups with 8 rats each, as follows.

Group I Control + PG (1.5 ml/kg; p.o.; Control)
Group II L-NAME + PG (1.5 ml/kg; p.o.; L-NAME)
Group III L-NAME + tangeretin (15 mg/kg; p.o.; L+T15)
Group IV L-NAME + tangeretin (30 mg/kg; p.o.; L+T30)
Group V L-NAME + captopril (5 mg/kg; p.o.; L+Cap)

Tangeretin, captopril, and PG were orally administered daily for the last 2 weeks of the experiments. The choice of concentrations of tangeretin used in the present study was influenced by previous reports[35-37] and a preliminary study. The results from the preliminary study showed that tangeretin at doses 30 or 50 mg/kg could reduce blood pressure in a similar manner. Therefore, it was decided to choose tangeretin at doses of 15 and 30 mg/kg to observe a dose-dependent antihypertensive effect.

2.3 BP and heart rate (HR) and cardiac function measurement

Systolic BP (SP) and HR were measured weekly in conscious rats using non-invasive tail-cuff plethysmography (IITC/Life Science Instrument model 229 and model 179 amplifier; Woodland Hills, CA, USA) for 5 weeks. For each rat, SP and HR were detected and the mean values from the three detections with 15 min intervals were expressed. After five weeks of the experiment, rats were anesthetized with pentobarbital sodium (60 mg/kg/BW). Their chests were shaved and cleaned and they were placed on one side on a specially designed apparatus. An echocardiogram was performed using Model LOGIQ S7 (GE Healthcare, WI, USA). LV structure and function were assessed from two-dimentional short-axis views and then M-mode tracings were recorded for LV internal dimensions at end-diastole (LVIDd), LV internal dimensions at end-systole (LVIDs), interventricular septum at end-diastole (IVSd), interventricular septum at end-systole (IVSs), LV posterior wall thickness at end-diastole (LVPWd), LV posterior wall thickness at end-systole (LVPWs), end-diastolic volume (EDV), end-systolic volume (ESV) and stroke volume (SV) from three consecutive cardiac cycles. The LV fractional shortening (% LVFS) was calculated from equation: $\%FS = [(LVIDd-LVIDs)/LVIDd] \times 100$. After the cardiac function study, direct measurement of BP was determined. Briefly, a femoral artery was separated from the femoral vein, and a polyethylene tube was inserted. SP, diastolic BP (DP), mean arterial pressure (MAP) and HR were constantly monitored and recorded using the Acknowledge Data Acquisition and Analysis Software (BIOPAC Systems Inc., California, USA).

2.4 Morphometric analysis of heart tissue

Wet heart weights (HW) and LV weights (LVW) were calculated, and LVW to body weight (BW) ratios (LVW/BW) were measured. The LV tissues were fixed in 4% paraformaldehyde for 24 hours, routinely embedded into paraffin and carefully cut to 5 μm of thickness. LV hypertrophy was assessed using H&E staining of LV sections. Images were assessed under a SMZ745T stereomicroscope (Nikon, Tokyo, Japan). The LV wall thickness was measured every 45° interval around the cardiac circumference. Cross-sectional areas were assessed using the differences between the values of the external circumferential areas of the hearts and the chamber areas. LV Fibrosis was measured using picrosirius red stained LV sections. All sections were depicted with an Eclipse LV100 POL polarized light microscope (Nikon, Tokyo, Japan). LV fibrosis was calculated as a percentage of the positively stained area to medial area. Morphometric assessments were measured using Image-pro plus software (Media Cybernetics, MD, USA) and ImageJ morphometric software (National Institutes of Health, Bethesda, MD, USA).

2.5 Western blot assay

AT_1R, pERK1/2, pJNK, pp38MAPK and eNOS protein expressions in LV tissue were detected using the Western blot method following a previously explained method with some adjustments.[38] The tissues were carefully homogenized on ice. A sodium dodecylsulfate polyacrylamide gel electrophoresis system was used for protein electrophoresis, and then the proteins were transferred onto a polyvinylidene difluoride membrane and blocked with 5% BSA for 2 hours at room temperature. Subsequently, proteins were incubated with mouse primary antibodies to pERK1/2 and pJNK (Cell Signaling, Danvers, MA, USA) and eNOS (BD Biosciences, CA, USA) or rabbit primary antibodies to AT_1R (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and pp38MAPK (Cell Signaling, Danvers, MA, USA) overnight at 4°C. The membranes were rinsed with tris-buffered saline with tween and then incubated with horseradish peroxidase conjugated secondary antibody for 2 hours at room temperature. The blots were seen in Amersham™ ECL™ Prime solution (Amersham Biosciences Corp., Piscataway, NJ, USA), and densitometric analysis was measured using an ImageQuant™ 400 (GE Healthcare Life Sciences, Piscataway, NJ, USA). The intensities of AT_1R, pERK1/2, pJNK, pp38MAPK and eNOS bands were compared to those of the loading controls, β-actin.

2.6 Measurement of plasma Ang II, ACE activity, NO metabolites (NOx), malondialdehyde (MDA) and vascular superoxide production

Blood samples were drawn from abdominal aorta to analyze plasma Ang II, ACE activity, NOx and MDA levels. In this study, an Ang II EIA kit (St. Louis, MO, USA) was used to detect plasma Ang II concentration. ACE activity was measured using the o-phthaldialdehyde (OPA)-chromogenic reaction for histidyl-leucine. In brief, 25 μL sample of plasma and 50 μL of 15 mM Hip-His-Leu solution were mixed in 100 μL buffer (20 mM sodium borate and 300 mM NaCl, pH 8.3), and incubated at 37 °C for 30 min. Background absorbance was determined from plasma samples diluted in 150 μL buffer. The colour reaction was formed by adding OPA reagent (1 mM OPA and 1 mM 2-meraptoethanol in buffer containing 0.1 M sodium borate and 0.2 M NaOH, pH 12). After incubation at room temperature for 20 mins, the absorbance was measured at 390 nm with a spectrophotometer (Ultrospec 6300 pro. Bichrom Ltd., U.K.). Results were calibrated according to a standard curve of ACE solution (15-120 mU/mL).[39] The end products of NO metabolism, nitrate/nitrite, in plasma were investigated using an enzymatic conversion method and Griess reagents as described in a previous method with some modification.[40] Plasma MDA was evaluated with thiobarbituric acid reactive substances assay as previously described.[41] Superoxide production in carotid arteries was detected by luciginin-enhanced chemiluminescence.[40]

2.7 Statistical analysis

Data are shown as means ± SEM. The differences between groups were analyzed using one-way ANOVA followed by Fisher's Least Significant Difference tests. A statistical significance was considered when a p-value is less than 0.05.

3. Results

3.1. Effects of tangeretin and captopril on SP in L-NAME-induced hypertensive rats.

3.1.1. Effects of tangeretin and captopril on SP

Rat-baseline SP did not differ in all groups. Rats treated with L-NAME for a week had significantly increased SP compared to normal control rats. Over 3 weeks of the experiment, SP in L-NAME treated rats gradually increased (Fig. 1A). Tangeretin (15 or 30 mg/kg) significantly reduced SP in hypertensive rats in a dose-response dependent manner (167.37 ± 1.80 and 147.98 ± 2.00 mmHg, $P<0.05$) compared with the untreated rats (196.99 ± 5.99 mmHg, $P<0.05$). Captopril significantly decreased SP (141.99 ± 2.53 mmHg, $P<0.05$) in L-NAME treated rats compared to untreated rats. Furthermore, no significant differences in SP were detected between rats treated with captopril and tangeretin at 30 mg/kg (Fig. 1B).

3.1.2. Effect of tangeretin and captopril on BP and HR in anesthetized L-NAME-induced hypertension in rats

Blood pressure data measured by the direct method were consistent with the results obtained by the indirect method since SP, DP, MAP and HR were significantly increased in L-NAME treated rats compared to control rats ($P<0.05$). Hypertensive rats that received tangeretin had significantly decreased BP compared to untreated rats ($P<0.05$). Subsequently, the SPs of hypertensive rats treated with captopril or tangeretin (30 mg/kg) were significantly lower than those of in the hypertensive rats treated with tangeretin (15 mg/kg) ($P<0.05$). There were no significant differences of blood pressures between the captopril group and tangeretin at 30 mg/kg (Table 1).

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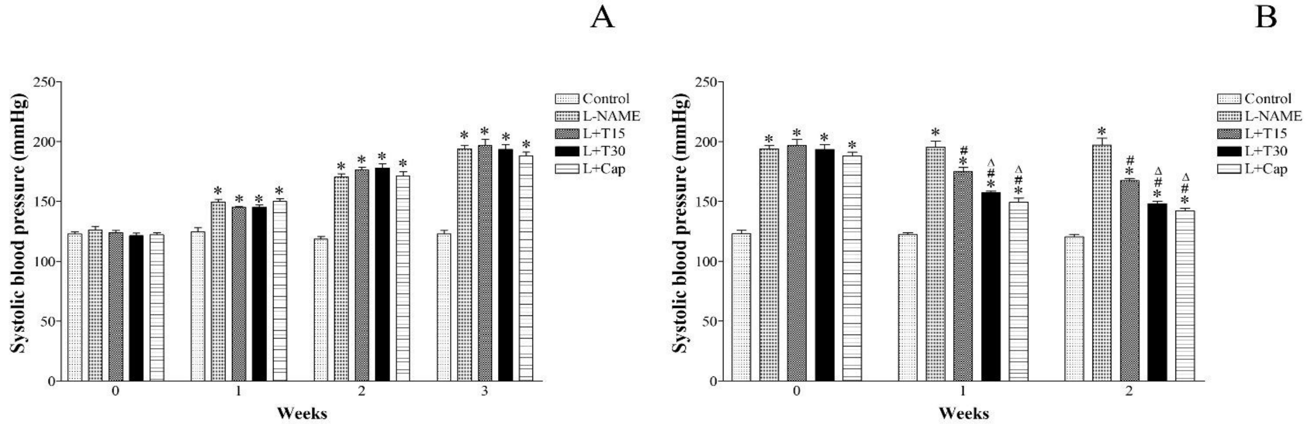

Fig. 1 The effect of 3-week-L-NAME treatment on systolic blood pressure (A) and the effect of 2-week-tangeretin and captopril treatment in L-NAME-treated rats (B). Results are shown as means ± SEM. (n = 8/group). *P<0.05 vs control, #P<0.05 vs L-NAME, ΔP<0.05 vs T15.

Table 1 Effects of tangeretin and captopril on SP. DP, MAP and HR in rats.

Parameters	Control	L-NAME	L+T15	L+T30	L+Cap
SP (mmHg)	120.39±2.33	197.31±6.64*	157.57±6.26*#	142.32±4.82*#Δ	140.03±3.70*#Δ
DP (mmHg)	74.59±2.95	137.48±5.31*	106.86±3.75*#	98.71±3.60*#	98.87±2.84*#
MAP (mmHg)	89.86±2.02	157.42±5.29*	123.76±4.43*#	113.25±3.94*#	112.59±2.95*#Δ
HR (beat/min)	363.57±15.02	413.52±15.02*	334.86±13.01#	339.81±15.69#	367.43±10.63#

SP: systolic blood pressure; DP: diastolic blood pressure; MAP: mean arterial pressure; HR: heart rate. Results are shown as means ± SEM. (n = 8/group). *P<0.05 vs control, #P<0.05 vs L-NAME, ΔP<0.05 vs T15.

Table 2 Effects of tangeretin and captopril on cardiac function in L-NAME-induced hypertensive rats.

Parameters	Control	L-NAME	L+T15	L+T30	L+Cap
IVSd (cm)	0.21±0.01	0.27±0.02*	0.23±0.01*#	0.20±0.01#	0.18±0.02#
IVSs (cm)	0.28±0.01	0.35±0.03*	0.32±0.02#	0.30±0.01#	0.28±0.02#
LVIDd (cm)	0.65±0.03	0.52±0.03*	0.56±0.02*#	0.59±0.01*#	0.59±0.02#
LVIDs (cm)	0.37±0.03	0.34±0.02	0.34±0.03	0.33±0.02	0.35±0.02
LVPWd (cm)	0.21±0.01	0.29±0.02*	0.25±0.02#	0.22±0.01#	0.21±0.01#
LVPWs (cm)	0.30±0.02	0.34±0.02	0.34±0.03	0.32±0.02	0.28±0.02#
EDV (ml)	0.67±0.08	0.35±0.06*	0.43±0.04*	0.48±0.03*	0.49±0.04*
ESV (ml)	0.14±0.03	0.10±0.02	0.11±0.02	0.10±0.02	0.11±0.01
EF (%)	79.51±2.94	70.21±2.74*	76.23±3.29	82.30±2.17#Δ	77.11±1.24#
SV (ml)	0.53±0.06	0.25±0.05*	0.32±0.03*#	0.38±0.02*#	0.38±0.03*#
FS (%)	44.03±3.50	34.95±1.99*	40.88±3.18	43.51±2.90#	40.63±1.14

IVSd: Interventricular septum thickness at end diastole; IVSs: Interventricular septum thickness at end systole; LVIDd: LV internal dimension at end-diastole; LVIDs: LV internal dimension at end-systole; LVPWd: LV posterior wall thickness in diastole; LVPWs: LV posterior wall thickness in systole; EDV: end-diastolic volume; ESV: end systolic volume; EF: ejection fraction; SV: stroke volume; FS: fractional shortening. Data are expressed as means ± SEM. (n = 7/group). *P<0.05 vs control, #P<0.05 vs L-NAME, ΔP<0.05 vs T15.

Table 3 Effects of tangeretin and captopril on cardiac mass indices in L-NAME-induced hypertensive rats.

Parameters	Control	L-NAME	L+T15	L+T30	L+Cap
BW (g)	476±14.15	445±16.25	453±5.48	470±7.93	468±7.48
HW/BW (mg/g)	2.65±0.03	2.97±0.02*	2.61±0.02#	2.46±0.05#	2.62±0.04#
LVW/BW (mg/g)	1.86±0.03	2.15±0.03*	1.85±0.02#	1.75±0.02#	1.75±0.03#

HW: heart weight; BW: body weight; LV: left ventricular; LVW: left ventricular weight. Data are expressed as means ± SEM. (n = 8/group). *P<0.05 vs control, #P<0.05 vs L-NAME, ΔP<0.05 vs T15.

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3.2. Effects of tangeretin and captopril on ventricular function in L-NAME-induced hypertension in rats

L-NAME rats showed significant increases in IVSd, IVSs and LVPWd with decreases in LVIDd, EDV, SV, %EF and %FS compared with the control rats (P<0.05, Table 2). These alterations of ventricular function were consistent with the development of LV remodelling (Table 3 and Fig. 2). Tangeretin or captopril alleviated EDV and SV alterations and fully recovered the other abnormal cardiac parameters in hypertensive rats (Table 3).

3.3. Effect of tangeretin and captopril on cardiac mass indices

BW among all groups was not significantly different, however, HW/BW and LVW/BW ratios were increased in rats that received L-NAME comparing to the control group (P<0.05; Table 3). Moreover, tangeretin or captopril restored HW/BW and LVW/BW ratios compared to untreated rats (P<0.05).

3.4. Effects of tangeretin and captopril on ventricular morphology in L-NAME-induced hypertensive rats

Histomorphometric analysis showed that long-term treatment with L-NAME caused significant increases in wall thicknesses and the cross-sectional areas and a reduction of luminal areas in the LV comparing to the control group (P<0.05, Fig. 2). These alterations could be restored by tangeretin. Captopril also alleviated these signs of LV hypertrophy in L-NAME hypertensive rats (P<0.05). Additionally, there was increased myocardial fibrosis calculated from the picrosirius red-stained LV sections by polarized light microscopy in hypertensive rats (P<0.05; Fig. 3). This fibrotic change presented in hypertensive rats was restored in tangeretin and mitigated in captopril treated groups (P<0.05).

3.5. Effects of tangeretin and captopril on RAS activation and AT_1R cascades in L-NAME-induced hypertensive rats

Plasma ACE activity and Ang II levels were significantly higher in hypertensive rats compared to control rats (P<0.05; Fig. 4). These abnormalities could be restored by tangeretin or captopril. The expressions of AT_1R, pERK1/2 and pJNK protein in myocardial tissue from L-NAME-induced hypertensive rats were significantly upregulated comparing to control rats (P<0.05). Supplementation of tangeretin markedly downregulated AT_1R, pERK1/2 and pJNK protein expression in L-NAME-induced hypertensive rats (P<0.05). Additionally, captopril also suppressed the upregulation of AT_1R and pERK1/2 protein expression in rats that received L-NAME (P<0.05; Fig. 5A, 5B and 5C). The expression of pp38MAPK, however, was not different among groups (Fig. 5D).

3.6. Effects of tangeretin and captopril on eNOS protein expression in LV tissues and plasma NOx from L-NAME-induced hypertensive rats

Suppression of eNOS protein expression was observed in L-NAME-induced hypertensive rats. Tangeretin and captopril significantly raised the expression of eNOS protein in L-NAME-induced hypertensive rats (P<0.05; Fig. 6A). These alterations of protein expression were accompanied with a reduction of plasma NOx concentration in L-NAME-induced hypertensive rats (P<0.05). Tangeretin and captopril could restore the low level of plasma NOx in hypertensive rats (P<0.05) (Fig. 6B).

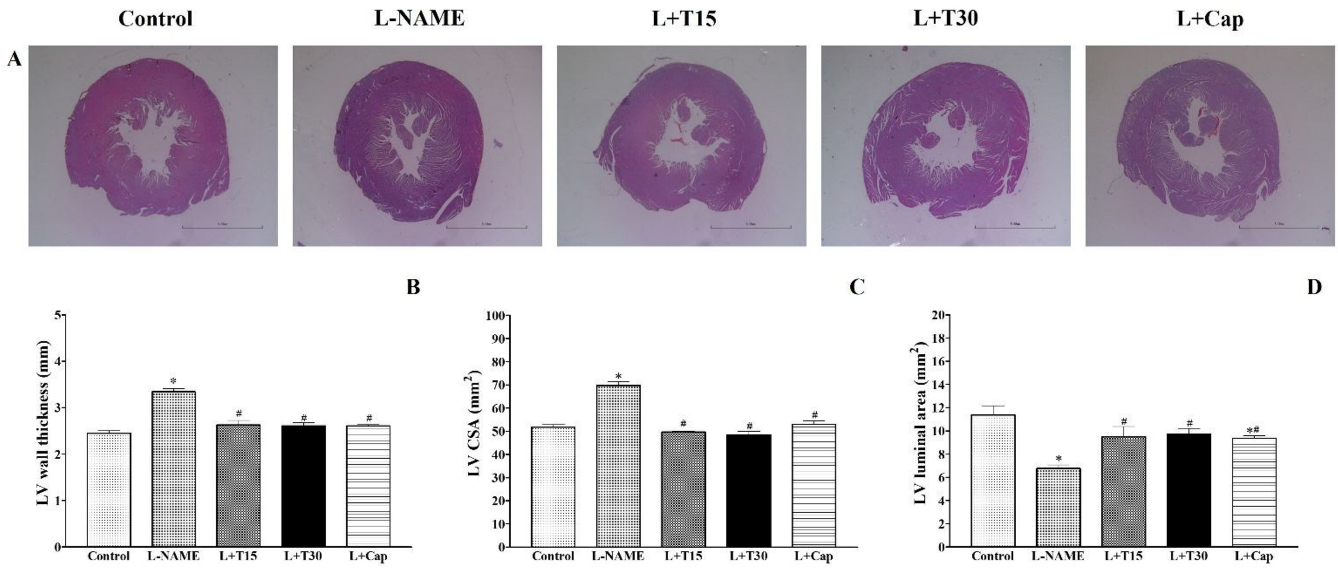

Fig. 2 Effect of tangeretin and captopril on LV morphology in rats. Representative figures of LV sections stained with hematoxylin and eosin under stereomicroscopy using a 1x objective lens, scale bars = 5.0 mm (A), and values of LV wall thickness (B), LV cross--sectional area (C) and LV luminal area (D). Results are shown as means ± SEM. (n = 8/group). *P<0.05 vs control, #P<0.05 vs L-NAME.

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3.7. Effects of tangeretin and captopril on plasma MDA and vascular superoxide production in L-NAME-induced hypertensive rats

An increase in plasma MDA levels in L-NAME-induced hypertensive rats was observed (P<0.05). Tangeretin restored plasma MDA concentrations in L-NAME-treated rats compared to untreated rats (P<0.05). The antioxidant effect was also demonstrated in hypertensive rats treated with captopril (P<0.05). There was a significant increase in superoxide production in carotid arteries isolated from L-NAME treated rats compared to control rats (P<0.05). This rise of superoxide production in hypertensive rats was fully recovered by tangeretin or captopril treatment compared to untreated rats (P<0.05) (Fig. 7).

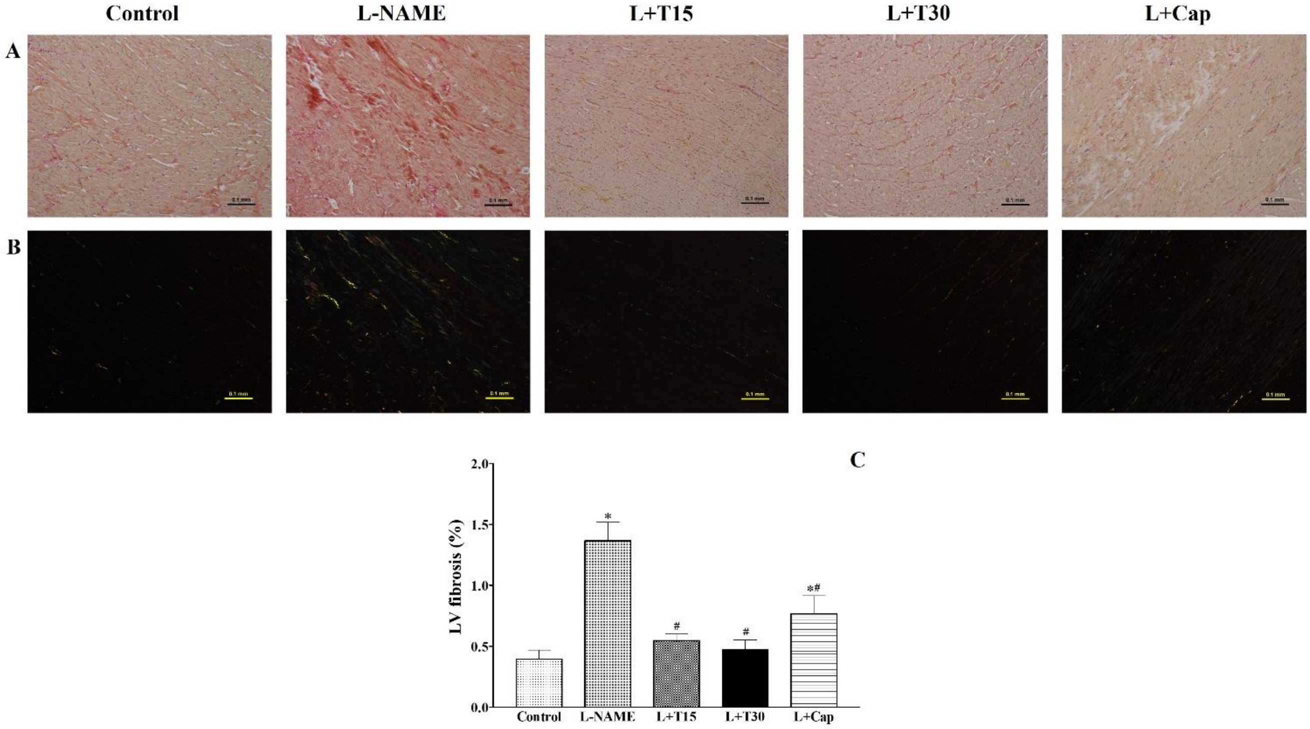

Fig. 4 Effect of tangeretin and captopril on myocardial fibrosis in L-NAME-induced hypertensive rats. Representative images of LV sections stained with picrosirius red under light microscopy (A) and polarized light microscopy (B) using a 10x objective lens, scale bars = 0.1 mm, and values of percentage areas of LV fibrosis (C). Data are expressed as means ± SEM. (n = 6-8/group). *P<0.05 vs control, #P<0.05 vs L-NAME.

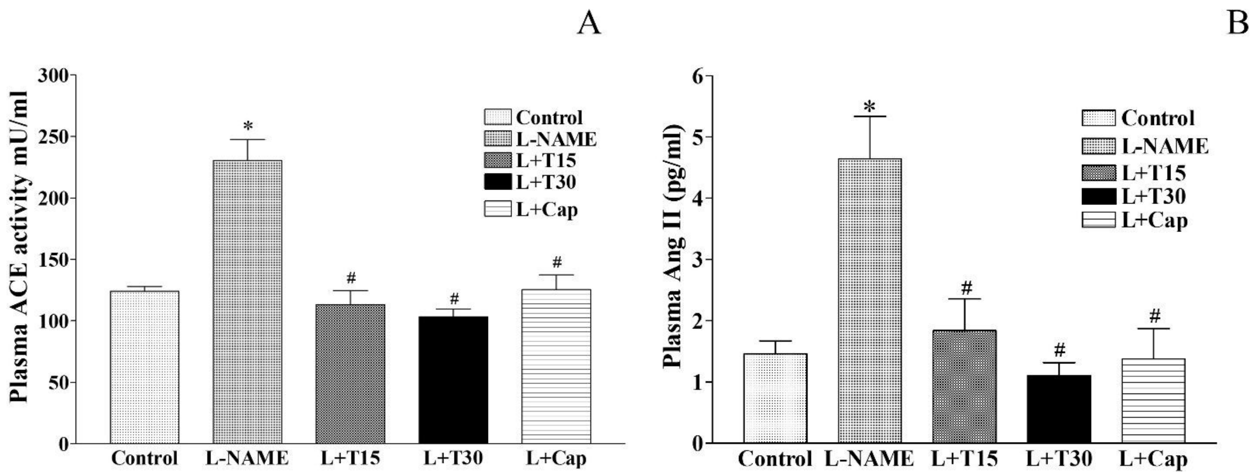

Fig. 3 Effect of tangeretin and captopril on plasma ACE activity (A) and Ang II levels (B) in L-NAME-induced hypertensive rats. Data are expressed as means ± SEM. (n = 6-8/group). *P<0.05 vs control, #P<0.05 vs L-NAME.

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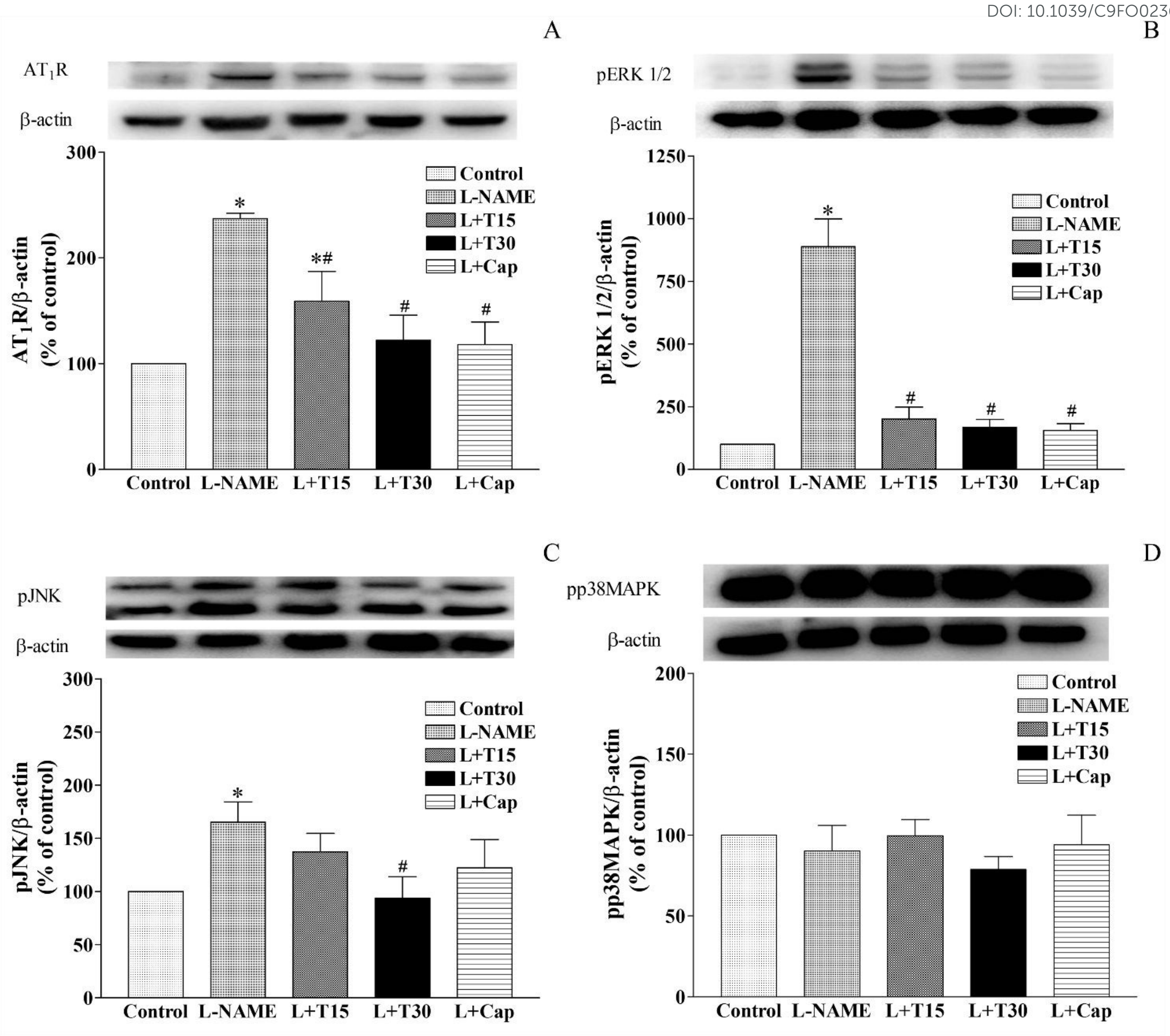

Fig. 5 Effects of tangeretin and captopril on AT1R (A), pERK1/2 (B), pJNK (C), and pp38MAPK (D) protein expression in cardiac tissue in L-NAME-induced hypertensive rats. Data are expressed as means ± SEM. (n = 4/group). *P<0.05 vs control, #P<0.05 vs L-NAME.

4. Discussion

The findings of this study are that in a rat model of L-NAME-induced hypertension, the animals exhibited high systemic blood pressure, impairment of LV function, and LV hypertrophy and fibrosis. These signs of cardiac alterations were related to overexpression of the cardiac AT_1R, pERK1/2 and pJNK signaling cascade and reduction of cardiac eNOS protein expression and plasma NO metabolites, along with increases in ACE activity, plasma Ang II levels, and oxidative stress markers in L-NAME treated rats. Tangeretin and captopril reduced BP and alleviated altered ventricular function and remodelling by suppressing the activation of RAS and expression of the AT_1R signaling pathway in L-NAME-induced hypertensive rats. Tangeretin and captopril increased cardiac eNOS protein expression. The reduction in oxidative stress by treatment with tangeretin or captopril was able to improve NO bioavailability in L-NAME-induced hypertensive rats.

LV dysfunction characterized by decreasing EF, SV and FS was shown in L-NAME hypertensive rats in the present study. Moreover, it was found that there was a reduction of EDV, indicating impairment of diastolic function in these hypertensive rats. It is well established that chronic changes in cardiac morphology subsequently result in cardiac dysfunction. Additionally, NO has clearly been known to influence cardiac performance since NO augments the Frank-Starling response and enhances myocardial relaxation.[42] Furthermore, intracardiac NO promotes ventricular diastolic distensibility and inhibits diastolic stiffness.[43, 44] Prendergast and co-workers indicated that preload-induced increases in cardiac output were significantly attenuated by NOS inhibition in the isolated ejecting guinea pig heart. They suggested involvement of direct effects of NO on myocardial diastolic and/or systolic function.[15]

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A recent study found that L-NAME significantly increased blood pressure, cardiac remodelling and dysfunction accompanied by a decrease in eNOS expression and NO bioavailability.[19] In addition, Kumar and co-workers reported that upregulation of eNOS expression attenuated cardiac remodelling and LV dysfunction in L-NAME-induced hypertensive rats.[45]

Rats that received L-NAME developed high BP associated with ventricular hypertrophy as evident by increases in HW/BW, LVW/BW ratios, the cross-sectional areas, wall thicknesses, and a decrease in luminal areas of the LV. Subsequently, LV fibrosis was accumulated in L-NAME hypertensive rats, confirming LV remodelling in this animal model. Since L-NAME has been developed to inhibit NO production resulting in hypertension, other consequences of hypertension in this animal model have been extensively studied.[46] Pechanova and coworkers found systemic high BP related to LV remodelling in NO-deficient hypertensive rats.[47] At least two possible mechanisms, hemodynamic and non-hemodynamic factors have been found to contribute to ventricular hypertrophy.[48] Hemodynamic overload from hypertension-induced cardiac adaptation to preserve normal cardiac output is well documented.[49] It was further reported that activation of RAS and depletion of NO production in an L-NAME-rat model are contributions of non-hemodynamic factors to mediate LV hypertrophy and remodelling.[2, 47] There is substantial evidence to show that increases in plasma Ang II, AT_1R expression, associated with cardiac remodelling were observed in L-NAME hypertensive rats.[11, 34] In this present study, the overactivation of RAS and AT_1R signalling downstream expressions, ERK1/2 and JNK but not pp38MAPK in ventricular tissues from L-NAME hypertensive rats were found. These results suggest that activation of the AT_1R/MAP kinases (ERK1/2/JNK) pathway could mediate the induction of ventricular hypertrophy and remodelling in NO deficient rats. It is noteworthy that activation of the AT_1R/MAP kinases signalling cascades is implicated in cell growth and hypertrophy.[12] Several studies confirmed the important role of AT_1R and its signalling cascade on cardiac morphology. For example, overexpression of AT_1R in the heart of transgenic mice was obviously related with cardiac hypertrophy, interstitial collagen deposition, myolysis, and cardiac remodelling.[50] Bueno and co-workers indicated that cardiac hypertrophy was mediated by the activation of the MEK1–ERK1/2 signalling pathway in transgenic mice.[51]

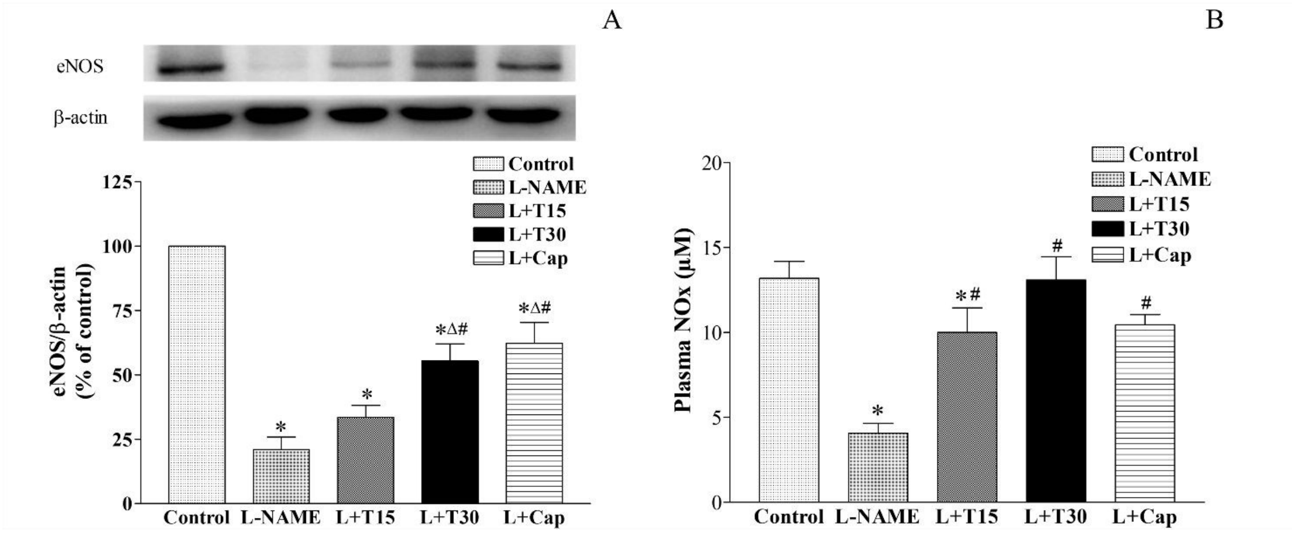

Fig. 6 Effects of tangeretin and captopril on eNOS protein expression (A) and plasma NOx (B) in cardiac tissue in L-NAME-induced hypertensive rats. Data are expressed as means ± SEM. (n = 4-6/group). *P<0.05 vs control, #P<0.05 vs L-NAME, ΔP<0.05 vs T15.

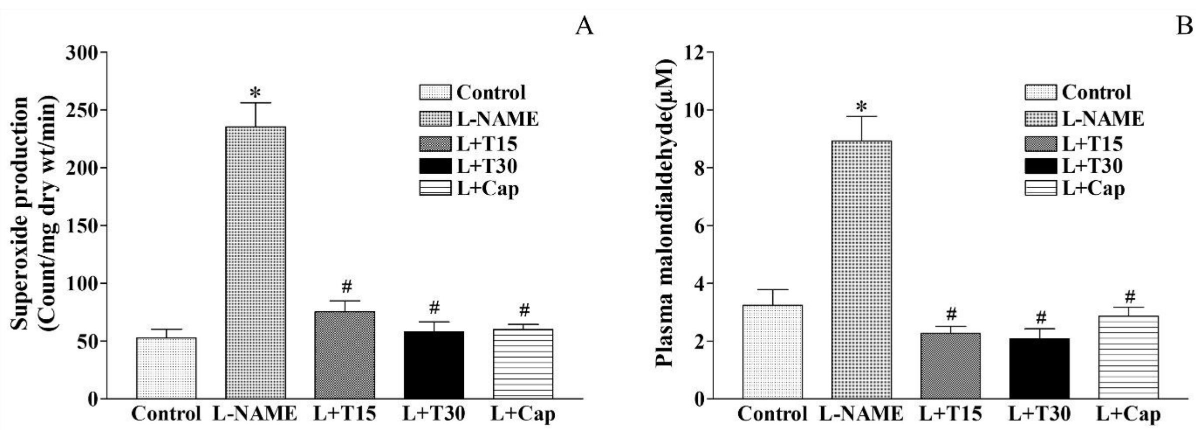

Fig. 7 Effects of tangeretin and captopril on vascular superoxide production (A) and plasma MDA levels (B) in L-NAME-induced hypertensive rats. Data are expressed as mean ± SEM. (n=6-8 /group). *P<0.05 vs control, #P<0.05 vs L-NAME.

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This study demonstrated low levels of eNOS protein expression in LV tissue and plasma NOx in L-NAME hypertensive rats. NO is one of the non-hemodynamic factors preventing maladaptive remodelling of hearts since upregulation of eNOS protein expression has been shown to reduce cardiac hypertrophy in mice.[52] Kobayashi and co-workers demonstrated that the downregulation of eNOS in the LV may participate in the myocardial structural changes in L-NAME rats.[53] It is well known that NO is able to inhibit cell growth and proliferation.[54] A previous study demonstrated that eNOS protects against myocardial remodelling and dysfunction in the rat myocardial infarction model.[55] NO can inhibit cell proliferation by modulating the expression of cell cycle regulatory proteins.[56] Therefore, NO is recognized to have beneficial effects on cardiac morphology and function. The current results are consistent with the previous study since this study also found the downregulation of eNOS protein expression in cardiac tissues and a decrease in plasma NOx concentration in L-NAME-induced hypertensive rats. Moreover, decreased NO bioavailability in this study was associated with increased oxidative stress markers, plasma MDA levels, and vascular superoxide production, however, tangeretin was able to decrease BP and improve LV dysfunction and hypertrophy in L-NAME-induced hypertensive rats. The anti-hypertensive effect of tangeretin in the present study was due to upregulation of eNOS protein expression, along with a rise in plasma NOx.

That tangeretin raised NO bioavailability might be mediated by its antioxidant property as high levels of oxidative stress markers in L-NAME hypertensive rats were attenuated in the tangeretin treated group. In the fact that superoxide is quick to react with NO to form peroxynitrite, resulting in reducing NO bioavailability.[57] There is direct evidence that tangeretin inhibited ERK1/2 phosphorylation in human T47D mammary cancer cells[58] and JNK phosphorylation in liver tissues of cisplatin-induced acute hepatic injury.[27] The results of the current study showed that tangeretin suppressed RAS activation and AT_1R signaling pathway expression in L-NAME hypertensive rats. The mechanism that is implicated in tangeretin reducing plasma Ang II level in the present study might be related to its inhibitory effect on ACE activity. It is possible that oxidative stress[59] and high circulating Ang II levels[60, 61] participated in the overexpression of AT_1R in hypertensive rats in the present study, thus, tangeretin reduced oxidative stress and plasma Ang II levels and subsequently suppressed AT_1R expression. Furthermore, there is strong evidence to support the suppressive effect of tangeretin on RAS since the highest accumulation of tangeretin was observed in the kidney compared to other vital organs.[37] Tangeretin alleviated L-NAME-induced LV remodelling in the present study; it may be the consequence of the antihypertensive effect of tangeretin to reduce the pressure load on the heart or its effect on non-hemodynamic factors or both. Furthermore, the improved cardiac function in tangeretin treated L-NAME hypertensive rats is related to its ability to reduce cardiac hypertrophy, which may be associated with increased eNOS protein expression and plasma NOx as discussed above. Interestingly, tangeretin produced a dose-dependent response in efficacy to reduce blood pressure and improve the other abnormal parameters in this study. It is possible that tangeretin at a dose 30 mg/kg BW is an optimal dose or an effective dose in this study Blood pressure, some parameters of cardiac function, LV diameter and eNOS protein expression, however, were not fully recovered by treatment, thus a longer treatment of captopril or tangeretin to restore these parameters might be more effective. The results of this study could suggest that tangeretin can be used as a food supplement to obtain a beneficial effect on reducing cardiovascular disease. There is evidence to support that the greatest tangeretin concentration was found in the flavedo of Florida citrus peel, especially King mandarin.[62] Therefore, consumption of the citrus peel, a good source of tangeretin, may provide more nutritional benefits on health promotion.

In the present study, captopril is an ACE inhibitor to treat hypertension. It was able to decrease blood pressure, improve cardiac alterations, suppress AT_1R/MAP kinase pathways, upregulate eNOS protein expression, reduce oxidative stress, and increase NO bioavailability in the L-NAME-induced hypertensive rats. The antihypertensive effect of captopril has been clearly recognized.[34,63] In addition, captopril exhibits antioxidant properties with its SH group.[32]

Conclusions

The results of the present study indicate that tangeretin can decrease BP and mitigate LV dysfunction and remodelling in NO-deficient hypertensive rats. These effects are associated with reducing RAS activation and the AT_1R/pERK/pJNK signaling cascade and raising NO availability along with reducing oxidative stress.

Conflicts of interest

There are no conflicts to declare.

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