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*Article*

# 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), or 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. Moreover, hesperidin or captopril alleviated L-NAME-induced cardiac remodeling; increases in wall thickness, cross sectional area (CSA) and fibrosis of left ventricular (LV), and vascular remodeling; increases in wall thickness, CSA, vascular smooth muscle cells and collagen deposition in the aorta. 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 over-expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) were 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 its 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$^{\omega}$-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-$\alpha$), a pro-inflammatory cytokine, to develop 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-$\alpha$ [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 [19]. 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 peroxide hydrogen [20], reduce plasma levels of lipid peroxidation markers and increase antioxidant enzyme activities in heart tissue in experimental ischemic myocardium rats [21]. Hesperidin has also exhibited an anti-inflammatory effect by reducing circulating inflammatory markers, i.e. TNF-$\alpha$, interleukin 6 (IL-6), and a high-sensitivity C-reactive protein (hs-CRP), in patients with type 2 diabetes [22] and suppressing inflammatory responses in lipopolysaccharide-induced RAW 264.7 cells [23]. Subsequently, a clinical study revealed that a combination of hesperidin, diosmin and troxerutin was effective to relieve the symptoms of acute hemorrhoidal disease [24]. Recently, the current authors have demonstrated an antihypertensive effect of hesperidin in renovascular hypertensive rats that involved the suppression of the renin-angiotensin system [25]. This study was aimed to further to 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 (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.

### *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 the HVAC (Heating, Ventilation and Air-Conditioning) System (25±2 °C) 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 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 were further divided into 4 following groups; L-NAME plus propylene glycol (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 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, 25].

### *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 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).

### *2.4 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.

*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 artery was 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 [26] with some modifications [27]. Plasma NOx was assayed using an enzymatic conversion method [28] with some modifications [27]. 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 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, MMP2 and MMP9 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, MMP2, MMP9 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 control group from the same gel.

*2.8 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.

## 3. Results

### *3.1 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 1). 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 ($p<0.05$).

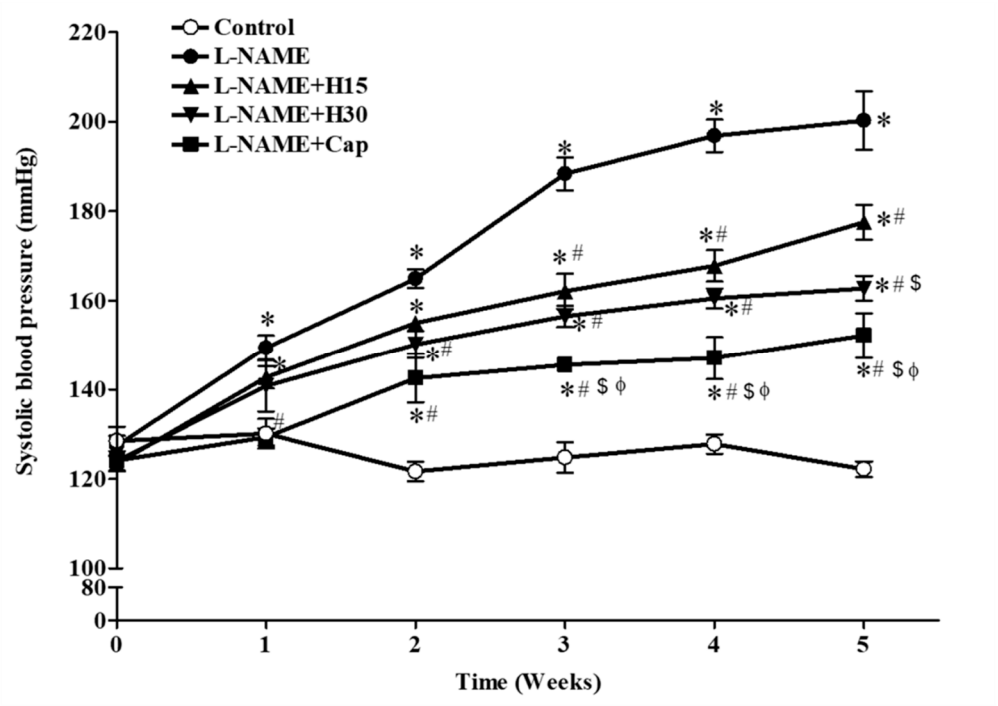


**Figure 1.** Time-course changes in systolic blood pressures of all experimental groups. Data are expressed as mean ± S.E.M (n = 7-8)/ group, * $p < 0.05$ vs. control, # $p < 0.05$ vs. L-NAME, $ $p < 0.05$ vs. L-NAME + hesperidin (15 mg/kg), ϕ $p < 0.05$ vs. L-NAME + hesperidin (30 mg/kg) group.

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

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, affected an 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, 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 2). 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 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 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 2B).

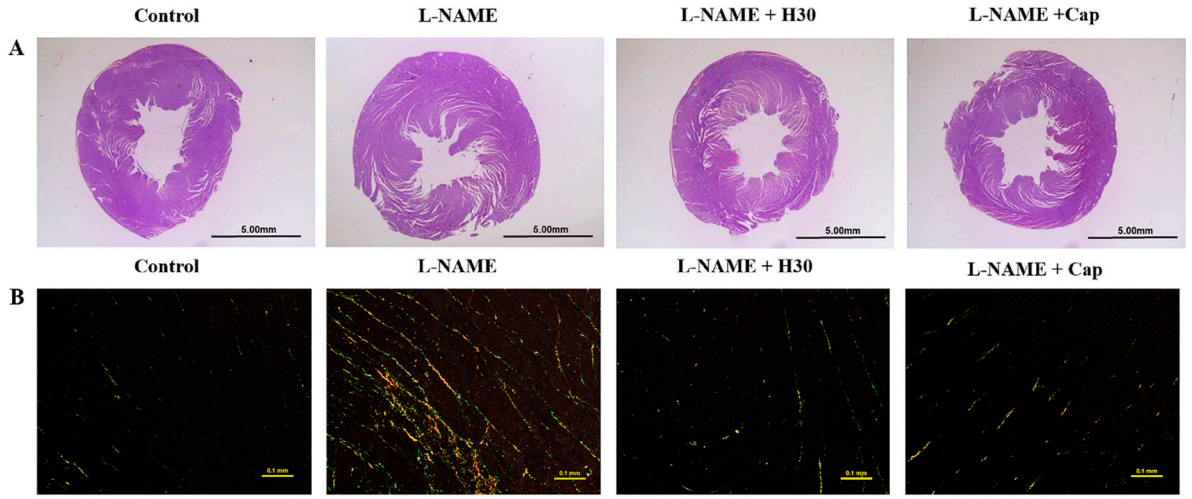


**Figure 2.** Effects of hesperidin and captopril supplementation on left ventricular morphometry (A) and fibrosis (B) in control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. Data are expressed as mean ± S.E.M (n = 7-8/group), * $p < 0.05$ vs. control, # $p < 0.05$ vs. L-NAME group.

**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 ($mm^2$) | 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 ($x10^3$ $\mu m^2$) | 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.

*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 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 ($p < 0.05$).

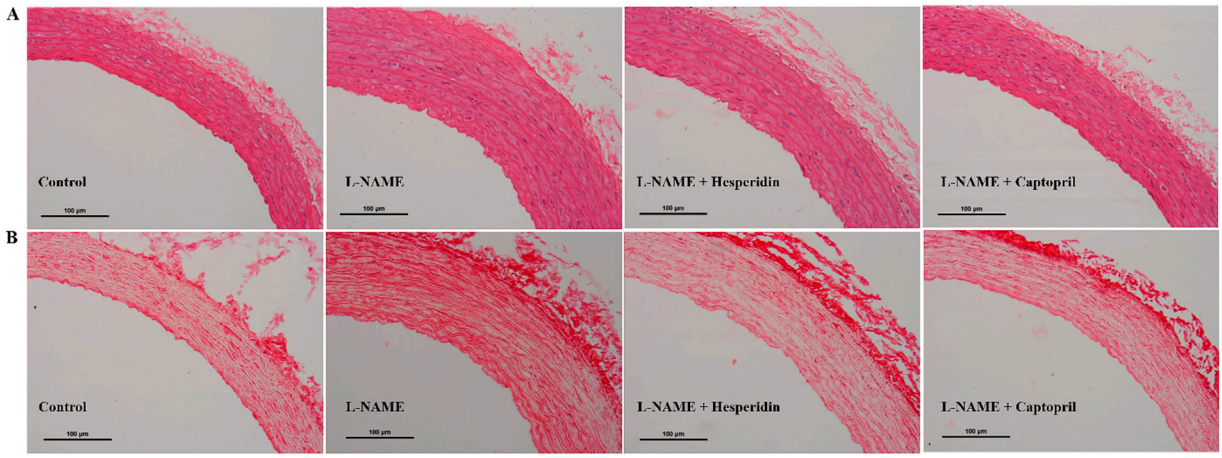


**Figure 3.** Effects of hesperidin or captopril supplementation on vascular morphology (A) and collagen deposition (B) in thoracic aortas of control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. Data are expressed as mean ± S.E.M (n = 6-8)/ group, * $p < 0.05$ vs. control, $^{\#}$ $p < 0.05$ vs. L-NAME group.

*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 a 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 4A 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 respectively, $p < 0.05$) (Figure 4C).

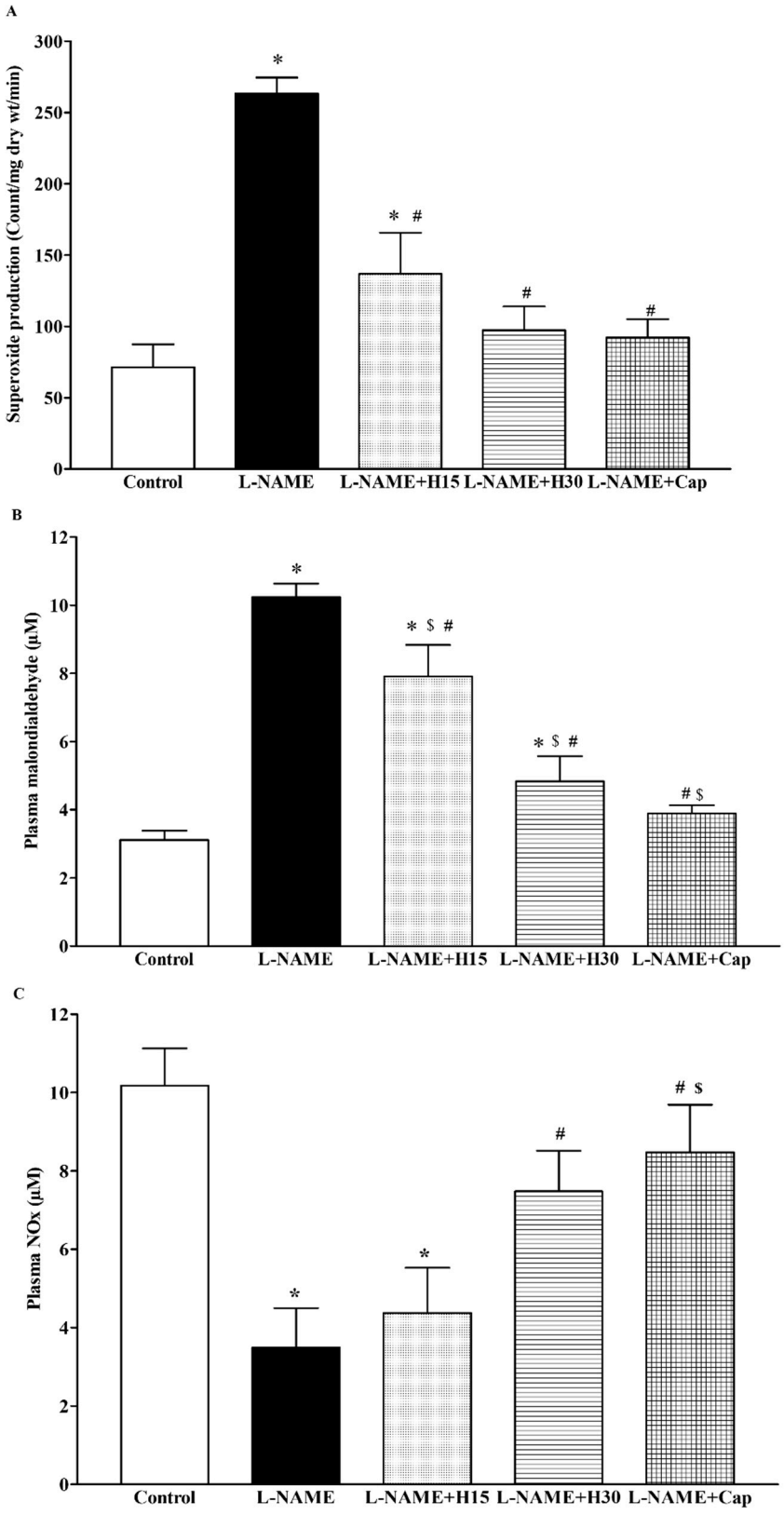


**Figure 4.** Effects of hesperidin and captopril supplementation on vascular $O_2^{\bullet -}$ production (A), plasma MDA (B) and plasma NOx (C) levels in control, L-NAME, L-NAME + hesperidin (15mg/kg), L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (5 mg/kg) groups. Data are expressed as mean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

*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*

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 5A and B). These 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 5C and 5D).

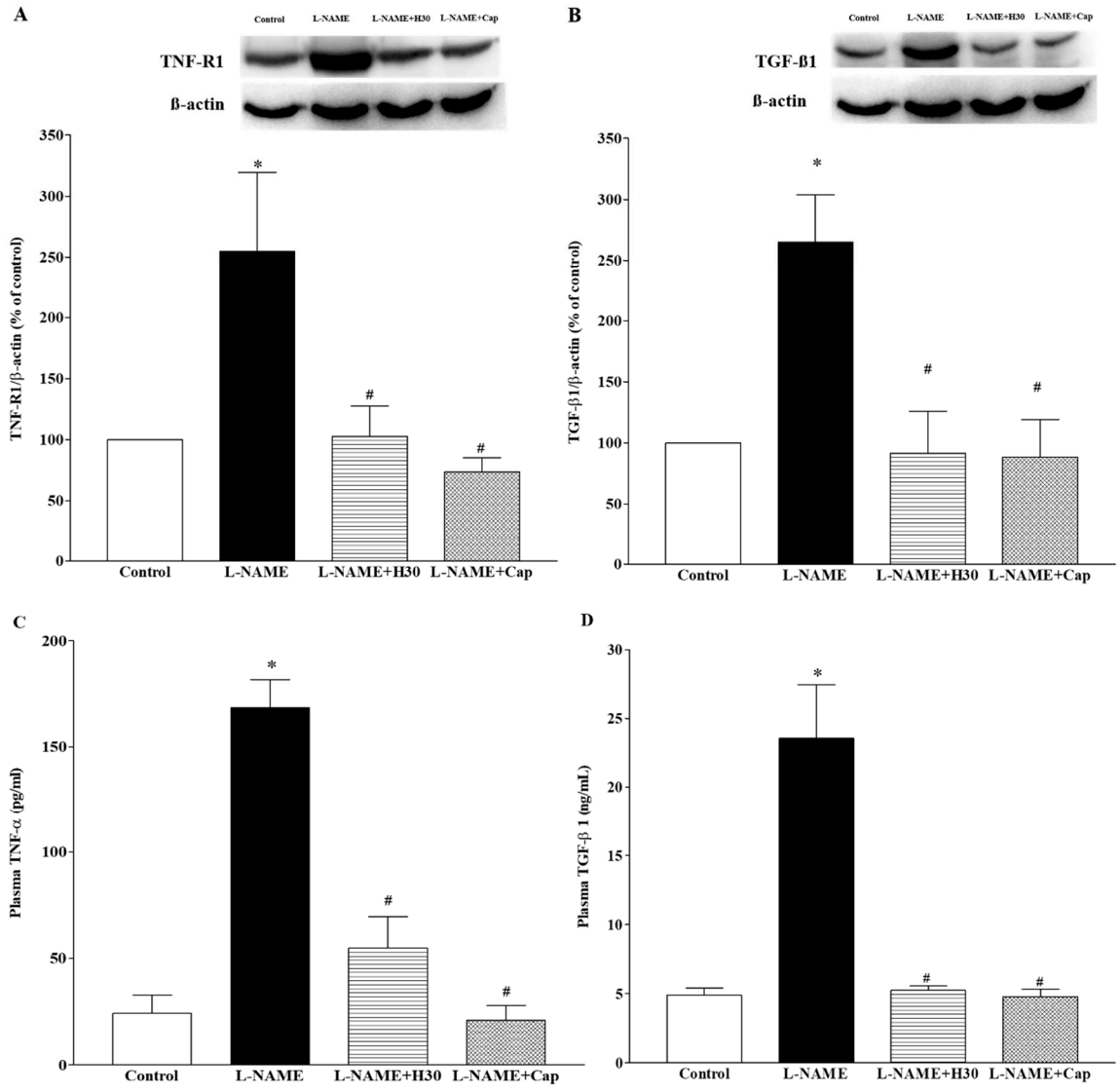


**Figure 5.** Effects of hesperidin and captopril on protein expression of TNF-R1 (A) and TGF- β1 (B) in heart tissue and on concentrations of plasma TNF-α (C) and TGF- β1 (D) collected from control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. The top panel shows 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 of MMP-2 and MMP-9 protein expression was observed in thoracic aortic tissue collected from the hypertensive group compared with the control group (Figure 6A and 6B, $p < 0.05$). Hesperidin or captopril treatment significantly suppressed the level of MMP-2 and MMP-9 protein expression compared with untreated rats, ($p < 0.05$).

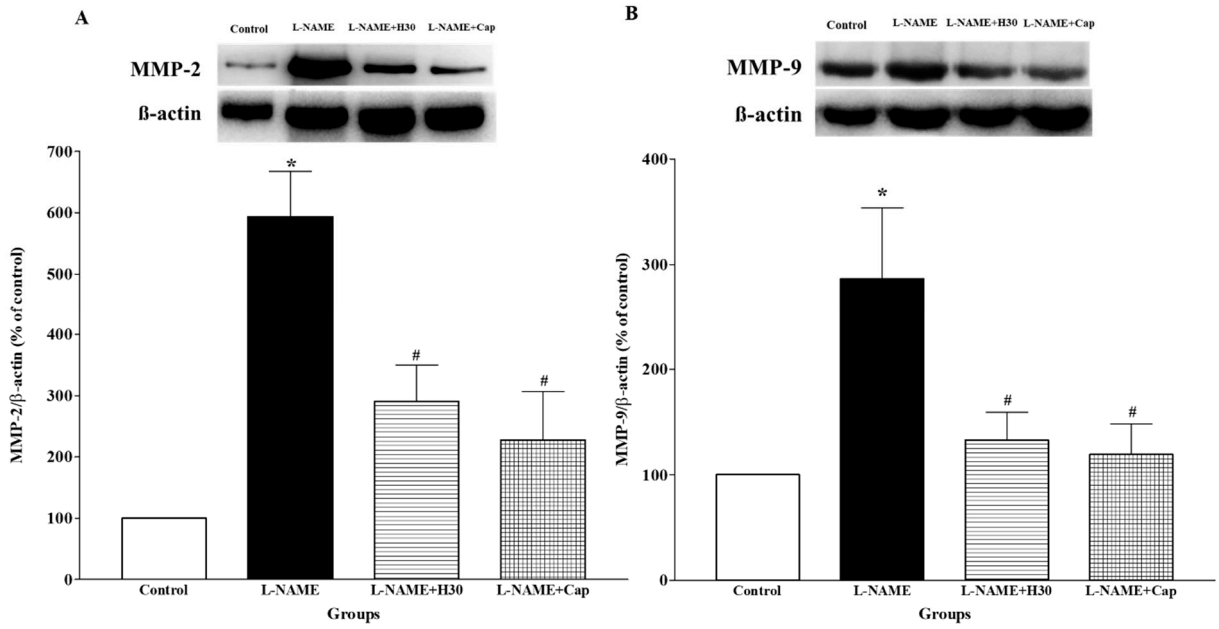


**Figure 6.** Effects of hesperidin and captopril on protein expression of MMP-2 (A) and MMP-9 (B) in aortic tissue collected from control, L-NAME, L-NAME + hesperidin (30 mg/kg) and L-NAME + captopril (2.5 mg/kg) groups. The top panel, shows representative bands of MMP-2 (A) and MMP-9 (B) protein expression 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.

## 4. Discussion

This study demonstrates that rats received that L-NAME developed hypertension and cardiovascular remodeling. Hesperidin mitigates 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 enhancing NO availability.

It was found that chronic treatment of L-NAME produced the development of NO-deficient hypertension as well as cardiovascular remodeling. These remodeling included increases in

LVW/HW ratio, LV wall thickness, LV CSA, LV fibrosis, aortic wall thickness, aortic cross sectional area, aortic smooth muscle cell number and collagen deposition. It has been well accepted that chronic inhibition of NO synthase using L-NAME resulted in NO depletion, increased vascular tone and high blood pressure [29]. Several studies demonstrated that cardiovascular remodeling occurred after chronic treatment with L-NAME (40 mg/kg) for 5 weeks [4, 10, 30]. The mechanisms involved in cardiac remodeling in an animal model of nitric oxide deficient hypertension is still unclear, however, two possible mechanisms related to hemodynamics and non-hemodynamic aspects have been described [31]. Hemodynamic overload in hypertension provoked left ventricular hypertrophy because of the adaptive response to conserve cardiac output [32]. 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 [33]. It is well documented that vascular remodeling in hypertension occurs in response to long-term modifications of hemodynamic conditions [34, 35]. 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, 36]. 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 [37] by the fact that superoxide can rapidly react with nitric oxide to form peroxynitrite [38]. This reaction results in reducing nitric oxide bioavailability [39]. In the present study, increases in plasma MDA levels and vascular superoxide production were accompanied by decreased plasma NOx 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 confirm that hesperidin has strong antioxidant activity [21, 40]. Hesperidin exhibits its antioxidant properties with two main mechanisms including, directly scavenging reactive oxygen species [41] and boosting cellular antioxidant defense [20]. Thus, one of the possible mechanisms of the cardiovascular protective effects of hesperidin in this study that might have involved its antioxidant capability 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 [42, 43]. Results of the current study proved that as in the previous studies, there were increases in level of the proinflammatory cytokine, TNF-$\alpha$, in plasma and expression of TNF-$\alpha$ protein in heart tissue in L-NAME hypertensive rats. Myocardial TGF-$\beta$ protein expression was also observed in L-NAME hypertensive rats. It is well established that TGF-$\beta$ plays and an important role in responses to inflammation to activate fibrogenesis, which is the important pathological process for cardiac remodeling [44, 45]. The present study has also shown that hesperidin attenuated cardiac remodeling accompanied with decreased systemic and heart inflammation in L-NAME hypertensive rats. The protein expression of TGF-$\beta$ 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-$\alpha$ [46]. Li and coworkers demonstrated that hesperidin decreased the production of IL-1$\beta$, IL-6, and TNF-$\alpha$ in a rat model of rheumatoid arthritis [47]. 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 [48, 49]. Overexpression of MMP mediated vascular remodeling was stimulated by oxidative stress and inflammatory cytokines [49]. 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 [50]. Interestingly, 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. These results are consistent with those previous studies in that captopril prevented high blood pressure, cardiac fibrosis and collagen deposition induced by L-NAME in rats [51-53]. They suggested that captopril may have antioxidant activity with thiol-groups in addition to its main action as an angiotensin converting enzyme activity inhibitor.

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

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