รายงานวิจัยฉบับสมบูรณ์

Effects of ellagic acid on the cardiac remodeling in rat with nitric oxide deficiency

ผลของอีลาจิก แอสิด ต่อการปรับเปลี่ยนโครงสร้างของหัวใจหนู
ที่มีภาวะพร่องไนตริกออกไซด์

โดย ผศ.ดร.ปาริฉัตร ประจะเนย์

พฤษภาคม 2561

สัญญาเลขที่ MRG5680036

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สังกัดภาควิชากายวิภาคศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
อำเภอเมือง จังหวัดขอนแก่น

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยขอนแก่น

กิตติกรรมประกาศ

งานวิจัยเรื่อง ผลของอีลาจิก แอสิด ต่อการปรับเปลี่ยนโครงสร้างของหัวใจหนูที่มีภาวะพร่องไนตริกออกไซด์ ได้รับทุนสนับสนุนจากสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยขอนแก่น ภายใต้ทุนพัฒนาศักยภาพในการทำวิจัยของอาจารย์รุ่นใหม่ ปี 2556 ผู้วิจัยจึงขอขอบพระคุณเป็นอย่างสูงมา ณ โอกาสนี้

คณะผู้วิจัย

พฤษภาคม 2561

รูปแบบ Abstract (บทคัดย่อ)

Project Code : MRG5680036

Project Title: Effects of ellagic acid on the cardiac remodeling in rat with nitric oxide deficiency

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Project Period : 2 years

Ellagic acid, a polyphenol found in a wide variety of nut as well as fruits such as strawberries, raspberries and pomegranates, has been reported the have biological effect such as antioxidant and anti-inflammation. This study aimed to investigate the preventive effect of ellagic acid (EA), on blood pressure, oxidative markers and cardiovascular changes in L-NAME induced-hypertensive rats. Male Sprague-Dawley rats were administrated with L-NAME (40 mg/kg/day) for five weeks. During L-NAME treatment, ellagic acid (7.5 and 15 mg/kg/day) or vehicle was given daily to hypertensive rats. At the end of experimental periods, hypertensive rats concurrent treatment with ellagic acid significantly improved hemodynamic parameters in L-NAME hypertensive rats by reducing high blood pressure, decreasing hindlimb vascular resistance and increasing hindlimb blood flow when compared to control rats ($p < 0.05$). Co-treatment with ellagic acid also significantly attenuated hypertension-induced oxidative stress by decreased vascular superoxide ($O2^{\bullet -}$) production, plasma malondialdehyde (MDA) levels, and down-regulation NADPH oxidase subunit p47phox ($p < 0.05$). Moreover, ellagic acid was associated with the restoration endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) expression as indicated by and increased plasma NO metabolite (NOx) levels ($p < 0.05$). Furthermore, the cardiovascular structural alterations found in hypertensive rats were prevented by ellagic acid (decreased collagen in left ventricle and thoracic aorta and decreased VSMCs in the thoracic aorta whereas increased elastin deposition in the thoracic aorta), which was consistent with reduced aortic matrix metalloproteinases-2 (MMP-2) and matrix metalloproteinases-9 (MMP-9) levels ($p < 0.05$). The present results indicate that ellagic acid attenuates hypertension by reducing NADPH oxidase subunit p47phox expression, which prevents oxidative stress and restores NO bioavailability.

Keywords : Ellagic acid, hypertension, oxidative stress, ventricle, aorta

บทคัดย่อ

รหัสโครงการ: MRG5680036

ชื่อโครงการ: ผลของอีลาจิก แอสิด ต่อการปรับเปลี่ยนโครงสร้างของหัวใจหนูที่มีภาวะพร่องไนตริกออกไซด์

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อีลาจิก แอสิด คือสาร polyphenol ที่พบในถั่ว และผลไม้หลายชิดเช่น สตอเบอรี่ ราสเบอรี่ และ ทับทิม มีรายงานว่าสารชนิดนี้มีคุณสมบัติต้านอนุมูลอิสระ และต้านการอักเสบ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลการป้องกันของอีลาจิก แอสิด ต่อความดันเลือด พลศาสตร์การไหลเวียนเลือด ภาวะเครียดออกซิเดชัน ภาวะอักเสบและภาวะการเปลี่ยนแปลงโครงสร้างของหลอดเลือดในหนูขาวความดันเลือดสูงที่ถูกเหนี่ยวนำด้วยสารแอลเนม หนูขาวเพศผู้พันธุ์ Sprague-Dewley ได้รับสารแอลเนม (40 มก./กก./วัน) เป็นเวลา 5 สัปดาห์ ในระหว่างที่ให้สารเอลเนม หนูขาวที่มีความดันเลือดสูงจะได้รับการป้อนด้วยอีลาจิก แอสิด (7.5 และ 15 มก./กก./วัน) หรือตัวทำละลาย หลังสิ้นสุดการทดลองพบว่าหนูขาวที่มีความดันเลือดสูงที่ได้รับกรดแอลลาจิคเพิ่มประสิทธิภาพพลศาสตร์การไหลเวียนเลือดในหนูขาวความดันเลือดสูงที่ถูกเหนี่ยวนำด้วยสารแอลเนม โดยลดความดันเลือด ลดความต้านทานของหลอดเลือดที่ไปเลี้ยงอวัยวะส่วนล่างและขาหลัง และเพิ่มปริมาณเลือดที่ไปเลี้ยงอวัยวะส่วนล่างและขาหลังอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับหนูทดลองความดันเลือดปกติ ($p < 0.05$) การให้อีลาจิก แอสิดร่วมด้วยยังสามารถลดภาวะเครียดออกซิเดชันและการอักเสบที่เกิดจากภาวะความดันเลือดสูง โดยลดการสร้างสารซุบเปอร์ออกไซด์ (superoxide; $O2^{\bullet -}$) ในหลอดเลือด ลดระดับของมาลอนไดอัลดีไฮด์ (malondialdehyde; MDA) ในพลาสมา และลดการแสดงออกของ NADPH oxidase subunit p47phox ($p < 0.05$) นอกจากนี้อีลาจิก แอสิด ยังมีความสัมพันธ์กับการปรับเปลี่ยนการแสดงออกของโปรตีน endothelial nitric oxide synthase (eNOS) ซึ่งบ่งบอกได้โดยการเพิ่มขึ้นของระดับพลาสมา NOx ($p < 0.05$) นอกจากนี้โครงสร้างของหลอดเลือดที่เปลี่ยนแปลงไปในหนูขาวที่มีภาวะความดันเลือดสูงสามารถป้องกันได้โดยอีลาจิก แอสิด ด้วยการลดการสะสมคอลลาเจนทั้งในผนังหัวใจห้องล่างข้างซ้าย และในหนังหลอดเลือดเลือดแดงใหญ่อก ร่วมถึงลดจำนวนเซลล์กล้ามเนื้อหลอดเลือด และเพิ่มการสะสมอิลาสติน ในหนังหลอดเลือดเลือดแดงใหญ่อก ซึ่งสอดคล้องกับการลดลงของระดับ matrix metalloproteinases-2 (MMP-2) และ matrix metalloproteinases-9 (MMP-9) ($p < 0.05$) ผลของการศึกษาครั้งนี้บ่งบอกว่าอีลาจิก แอสิด สามารถลดภาวะความดันเลือดสูงโดยลดการแสดงออกของ p47phox NADPH oxidase subunit ซึ่งป้องกันภาวะเครียดออกซิเดชัน และฟื้นฟูปริมาณ NO ที่ไปออกฤทธิ์ นอกจากนี้กรดแอลลาจิคยังมีบทบาทในการเปลี่ยนแปลงโครงสร้างของผนังหัวใจและหลอดเลือดโดย ลดระดับของ MMP-2 และ MMP-9

คำหลัก: อีลาจิก แอสิด, ความดันเลือดสูง, ภาวะเครียดออกซิเดชัน, หัวใจห้องล่าง, หลอดเลือดแดงใหญ่

(1) วัตถุประสงค์ของโครงการ

1.1 To examine the effect of ellagic acid on blood pressure in rat treated with L-NAME

1.2 To evaluate an oxidative maker in rat treated with L-NAME and ellagic acid

1.3 To study geometry of left ventricular wall of rat treated with L-NAME and ellagic acid

1.4 To evaluate the amount of fibrosis of heart in rat treated with L-NAME and ellagic acid

1.5 To study vascular wall remodeling in rat treated with L-NAME and ellagic acid

Experimental design for objective No 1.1.1. To examine effect of ellagic acid on blood pressure in rat treated with L-NAME

After 1 week of acclimatization, the animals were randomly divided into major experimental studies as follows;

Group of experiment

Rats were received L-NAME 40 mg/kg/day in their drinking water for 5 weeks together with daily intragastrically administration with a vehicle, distilled water (DW), and ellagic acid (EA) at dose of 15 mg/kg BW/day and 7.5 mg/kg/day. The animals were randomly divided into 4 groups with 10 animals in each group.

Group I Control + DW 1.5 ml/kg BW/day; p.o. for 5 weeks

Group II Control + EA 7.5 mg/kg/day; p.o. for 5 weeks

Group III Control + EA 15 mg/kg/day; p.o. for 5 weeks

Group IV L-NAME + DW 1.5 ml/kg BW/day; p.o. for 5 weeks

Group V L-NAME + EA 15 mg/kg/day; p.o. for 5 weeks

Group VI L-NAME + EA 15 mg/kg/day; p.o. for 5 weeks

At the end of experiment, the body weight was measured before anaesthetized with and intraperitoneal injection of Pentobarbital at the dose of 60 mg/kg. A tracheotomy were performed for spontaneous of breathing, and left femoral artery was cannulated with polyethylene catheter connected to a pressure transducer for continuous monitoring of blood pressure and heart rate (HR), using the Acqknowledge data acquisition analysis software (BIOPAC Systems Inc., California, USA). The catheter was filled with heparinized saline to prevent clotting. Baseline BP and HR values were monitored in animals for 10 minutes. After direct blood pressure measurement, rats were sacrificed by over dosage of the anesthetic drug.

Experimental design for objective No 1.1.2 To evaluate an oxidative maker in rat treated with L-NAME and ellagic acid

Carotid arteries were collected and immediately measured for superoxide ($O_2^{\bullet -}$) production assay, which blood sample was collected for the determination of plasma MDA.

1. Assay of vascular $O_2^{\bullet -}$ production

Productions of $O_2^{\bullet -}$ in vascular tissue were determined by lucigenin-enhanced chemiluminescence as previously described with some modification (Kukongviriyapan, 2007). The carotid artery were excised rapidly from animal after sacrified and placed in ice-cold saline and dissected free of fat and adhering tissue. Vessel segments were cut into 1 cm in length and incubated with 1 ml oxygenated Krebs-Ringer bicarbonate solution at pH 7.4, 37 °C for 30 min. Lucigenin 100 µM were added in sample tube and place in a luminometer (Turner Biosystems, CA, USA.). Luminometer counts were integrated every 30 sec for 5 min and averaged. The vessels were dried at 45 °C for 24 hr and weighed. Vascular tissue $O_2^{\bullet -}$ productions were expressed as relative light unit count per min per dried weight of vascular tissues.

2. Assay of plasma malondialdehyde (MDA)

Malondialdehyde is one of the end products of unsaturated fatty acid peroxidation. Levels of MDA are often measured as an index of lipid peroxidation. It can react with thiobarbituric acid (TBA) in boiling water temperature to form a colored complex called thiobarbituric acid-reactive substance (TBARS), which can be detected by spectrophotometric assay. The level of MDA was assayed following a previous described method of Luangaran and coworkers (2007). In brief; 150 µl of plasma was reacted with 10 % trichloroacetic acid (TCA), 125 µl of 5 mM ethylenediamine tetraacetic acid (EDTA), 125 µL of 8 % sodium dodecylsulfate (SDS) and 10 µl of 0.5 µl/ml of butylated hydroxytoluene (BHT). The mixture was left for 10 min, then 0.6 % TBA was added in an equal volume and the mixture was heated for 30 min in a boiling water bath. After cooling to room temperature, the mixture was centrifuged 10,000 g for 5 minutes at 25 °C. The absorbance of the supernatant was measured at the wavelength of 532 nm by spectrophotometry. A standard curve was generated using appropriate concentrations of standard tetraethoxypropane (0.3-10 µmol/)

3. Assay of plasma nitric oxide metabolites or nitrate/nitrite

Accumulation of nitrate/nitrite, the end products of NO metabolism was used as indices of NOS activity by using Griess reagents as previously described (Nakmareong et al.). The resulting reaction product, azoic compound, can be measured spectrophotometrically at wavelength 540 nm. Briefly, plasma samples were deprotenized by ultrafiltration using centrifugal concentrators (NANOSEPTM, Pl Filtration, USA). The supernatant was mixed with 1.2 µM NADPH, 4 mM glucose-6-phosphate disodium (G-6-P), 1.28 unit/ml glucose-6-phosphate dehydrogenase (G-6-PD) and 0.8 unit nitrate reductase, and then incubated at 30 °C for 30 minutes. Then, the mixture was reacted with a Griess solution (4% sulfanilamide in 0.3% naphtylenediamine dichloride, NED) for 15 minutes. The absorbance of samples was measured by an enzyme-linked immunosorbent assay (ELISA) plate reader with a filter wavelength of 540 nm (Tecan GmbH., Groding, Australia). A standard curve was established with a set of a serial dilution of NaNO2.

4. Western blot analysis in the aortic tissues of p47phox NADPH oxidase subunit and eNOS protein

Expressions of the p47phox NADPH oxidase subunit and eNOS proteins in aortic homogenates were determined following a previously described method with some modifications (Mukai and Sato, 2009; Nakmareong et al., 2011). In brief, the thoracic aortas were homogenized in cell lysis buffer (Cell Signaling Technology, Inc., MA, USA) and centrifuged at 4°C and 12000 rpm for 30 minutes. The supernatant was collected and the protein content was analyzed by the Bradford dye-binding method (Bradford, 1976). A total of 15 µg of protein per sample was separated on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) by an electrophoresis system and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% skimmed milk in phosphate buffered saline with 0.1% Tween-20 (PBST) at room temperature for 2 hours and incubated overnight at 4°C with primary antibody of mouse monoclonal anti-p47phox (1:2500, Santa Cruz Biotechnology, CA, USA) or mouse monoclonal anti-eNOS (1:2500, BD Biosciences, CA, USA). The membranes were washed with PBST 3 times for 7 minutes before being incubated with the secondary antibody, horseradish peroxidase goat anti-mouse IgG (1:2500, Santa Cruz Biotechnology, CA, USA), for 2 hours at room temperature. The blots were developed in Amersham™ ECL™ Prime solution (Amersham Biosciences Corp., Piscataway, NJ,USA), and densitometric analysis was performed using an ImageQuant™ 400 imager (GE Healthcare Life Sciences, Piscataway, NJ, USA). Goat polyclonal IgG to β-actin (1:5000, Santa Cruz

Biotechnology, CA, USA) was used as a loading control. The intensity of the specific p47phox and eNOS protein bands were normalized to that of β-actin, and data were expressed as a percentage of the values determined in control aorta from the same gel.

Experimental design for objective No 1.1.3 To study geometry of left ventricular wall of rat treated with L-NAME and ellagic acid

The hearts were rapidly removed and left ventricle was isolated and bisected coronally at the midventricular position, equidistant between base and apex. The clotted blood in the heart chamber was removed. Both pieces were dried with fitter paper before weighing. The atria and the right ventricle were removed. The left ventricle was weighed. Then, the heart was fixed 24 h in 10% formalin and dehydrated in graded series of ethanol (70%, 80%, 95% and 100%) for 30 minutes in each step. The clearing processes were performed with 3 changes of xylene for 30 minutes each. After clearing with xylene, the specimens were infiltrated in the mixture of xylene and paraffin at the ratio of 2:1, 1:1, 1:2 and pure paraffin for 1 h per each, respectively. The specimens were embedded in paraffin block. Specimen blocks were serially cut at 5 μm thickness from the midventricular surface, either to the base or to the apex. The sections were stained with Hematoxylin and Eosin (H&E) to investigate the general appearance in the heart ventricle. Picrosirius red staining was applied to investigate the fibrosis of the left ventricle.

Morphometric evaluation of the heart was performed by capturing heart images with stereoscope (Nikon SMZ745T with NIS-elements D 3.2) at 1x objective lens. After that Image-J (NIH image analysis software) was used for morphometric evaluation as follows:

1. The left ventricular wall thickness was measured every 45° interval around the cardiac circumference as shown in figure 6. The average value was calculated for each section.

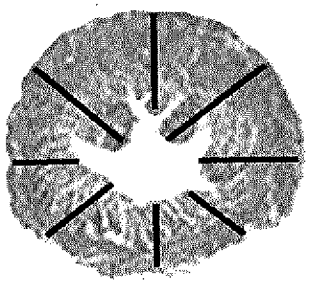

Figure 1 A scheme shows the method for measuring the left ventricular wall thickness.

2. The whole heart section and a known length of calibrator were captured by using stereoscope. Cross sectional area (CSA) (Figure 2C) was calculated by using the difference between the value of the external circumferential area of the heart (Figure 2A) and the chamber area (Figure 2B).

3. The ventricular luminal area was measured by using the value of the chamber area of the heart (Figure 2B).

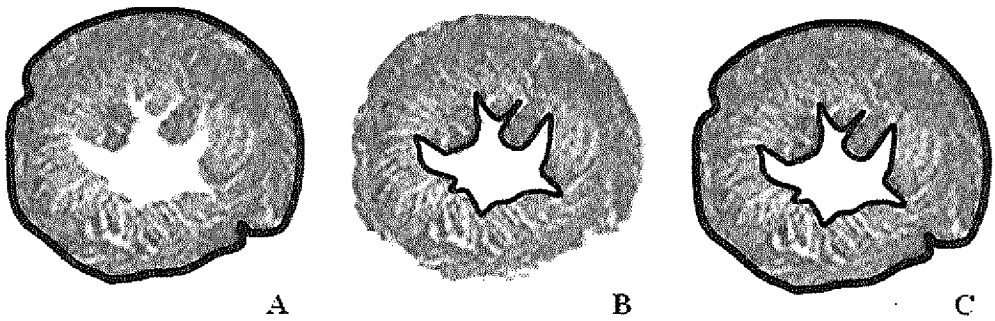

Figure 2 A scheme shows the method for measuring the cross sectional area of the left ventricle.

Experimental design for objective No 1.1.4 To evaluate the fibrosis amount of heart in rat treated with L-NAME and ellagic acid

The area of collagen fibers from four quadrants of the left ventricular cross section were observed under normal and polarized light microscope (Nikon ECLIPSE LV100POL with NIS-elements AR 3.1). Images from each quadrant which captured with normal light microscopy was used for analyzing the total area of heart tissue by using Image J software. Whereas images from each quadrant which captured with polarized light microscope were used for evaluation the amount of collagen fibers in the interstitial area of the heart section by Photoshop CS2 software and Image J software.

The percentage of interstitial fibrosis of left ventricular myocardium was calculated using the formula:

$$\text{Interstitial fibrosis (\%)} = \frac{Area\ of\ collagen\ fibers}{Total\ area\ of\ herat\ tissue} \text{ X } 100$$

Experimental design for objective No 1.1.5: To study vascular wall remodeling in rat treated with L-NAME and ellagic acid

1. Tissue preparation

After hemodynamic measurements as described above, the thoracic cavity was opened. The thoracic aorta was fixed by immersed in 4% phosphate-buffered paraformaldehyde solution for 24 hours. The specimens were dehydrated in graded series of ethanol (70%, 80%, 95% and 100%) for 30 minutes in each step. The clearing processes were performed with 3 changes of xylene for 30 minutes per each. After clearing with xylene, the specimens were infiltrated in the mixture of xylene and paraffin at the ratio of 2:1, 1:1, 1:2 and pure paraffin for 1 hour per each, respectively. The specimens were embedded in a paraffin block. Five- micrometer thick sections were prepared for staining. After that the specimens were stained by 3 different dyes for investigating the characteristic of vascular smooth muscle, elastic fibers and collagen fibers as follows;

1. Hematoxylin and Eosin staining were used to investigate vascular smooth muscle cells (VSMCs) and their general appearance in the vascular wall.

2. Miller's elastic staining was used to investigate elastic fibers in the vascular wall.

3. Picrosirius Red staining was used to investigate collagen fibers in the vascular wall.

2. Morphometric evaluation of thoracic aorta

Morphometric evaluation of both thoracic aorta was performed by captured artery images with Digital sight DS-2MV, Nikon, Japan at x200. Media cross-sectional area (CSA) was measured in tissue sections (40x), which was calculated by the external area (Ae) minus the lumen internal area (Ai). The external radius (Re) and the internal radius (Ri) were calculated as the square root of Ae/π and Ai/π, respectively. The medial thickness (M) of the vascular wall (tunica intima+tunica media) was calculated by subtracting the Re from the Ri. Finally, media to lumen ratio (M/L) was calculated by using the wall thickness divided by the radius of the lumen (Castro et al., 2009).

The number of VSMCs was obtained by counting their nuclei in the sections stained with H&E. The area fraction of elastin and collagen in aortic wall was evaluated by automatically counting threshold pixels stained with Miller's elastic staining or Picrosirius Red and dividing by a total number of medial pixels. These methods were assessed by means of image analysis software (Image-Pro Plus, Media Cybernetics, MD, USA) from tree consecutive sections (twelve aortic wall images) per animal. All measurements were made by one observer and preliminary observations

of intra-observer repeatability showed a coefficient of variation of less than 5% for all estimations of threshold area.

3. Immunohistochemistry study

To determine the expression of MMP-2 and MMP-9 in the thoracic aortas, paraffin-embedded tissue sections were placed on silane-treated slides, and heated at 60°C for 1 hour in the oven. After heated, the aortic sections were stained with specific antibodies (AB37150; Abcam, AB19016; Millipore for MMP-2 and MMP-9, respectively) and the R.T.U. Vectastain ABC kit (Vector Laboratories) as recommended by the manufacturer.

Briefly, the arterial sections were sequentially deparaffinized with 2 changes of xylene for 3 minutes per each. Then remove the xylene in alcohol for 2 changes of 3 minutes per each. After removing xylene with alcohol, the arterial sections were placed into the sink and wash in tap water for 5 minutes. Thereafter, MMP-2, the arterial sections were treated with the antigen retrieval using microwave full power in Tris-EDTA-citrate unmasking solution, pH 8.1 for 35 minutes. Whereas, MMP-9 were treated with antigen retrieval using microwave full power in Vector unmasking solution, pH 6 for 35 minutes. MMP-2 and MMP-9 sections were covered with 3% endogenous peroxidise for 15 minutes and normal blocking serum for 20 minutes (Normal Horse Serum from the R.T.U. kit). Then MMP-2 and MMP-9 sections were incubated with primary antibody solution dilution using antibody diluents, whereas negative control sections apply antibody diluents alone for 3 hours at room temperature (MMP-2 1:150 and MMP-9 1:25). The Universal Biotinylated Secondary Antibody (Biotinylated Anti Rabbit and Mouse from the R.T.U. kit) were used to cover each section for 30 minutes, then applied ABC reagent (the R.T.U. kit) for 30 minutes. Finally, the slide was covered with DAB solution for 5 minutes. The binding was displayed as a dark brown color. The images were captured using light microscopy (40X). MMP-2 and MMP-9 expressions will be assessed by quantifying the area fractions from 4 fields for each slice using the image analysis program.

The percentage of immune-stained MMP-2 and MMP-9 in the aortic wall was quantified in a similar way as collagen and elastin, by counting the threshold pixels stained for MMP-2 and MMP-9 using the Image-Pro Plus Program.

Immunohistochemical staining of MMP-2 and MMP-9 were semi-quantified intensity score by an arbitrary scale of weakness staining as 1, moderate staining as 2 and strong staining of brown color as 3. MMP-2 and MMP-9 were calculated from the percentage of area fraction multiply by intensity score and expressed as an arbitrary unit.

(3) ผลงานวิจัยที่ได้รับ

1. Effect of ellagic acid on blood pressure in rat treated with L-NAME

At the end of the experiment, arterial blood pressure and heart rate was measured. SBP, mean arterial pressure (MAP), DBP, pulse pressure (PP) and HR in rat treated with L-NAME only significantly increased when compared to those in the control rats. In contrast, rats receiving L-NAME together with EA at doses 7.5 and 15 mg/kg/day showed a significantly decreased in SBP, MAP, DBP, PP and HR when compared to L-NAME rats. Administration at high dose of EA more effectively reduced in SBP, MAP and DBP than low dose of EA. However, there were no significantly different in PP and HR between these two. Control rats treated with the same concentration of EA in the group treated with L-NAME had no a significant change in all parameters when compared to the control group (Table 1).

2. Effect of ellagic acid on oxidative stress markers

2.1. Vascular $O_2^{\cdot-}$ production

The vascular $O_2^{\cdot-}$ production in L-NAME-induced hypertensive rats (176.46±14.08 counts/min/mg dry weight) was significantly higher than control rats (64.36±8.91 counts/min/mg dry weight) (p<0.05). Treatment with EA at doses 7.5 mg/kg/day (113.22±12.96 counts/min/mg dry weight) and 15 mg/kg/day (77.85±5.27 counts/min/mg dry weight) for 5 weeks significantly decreased the vascular $O_2^{\cdot-}$ production when compared to hypertensive rats (p<0.05). Administration of EA inhibited a progressive increase in vascular $O_2^{\cdot-}$ production dose dependently, especially at high dose more effectively decreased of vascular $O_2^{\cdot-}$ production than low dose (Figure 1).

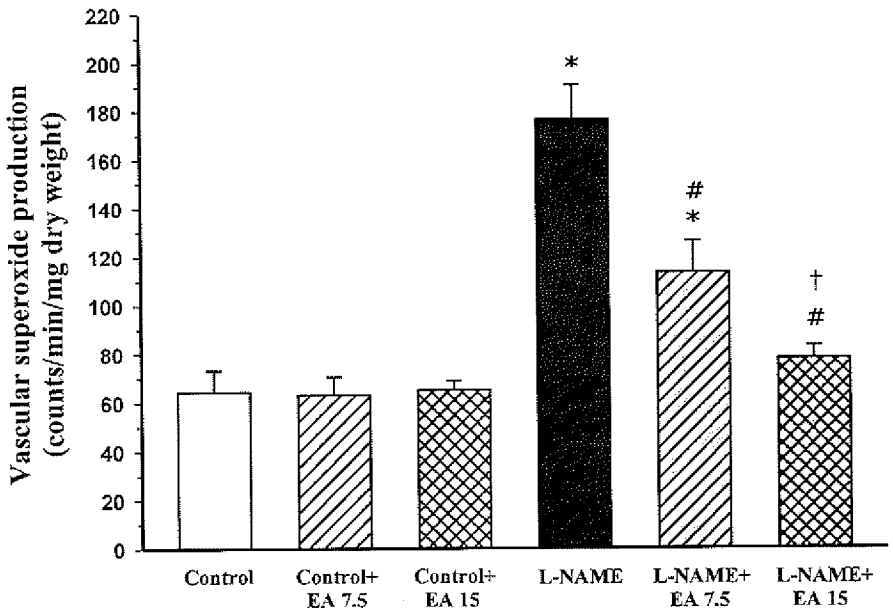

Figure 1 Effect of 5-week L-NAME and EA on superoxide production in the carotid arteries. Results are expressed as mean±SEM. Each group contains 10 animals. *p<0.05 vs. control group, #p<0.05 vs. L-NAME group

Table 1 Effect of EA on direct blood pressure, mean pulse pressure and heart rate

	Control	Control+EA 7.5 mg/kg	Control+EA 15 mg/kg	L-NAME	L-NAME+EA 7.5 mg/kg	L-NAME+EA 15 mg/kg
SBP (mmHg)	118.23±1.65	118.28±0.98	120.33±0.62	203.01±5.22*	169.26±3.23*#	159.18±3.37*#†
MAP (mmHg)	92.99±1.12	92.24±0.79	93.49±1.20	167.92±3.61*	138.65±3.21*#	128.52±2.90*#†
DBP (mmHg)	76.65±1.41	77.01±1.20	76.42±1.13	144.00±3.08*	115.28±3.17*#	107.25±2.42*#†
PP (mmHg)	41.61±1.73	42.26±0.82	43.95±0.77	59.21±3.25*	53.99±1.15*#	51.93±1.71*#
HR (beats/min)	357.90±6.04	357.10±8.37	354.99±5.80	432.64±2.56*	371.72±8.15#	363.91±4.55#

Values are mean±SEM. Each group contains 10 animals.

*p<0.05 vs. control group, #p<0.05 vs. L-NAME group, †p<0.05 vs. L-NAME+EA 7.5 mg/kg/day group

2.2. Plasma malondialdehyde

Plasma MDA level was higher in L-NAME-induced hypertensive rats (10.49±1.00 μM) comparing to that of control rats (3.31±0.10 μM) (p<0.05). Administration of EA at doses 7.5 mg/kg/day (5.67±0.23 μM) and 15 mg/kg/day (4.85±0.52 μM) significantly decreased the oxidative stress by reducing the level of plasma MDA when compared to hypertensive rats (p<0.05). Administration high dose of EA more effectively to reduce in plasma MDA level than low dose of EA. However, there were no significantly different between these two (Figure 2).

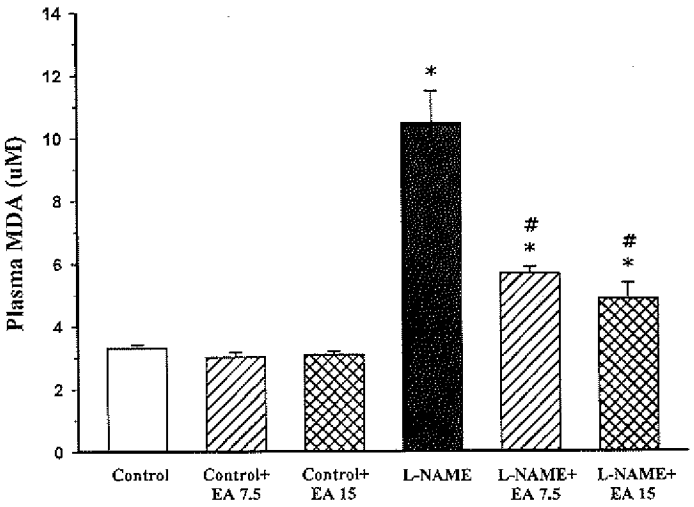

Figure 2 Effect of 5-week L-NAME and EA on plasma MDA. Results are expressed as mean±SEM. Each group contains 10 animals. *p<0.05 vs. control group, #p<0.05 vs. L-NAME group

2.3 Effects of ellagic acid on nitrate/nitrite production and eNOS protein expression

The concentration of nitrate/nitrite NO metabolites in plasma is shown in Figure 3A and the expression of eNOS protein in isolated rat aortas are shown in Figure 10B. The levels of NO metabolites in plasma were significantly reduced in L-NAME rats when compared with normal control rats (2.9 ± 0.2 vs.11.2 ± 0.6 μmol/L, $p < 0.05$) and administration of L-NAME was associated with down-regulation of eNOS protein expression compared to control rats ($p < 0.05$). There was no change in plasma nitrate/nitrite levels or eNOS protein expression in control rats treated with ellagic acid alone (Figure 3B). Co-

treatment of L-NAME administered rats with both 7.5 mg/kg or 15 mg/kg ellagic acid for five weeks significantly restored nitrate/nitrite production (6.4 ± 0.5 vs. 7.1 ± 0.4 μmol/L, $p < 0.05$, respectively) and eNOS protein expression, $p < 0.05$, respectively).

2.4 Effect of ellagic acid on p47phox protein expression in aortic tissues

The expression of p47phox protein in L-NAME treated rats was significantly up-regulated compared to control rats ($p < 0.05$, Figure 13A). Positive correlation of p47phox protein expression and the values of the vascular $O2^{\bullet -}$ production was observed ($R = 0.770$; Figure 13B). Treatment of the L-NAME group with 7.5 mg/kg ellagic acid reduced the over-expression of p47phox protein ($p < 0.05$) and treatment with 15 mg/kg ellagic acid restored p47phox protein levels to normal (Figure 4).

3. Effect of EA on morphometry of the heart

3.1 Effect of EA on the left ventricular wall thickness

L-NAME administration produced the development of ventricular structural change as indicated by a significant increase in wall thickness of left ventricle (3.93±0.05 mm) when compared to the control group (2.92±0.05 mm) ($p<0.05$). Interestingly, treatment with EA at doses 7.5 mg/kg/day (3.12±0.04 mm) and 15 mg/kg/day (3.03±0.04 mm) showed a significant decrease in left ventricular wall thickness when compared to the L-NAME-induced hypertensive rats ($p<0.05$). Administration high dose of EA trended to decrease in left ventricular wall thickness than low dose of EA-treated group. However, there were no significantly different between these two (Figure 5).

3.2 Effect of EA on the left ventricular cross-sectional area

The cross sectional area in L-NAME-induced hypertensive rats showed a significant increase (86.32±1.91 mm^2) when compared to the control rats (66.38±1.12 mm^2) ($p<0.05$). Furthermore, L-NAME administration and treated with EA at doses 7.5 mg/kg/day (68.22±0.82 mm^2) and 15 mg/kg/day (66.45±1.33 mm^2) showed to be effectively reduce in cross sectional area of left ventricle when compared to the hypertensive and control rats ($p<0.05$). Administration high dose of EA trended to decrease in cross sectional area than low dose of EA-treated group. However, there were no significantly different between these two doses of EA treated group (Figure 6).

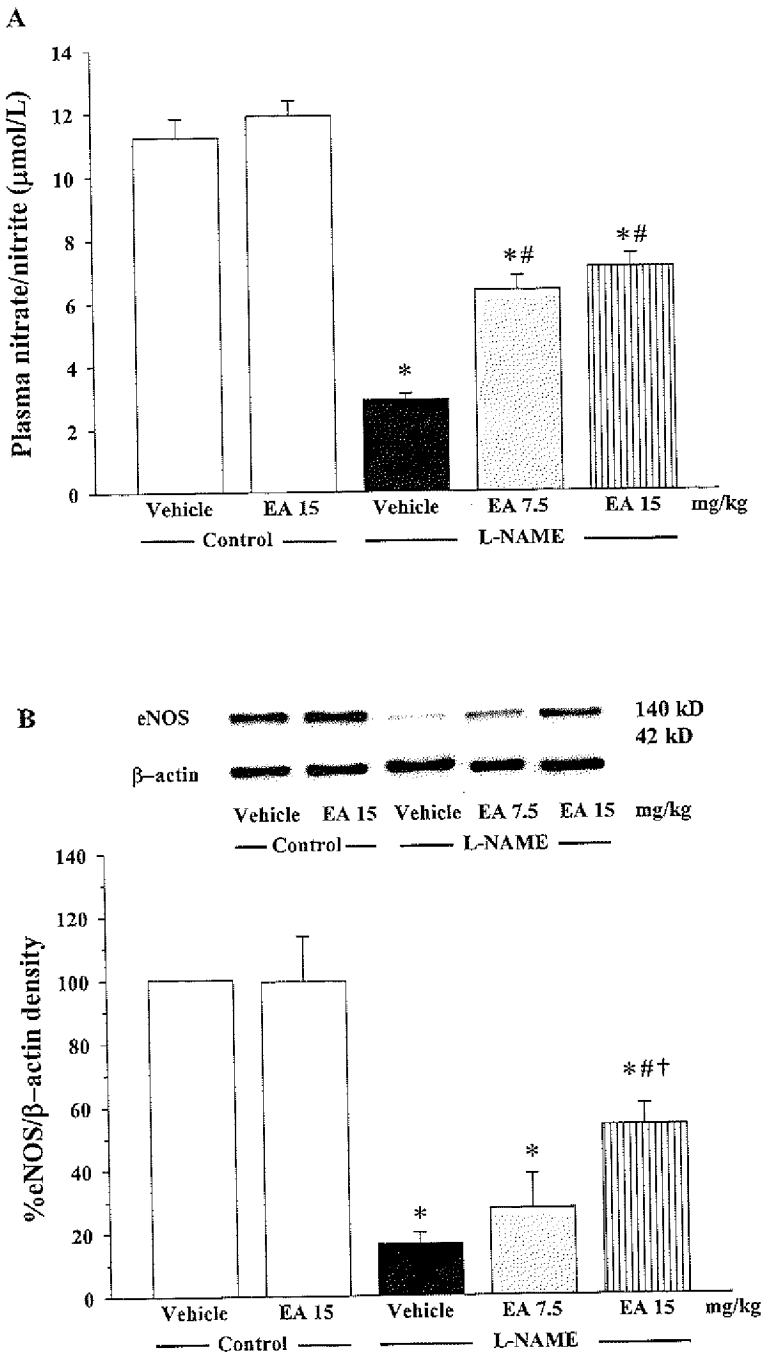

Figure 3 Effect of ellagic acid (EA) on **(A)** nitrate/nitrite production (n = 7/group) and **(B)** eNOS protein expression (n = 5/group). Values are expressed as mean ± SEM, * $p < 0.05$ when compared to control group, # $p < 0.05$ when compared to L-NAME group and † $p < 0.05$ when compared to L-NAME+EA 7.5 mg/kg group.

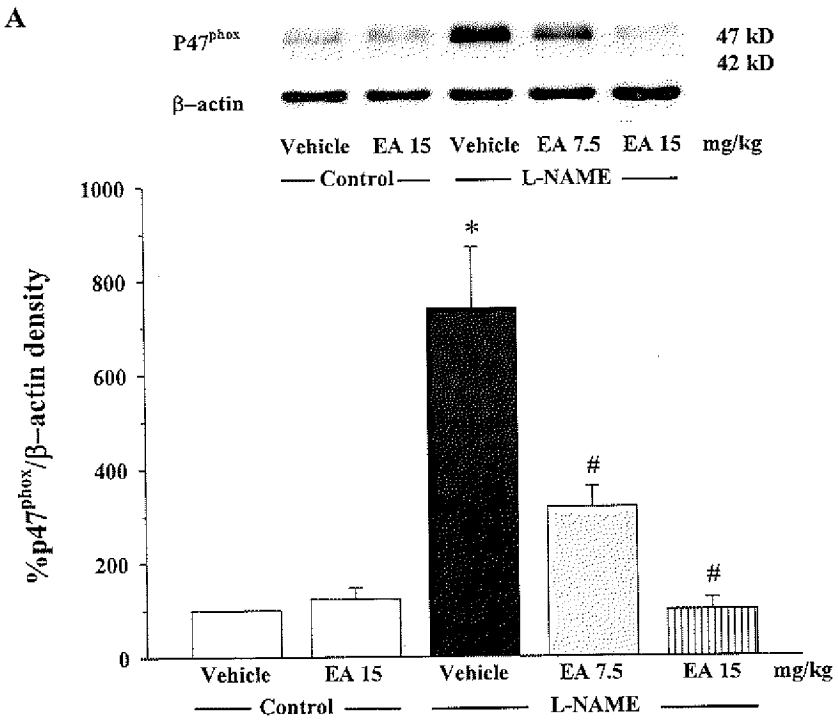

Figure 4 Effect of ellagic acid (EA) on (A) p47phox protein expression in aortic tissues. Values are expressed as mean ± SEM, (n = 5/group). * $p < 0.05$ when compared to control group and # $p < 0.05$ when compared to L-NAME group.

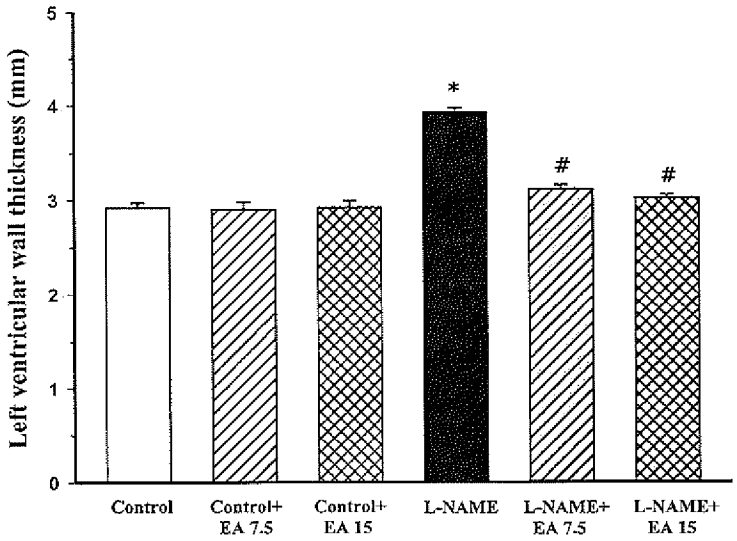

Figure 5 Effect of 5-week L-NAME and EA on left ventricular wall thickness. Results are expressed as mean±SEM. Each group contains 10 animals. *p<0.05 vs. control group, #p<0.05 vs. L-NAME group

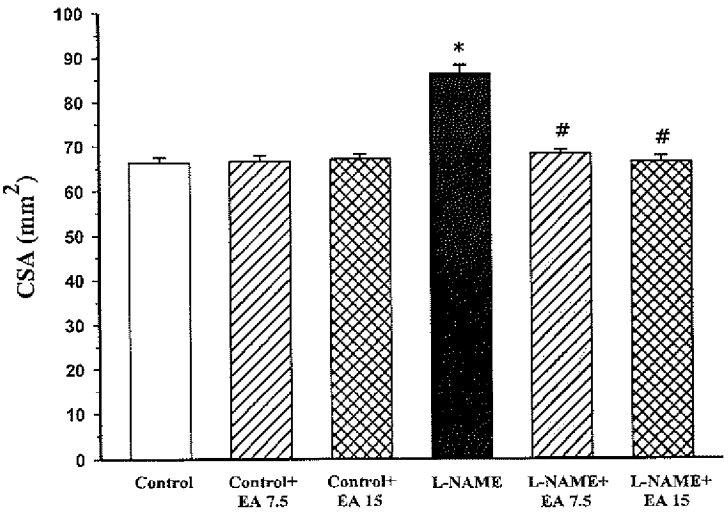

Figure 6 Effect of 5-week L-NAME and EA on cross sectional area. Results are expressed as mean±SEM. Each group contains 10 animals. *p<0.05 vs. control group, #p<0.05 vs. L-NAME group

3.3 Effect of EA on the left ventricular luminal area

After 5 weeks of L-NAME administration, the ventricular luminal area was significantly decreased (6.82±0.28 mm^2) when compared to the control group (11.47±0.49 mm^2) ($p<0.05$). L-NAME-treated with EA at doses 7.5 mg/kg/day (8.83±0.28 mm^2) and 15 mg/kg/day (9.83±0.43 mm^2) were able to improve the ventricular luminal area when compared to the hypertensive groups ($p<0.05$). High dose of EA supplementation trended to decrease in cross sectional area than low dose of EA supplementation. However, there were no significantly different between these two doses of EA treated group (Figure 7).

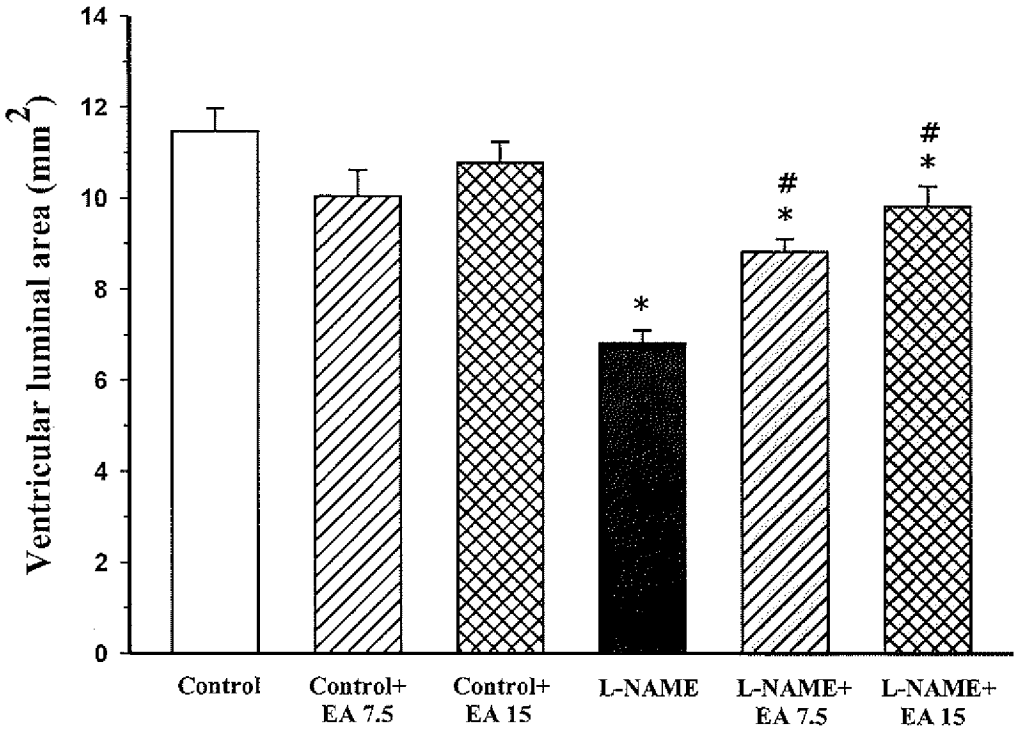

Figure 7 Effect of 5-week L-NAME and EA on ventricular luminal area. Results are expressed as mean±SEM. Each group contains 10 animals. *$p<0.05$ vs. control group, #$p<0.05$ vs. L-NAME group

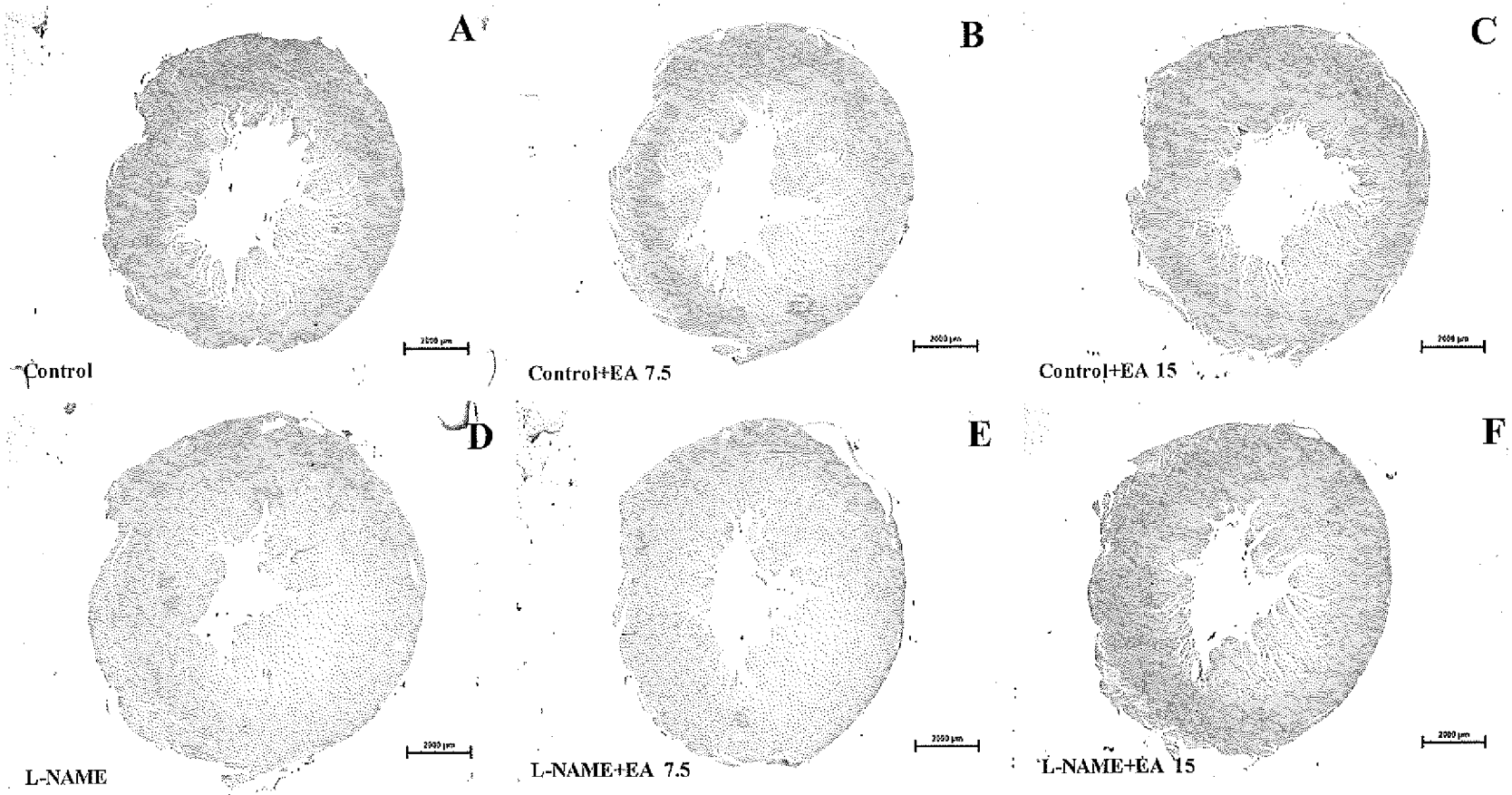

Figure 8 Morphology of left ventricle stained with H&E. Control (A), Control+EA 7.5 mg/kg/day (B), Control+EA 15 mg/kg/day (C), L-NAME (D), L-NAME+EA 7.5 mg/kg/day (E) and L-NAME+EA 15 mg/kg/day (F). The heart sections were captured by stereoscope at 1x objective lens.

4. Effect of ellagic acid on fibrosis amount of heart in rat treated with L-NAME

Chronic administration of L-NAME produced the development of myocardial fibrosis in left ventricle as indicated by a significant enhance interstitial fibrosis (5.02±0.38 %) when compared to the normotensive control rats (1.18±0.10 %) (p<0.05). Treatment with EA at doses 7.5 mg/kg/day (1.55±0.10 %) and 15 mg/kg/day (1.26±0.10 %) showed to be an effective for decrease in myocardial fibrosis of left ventricle when compared to the L-NAME-induced hypertensive rats (p<0.05). Interestingly, high dose of EA supplementation trended to decrease in myocardial fibrosis of left ventricle than low dose of EA supplementation (Figure 9, 10 and 11). Furthermore, L-NAME administration presented perivascular collagen accumulation higher than the normotensive control rats. In contrast, administration of L-NAME and treated with two doses of EA presented perivascular fibrosis lower than those in hypertensive rats.

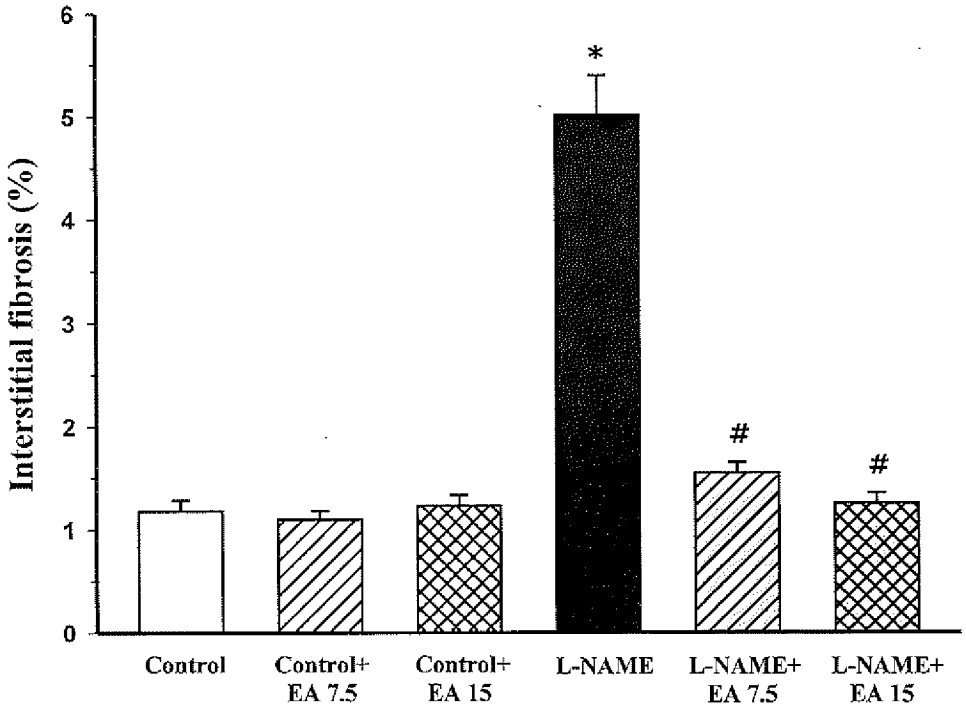

Figure 9 Effect of 5-week L-NAME and EA on myocardial fibrosis in interstitial regions of the left ventricle. Results are expressed as mean±SEM. Each group contains 10 animals. *p<0.05 vs. control group, #p<0.05 vs. L-NAME group

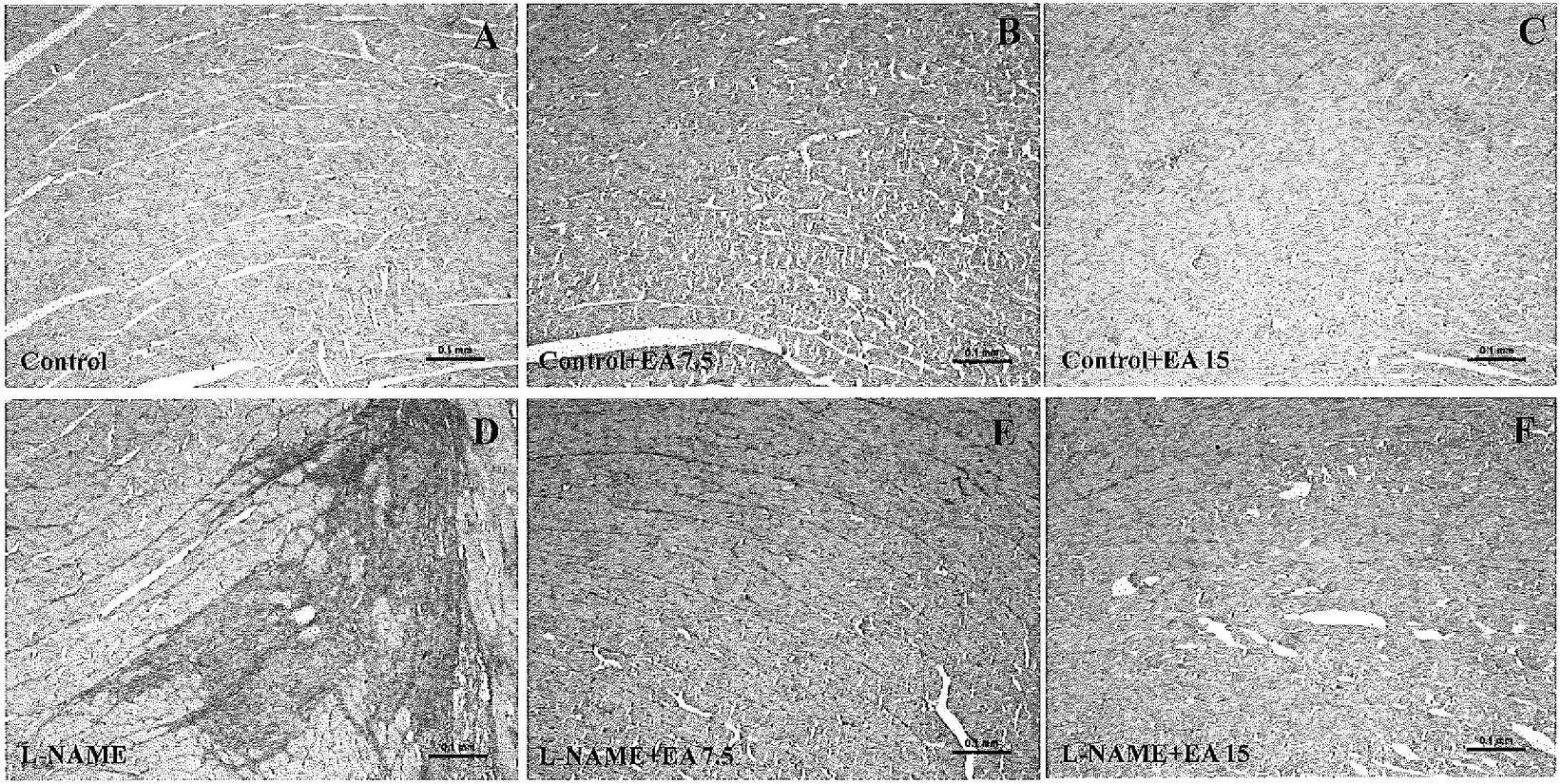

Figure 10 Myocardial fibrosis in interstitial regions of the left ventricle. Control (A), Control+EA 7.5 mg/kg/day (B), Control+EA 15 mg/kg/day (C), L-NAME (D), L-NAME+EA 7.5 mg/kg/day (E) and L-NAME+EA 15 mg/kg/day (F). Picrosirius red counterstained with Hematoxylin, polarized light microscopy (10x).

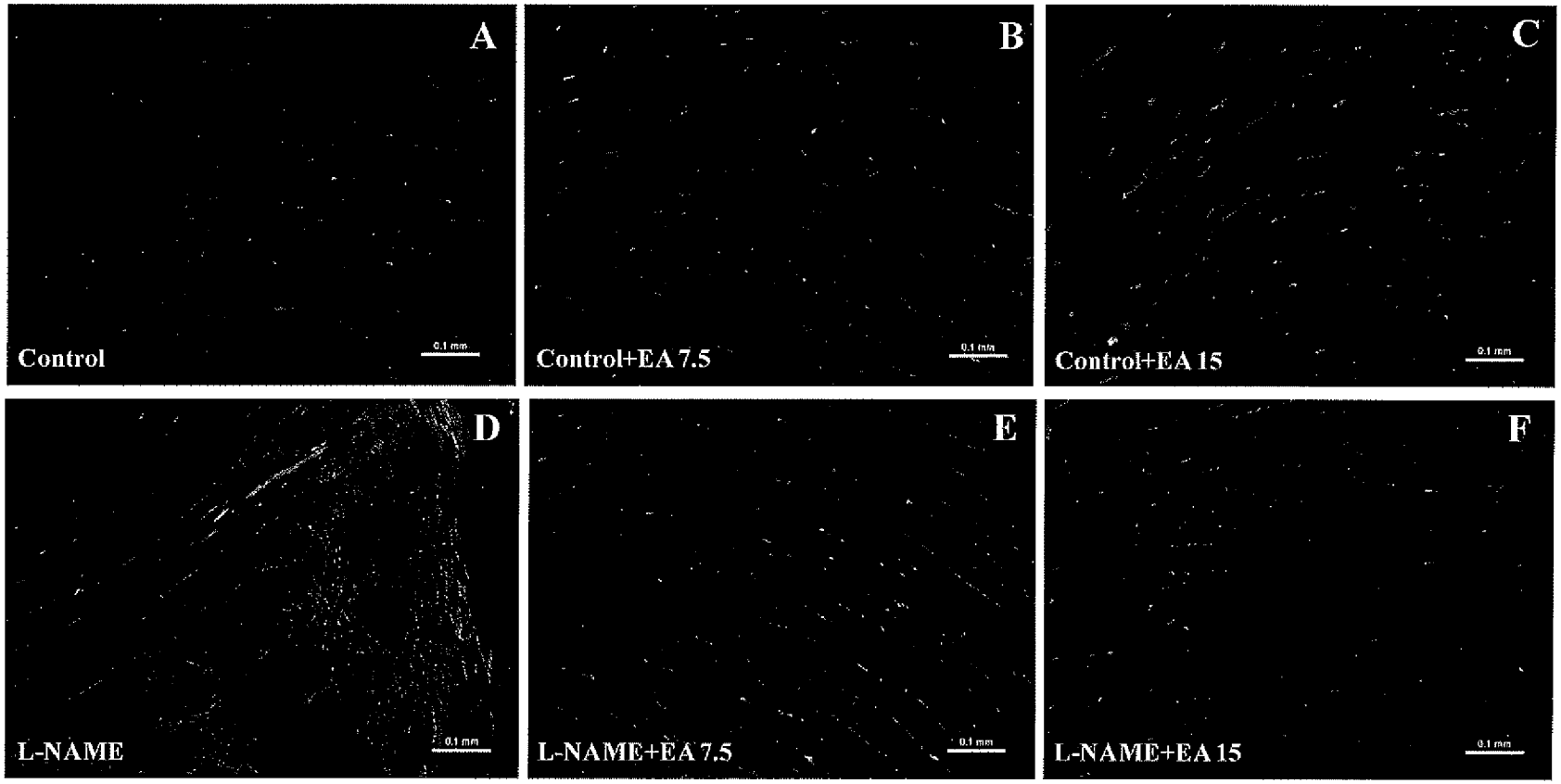

Figure 11 Analysis of picrosirius red-stained sections revealed myocardial fibrosis in interstitial regions of the left ventricle. Control (A), Control+EA 7.5 mg/kg/day (B), Control+EA 15 mg/kg/day (C), L-NAME (D), L-NAME+EA 7.5 mg/kg/day (E) and L-NAME+EA 15 mg/kg/day (F). Picrosirius red stain, polarized light microscopy (10x).

5. Effect of ellagic acid on vascular remodeling

Arterial wall hypertrophic remodeling was associated with the L-NAME hypertensive group, with significant increases in medial thickness, media CSA and M/L ratio ($p < 0.05$, Table 2), whereas there were no significant differences in the lumen areas (Table 2) after 5 weeks of L-NAME administration compared to control group. Moreover, the animals in the L-NAME group showed an increased number of VSMCs and collagen fibers per aortic CSA, whereas elastin fibers were decreased, suggesting medial hyperplasia were occurred in the L-NAME group ($p < 0.05$, Figure 12-14). After treatment with EA was prevented the morphological changes of the aortic wall in hypertensive rats, with significantly lower wall thickness, aortic CSA, M/L ratio, the number of VSMCs and collagen fibers, and higher elastin fibers in the L-NAME hypertensive rats ($p < 0.05$, Table 2 and Figure 12-14).

To confirm arterial wall hypertrophic remodeling findings, the immunohistochemical stained MMP-2 and MMP-9 in the aortic wall was quantified. The MMP-2 (Table 3, Figure 15) and MMP-9 (Table 3, Figure 16) levels in the aortas significantly increased in the L-NAME group compared with control group ($p < 0.05$). The increased MMP-2 and MMP-9 in L-NAME hypertensive group were significantly attenuated by EA ($p < 0.05$), whereas the MMP levels did not change in the control+EA treated group (Table 3, Figure 15 and 16).

Table 2 Effect of ellagic acid (EA) on the vascular structural modifications in the thoracic aorta. (Remodeling hypertrophy)

Parameters	Control		L-NAME		
	Vehicle	EA (mg/kg/day)	Vehicle	EA (mg/kg/day)	
		15		7.5	15
Wall thickness (µm)	104.13±1.67	108.96±1.33	149.91±1.77*	142.17±5.71*	128.86±1.18*,#
CSA (x10^3 µm^2)	604.78±14.58	632.32±15.69	820.37±4.99*	817.27±25.37*	757.40±16.99*,#
M/L ratio (%)	0.13±0.005	0.13±0.002	0.19±0.006*	0.18±0.009*	0.16±0.005*,#
Lumen area (x10^3 µm^2)	1917.19±102.24	2232.52±78.68	1915.48±83.31	2007.19±59.57	1967.15±92.02
VSMCs (cells/CSA)	1164.44±90.13	1140.90±51.89	2134.53±58.83*	1922.51±71.33*	1623.56±52.17*,#,†
Collagen contents (% area fraction)	12.55±1.29	11.52±0.49	24.66±0.68*	17.00±0.67*,#	13.06±0.70#,†
Elastin contents (% area fraction)	39.01±1.11	36.46±0.10	29.55±1.01*	30.76±0.84*	34.42±0.46*,#

Values are expressed as mean ± SEM, (n = 5/group). *$p < 0.05$ when compared to control group, # $p < 0.05$ when compared to L-NAME group and †$p < 0.05$ when compared to L-NAME+EA 7.5 mg/kg group.

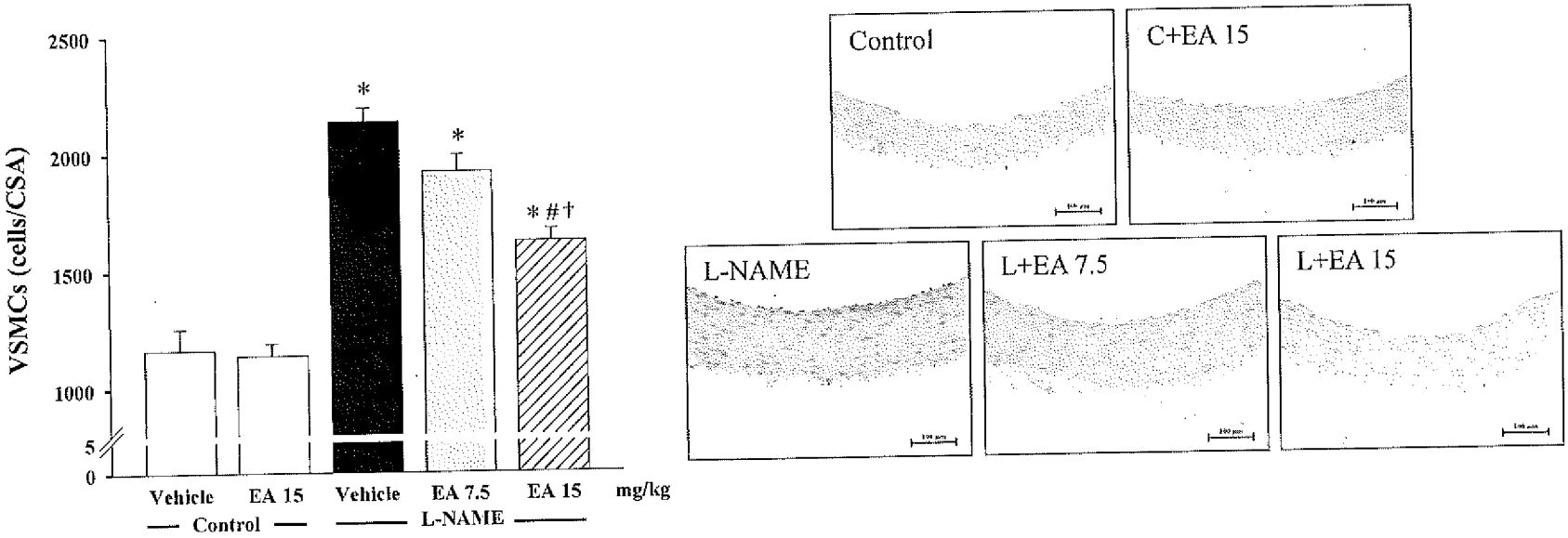

Figure 12 Effect of ellagic acid (EA) on vascular smooth muscle cells in the aortas of all experimental groups. The representative photographs of the aortic samples (x200) were stained with H&E stain for assessment of the number of vascular smooth muscle cells per cross-sectional area in the tunica media. Values are expressed as mean ± SEM, (n = 5/group). *$p < 0.05$ when compared to control group, # $p < 0.05$ when compared to L-NAME group and †$p < 0.05$ when compared to L-NAME+EA 7.5 mg/kg group.

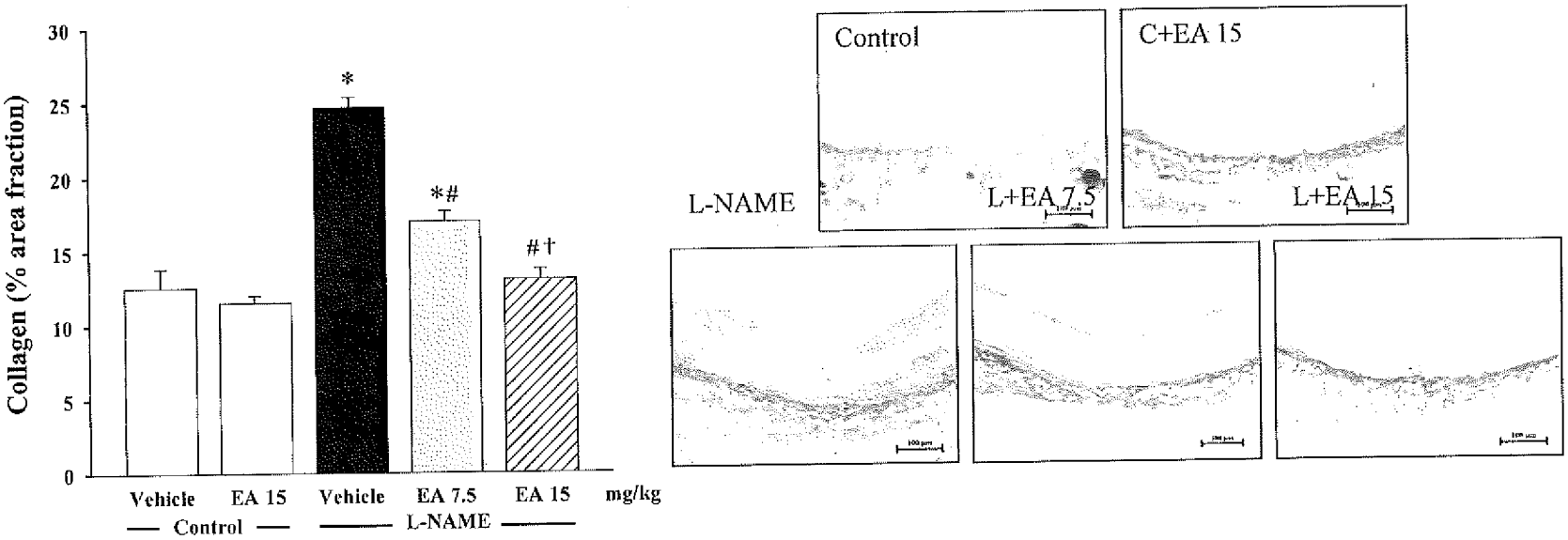

Figure 13 Effect of ellagic acid (EA) on collagen content in the aortas of all experimental groups. The representative photographs of the aortic samples (x200) were stained with Picrosirius Red stain for assessment of area fractions of collagen in the tunica media. Values are expressed as mean ± SEM, (n = 5/group). *p < 0.05 when compared to control group, # p < 0.05 when compared to L-NAME group and †p < 0.05 when compared to L-NAME+EA 7.5 mg/kg group

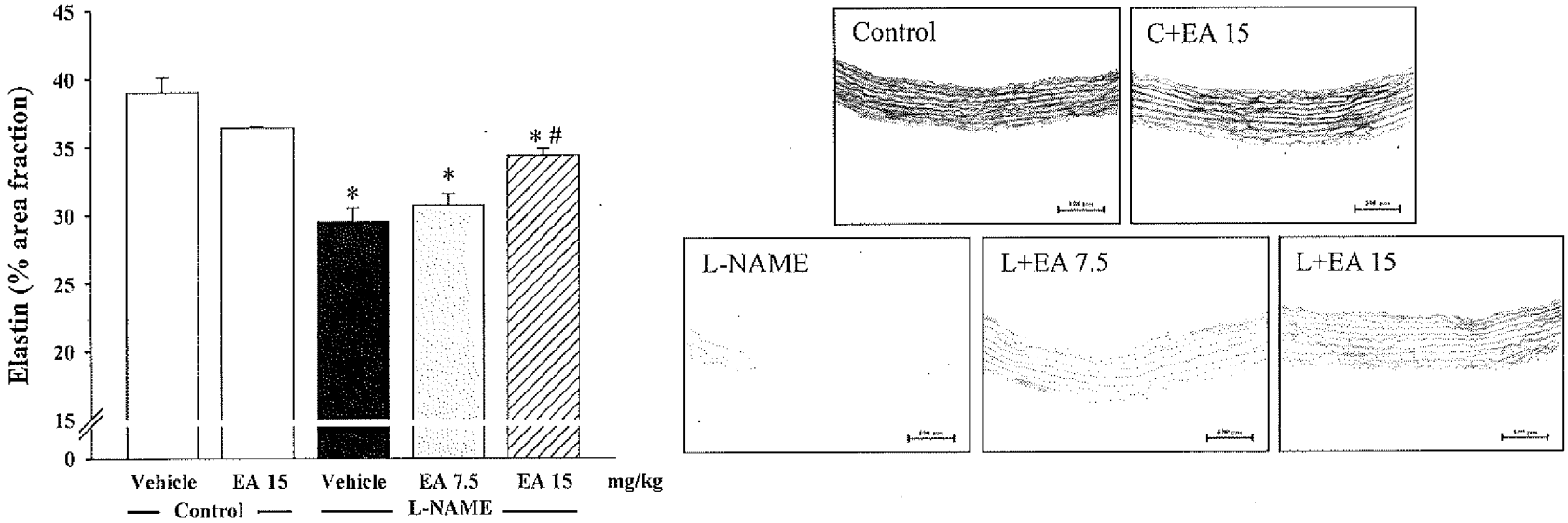

Figure 14 Effect of ellagic acid (EA) on elastin content in the aortas of all experimental groups. The representative photographs of the aortic samples (x200) were stained with Miller's elastic stain for assessment of the area fractions of elastin in the tunica media. Values are expressed as mean ± SEM, (n = 5/group). *p < 0.05 when compared to control group, # p < 0.05 when compared to L-NAME group and †p < 0.05 when compared to L-NAME+EA 7.5 mg/kg group.

Table 3 Effect of ellagic acid (EA) on MMP-2 and MMP-9 in the thoracic aorta.

Groups	Parameters	
	MMP-2 (Arbitrary Units)	**MMP-9** (Arbitrary Units)
Control	7.48±0.36	7.36±0.66
Control + EA 15 mg/kg	6.23±0.73	6.63±1.21
L-NAME	14.20±2.61*	14.78±2.71*
L-NAME + EA 7.5 mg/kg	7.89±0.60#	7.47±0.77#
L-NAME + EA 15 mg/kg	5.53±0.49#	4.25±0.39#

Values are expressed as mean ± SEM, (n = 5/group). *$p < 0.05$ when compared to control group and # $p < 0.05$ when compared to L-NAME group.

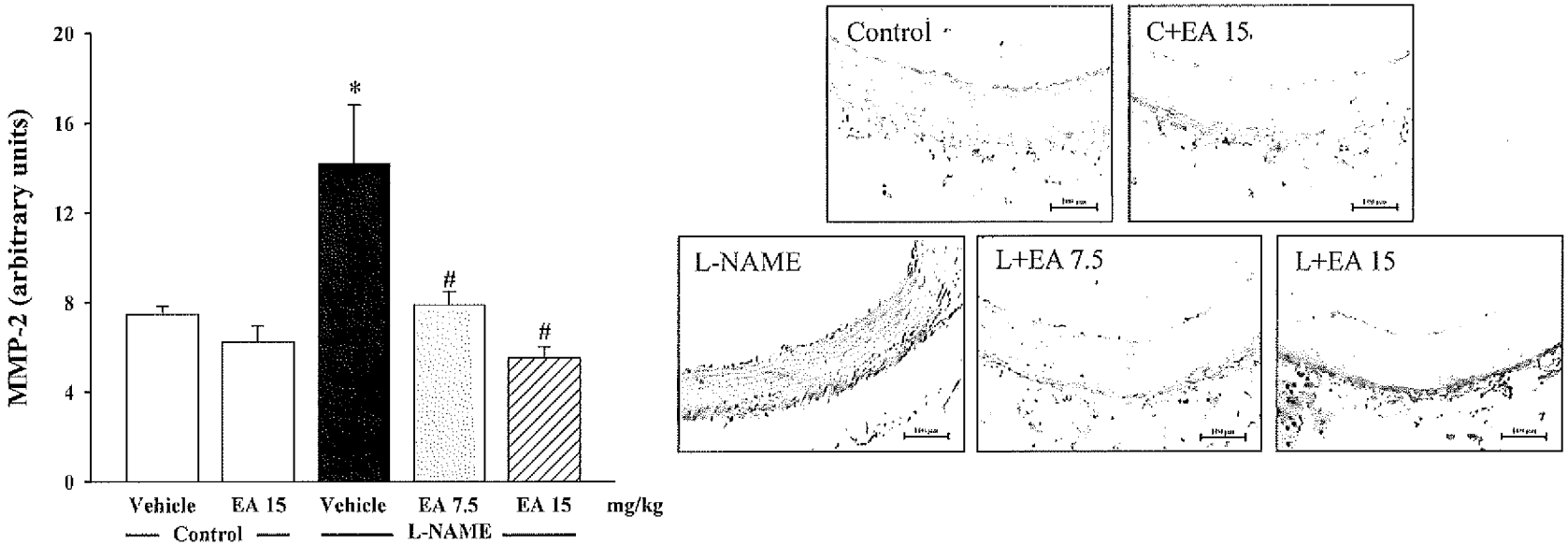

Figure 15 Effect of ellagic acid (EA) on MMP-2 localization in the aortas of all experimental groups. Values are expressed as mean ± SEM, (n = 5/group). *p < 0.05 when compared to control group and # p < 0.05 when compared to L-NAME group.

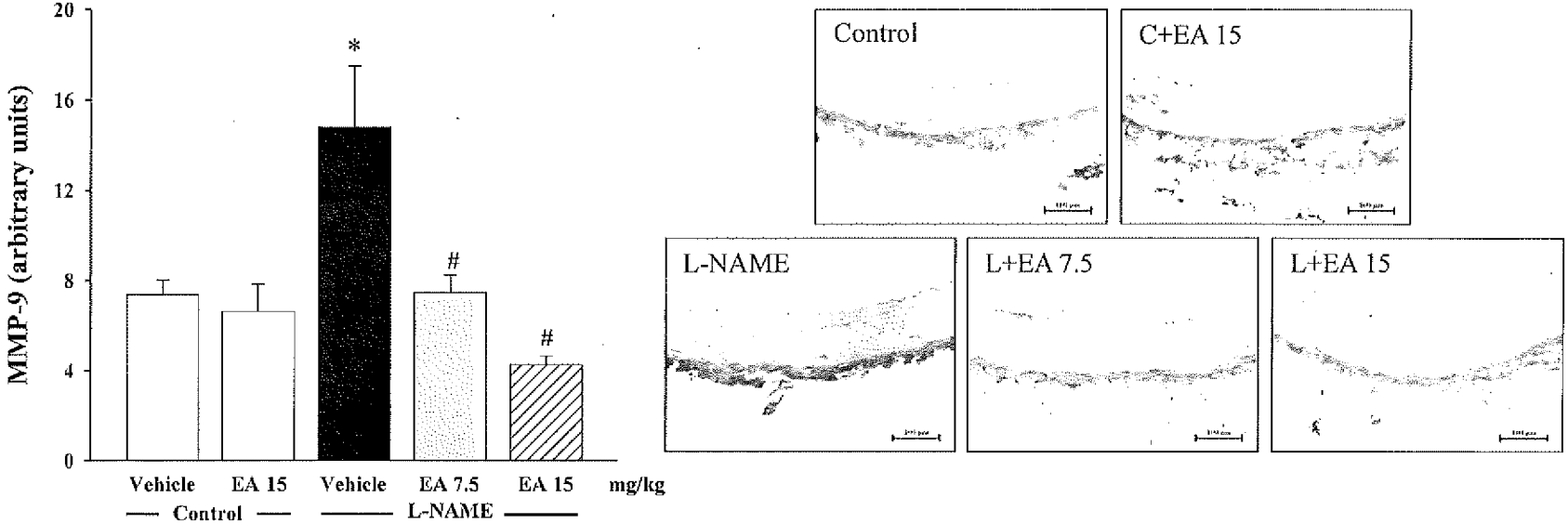

Figure 16 Effect of ellagic acid (EA) on MMP-9 localization in the aortas of all experimental groups. Values are expressed as mean ± SEM, (n = 5/group). *p < 0.05 when compared to control group and # p < 0.05 when compared to L-NAME group.

(4) สรุปและวิจารณ์ผลการทดลอง

Chronic blockade of NO synthesis by L-NAME is a well-known model of hypertension. Although this model cannot be easily extrapolated to human hypertension conditions, it provides the possibility of reducing the causes of increased blood pressure to a single factor, which is a decrease in NO bioavailability. This study investigated the antihypertensive effects of ellagic acid and possible mechanisms involved in L-NAME-induced hypertensive rats.

The findings demonstrated that L-NAME-induced hypertension exhibited hemodynamic alterations, including high blood pressure, high HVR, and low HBF. There was a reduction of plasma NOx that was consistent with down-regulation of eNOS expression in hypertensive rats. Oxidative stress markers were significantly increased in this animal model of experimental hypertension. The protein expression of p47phox NADPH oxidase subunit was markedly increased in hypertensive rats. Administration of ellagic acid reduced blood pressure, HR, HVR as well as increased HBF in L-NAME hypertensive rats. Decreased plasma NOx and eNOS expression in hypertensive rats were normalized by daily treatment with ellagic acid. Furthermore, ellagic acid also reduced plasma MDA, vascular $O2^{\bullet -}$ production and alleviated the expression of the NADPH oxidase subunit in these rats.

It is well established that long-term administration of the L-arginine analogue, L-NAME to normotensive rats can induce NO-deficient hypertension. The precise mechanism is based on the fact that NO is synthesized and released from endothelial cells to mediate vasorelaxation (Ignarro, 2002) and L-NAME reduces NO production resulting in increased total peripheral resistance and high blood pressure (Gardiner et al., 1990; Rees et al., 1989). This mechanism is supported by findings that disruption of plasma NOx level and reduced protein eNOS expression have been reported in long-term L-NAME treatment (Nakmareong et al., 2012; Nyadjeu et al., 2013; Pakdeechote et al., 2014).

The present results demonstrate that L-NAME treatment for five weeks caused a progressive increase in blood pressure resulting from increased HR and HVR. These observations were also associated with a reduction in plasma NOx levels and down-regulation of protein eNOS expression. The doses of ellagic acid (7.5 and 15 mg/kg/day) chosen for this study represent pharmacological doses. The higher dose of 15 mg/kg/day would equate to human consumption of approximately 850 mL/day of pomegranate juice (Reagan-Shaw et al., 2008; Seeram et al., 2004). However, this dose did not lead to any evident toxicity as indicated by the fact that the body weight of these rats was not different to that of the vehicle-treated rats. Moreover, our results showed that ellagic acid markedly improved the symptoms of hypertension in L-NAME treated rats. The protective effect of ellagic acid was characterized by reductions in HR and HVR and augmentation of HBF. While this report is the first to

directly demonstrate the protective effect of ellagic acid on L-NAME-induced hypertension, several previous studies have shown beneficial effects of consumption of pomegranate juice, which contains a high concentration of ellagic acid (Aviram et al., 2004; Ding et al., 2014; Mohan et al., 2010). For example, Aviram and coworkers found that consumption of pomegranate juice (50 mL/day) for a year was able to reduce SBP in atherosclerotic patients and they suggested that the pomegranate juice had a potent antioxidative capacity as one possible mechanism (Aviram et al., 2004). In another study, NOS activity was improved in diet-induced atherosclerosis in mice after ellagic acid supplementation (Ding et al., 2014). Finally, in diabetic Wistar rats, chronic administration of pomegranate juice extract (100 and 300 mg/kg/day) for four weeks caused a reduction in MAP presumably related to its antioxidant capacity (Mohan et al., 2010).

Oxidative stress is an imbalance between the production of reactive oxygen species and the antioxidant capacity and has been found to link with the pathogenesis of hypertension (Broadhurst, 1997; Eftekhari et al., 2011; Houston, 2011). The association between oxidative stress and hypertension has been extensively demonstrated ever since the plasma level of MDA, a biomarker of lipid peroxidation, was found to be increased in patients with hypertension (Higashi et al., 2002; Lacy et al., 1998; Lip et al., 2002). In L-NAME hypertensive rats, an increased in the level of plasma MDA and vascular $O2^{\bullet -}$ are found. In addition, a positive correlation between SBP and vascular $O2^{\bullet -}$ production was shown in this study, suggesting that $O2^{\bullet -}$ rapidly reacts with NO to produce peroxynitrite ($ONOO^-$) resulting in impaired NO bioavailability (Pryor and Squadrito, 1995). These findings are supported by several studies in L-NAME hypertensive rats (Pakdeechote et al., 2014; Tsukahara et al., 2000; Xu and Touyz, 2006). The increase in $O2^{\bullet -}$ production in L-NAME hypertensive rats is probably due to the augmentation of NADPH oxidase activity, a major source of vascular $O2^{\bullet -}$ production. It has been well documented that $O2^{\bullet -}$ production is primarily produced from NADPH oxidase, which has several subunits (Litterio et al., 2012). In this study, we found the up-regulation of p47phox protein expression, an NADPH oxidase subunit, in the vascular tissues. This observation has been reported that in nitric oxide-deficient hypertensive rats showed an overexpression of NADPH oxidase subunit p47phox with lipid peroxidation and high levels of vascular $O2^{\bullet -}$ production (Bunbupha et al., 2014; Harrison et al., 2010). Thus, our findings suggested that increased vascular $O2^{\bullet -}$ level observed in L-NAME hypertensive rats play an important role in decreasing NO bioavailability as it was confirmed by the low level of plasma NOx. In this study, ellagic acid reduced the development of L-NAME-induced hypertension in rats by significantly reducing oxidative stress

markers, and NADPH oxidase subunit p47phox expression. There is substantial evidence to show that ellagic acid has potent antioxidative effects. Goswami and coworkers found that dietary supplementation with ellagic acid reduced oxidative stress and improved sexual dysfunction in type 1 diabetic rats (Goswami et al., 2014). In addition, ellagic acid was reported to prevent diet-induced atherosclerosis in WT mice and this was related to improvements in NO availability, reduced oxidative stress and activation of the Nrf2 antioxidant transcription factor (Ding et al., 2014).

In addition, in this study, cardiovascular geometry including the wall thickness, CSA and luminal area of left ventricle, the wall thickness, CSA and luminal area of the thoracic aorta were examined. This present study revealed that not only did the wall thickness and CSA of left ventricle increased, but ventricular luminal area in the L-NAME-induced hypertensive rats did decrease also. Regarding HW and the relative HW, there was significant difference among groups. These results showed that administration L-NAME at the dose of 40 mg/kg/day for 5 weeks causes an increased in the HW, LVW and the relative LVW. The structural alterations of heart ventricle are concordance with the elevation of blood pressure and heart rate. These results indicated that the left ventricle of L-NAME-induced hypertensive rats have been remodeled by hypertrophy as cardiac adaptation to maintain the normal cardiac output. The cardiac remodeling which was observed in this study could be classified as an inward hypertrophic (Feihl et al., 2008). Not only are the cardiac remodeling found in this study but also the thoracic wall remodeling. Similarly to previous reported, hypertension causes cardiovascular remodeling and hypertrophy. This alteration helps to normalized left ventricular and arterial wall stress and compensated for a reduction in myocardial function to preserve a normal cardiac output (Mayet and Hughes, 2003). Evidence shows that the functional performance of hypertrophied cardiomyocytes is impaired, and that additional alterations develop in cardiomyocytes themselves, the ECM and the intramyocardial vasculature, leading to myocardial remodeling and providing the basis for the adverse prognosis associated with pathological LVH in hypertensive condition (Frohlich et al., 2011). In NO deficiency condition, the increased accumulation of superoxide generation occurred and led to an inflammation, vascular cell growth and increased collagen deposition in both heart and vessel (Moens et al., 2008; Simko et al., 2010; Xu and Touyz, 2006). The NOS is also involved in the pathophysiology of hypertrophy by sustained pressure overload activated by ROS. A decreased in the bioavailability of NOS has been shown to cause advanced hypertrophy (Moens et al., 2008). The decrease of NOS activity in L-NAME-induced hypertensive model was associated with the development of hypertension and cardiovascular remodeling, including LVH with increased wall thickness and CSA of the aorta (Simko et al., 2007). Hu and coworkers reported that administration of L-NAME marked myocardial

hypertrophy and fibrosis which were supported by increases LVW and LVW/BW ratio, enlargement of cardiomyocytes and hyperchromitic nuclei with infiltration of fibroblasts. The development of myocardial hypertrophy may also involve the decreases in left ventricular cGMP and NO formation (Hu et al., 2005). Moreover, L-NAME administration diminished NOS activity, enhanced EDCF formation and cardiovascular structural remodeling by reduced internal diameter of vascular wall and increased relative LVW (Paulis et al., 2008).

It is well established that matrix metalloproteinases (MMPs), a family of proteolytic enzymes that degraded various components of the ECM, are crucial for cell migration and proliferation. In addition, it possesses a major function in degradation and removal of ECM components from the tissue (Boonla et al., 2014; Galis and Khatri, 2002; Newby, 2006; Sangartit et al., 2014). MMPs activity not only involved in cardiac remodeling but also associated with vascular remodeling. ProMMPs are cleaved into active forms that promoted degradation of ECM proteins. Recent evidence suggested that direct or indirect effects of MMPs on ion channels in the endothelium and vascular smooth muscle, and on other mechanisms of vascular relaxation/contraction were associated with control the blood pressure. Also, endogenous tissue inhibitors of MMPs (TIMPs) provide a balancing mechanism to prevent excessive degradation of ECM. An imbalance between MMPs and TIMPs could cause large increases in MMP activity and may lead to pathological changes in the vessel wall structure associated with development of hypertension (Raffetto and Khalil, 2008). Previous studies addressed the question of whether myocardial fibrosis during NO synthase inhibition is caused by the consequent hypertension or by the lack of NO. Evidence from Rossi and coworkers (2003) supported the idea that fibrotic changes in the myocardium of rats treated with NO synthase inhibitor are directly related to NO insufficiency. Furthermore, NO negatively regulates the production of extracellular components through the modulation of TGF-β3 mRNA expression in cardiac fibroblasts (Rossi et al., 2003). There is evidence to support that ROS can be regulated collagen metabolism in cardiac fibroblasts, which are the major cell type responsible for collagen synthesis and degradation in the myocardium. Collagen degradation is regulated by the activity of extracellular MMPs (Siwik et al., 2001). ROS also have potent effects on the ECM, stimulating cardiac fibroblast proliferation and activating MMPs, effects central to fibrosis and ECM remodeling (Takimoto and Kass, 2007). Interestingly, treatment with EA showed to be effective for reduced myocardial fibrosis in interstitial regions and presented perivascular fibrosis lower than L-NAME-treated rats. There is evidence to support the effect of EA on fibrotic markers (MMPs and TIMPs) during alcohol-induced hepatotoxicity from Devipriya and colleagues (2007). They found that co-administration of EA with alcohol effectively decrease the expression of fibrotic markers during alcohol-

induced tissue injury (Devipriya et al., 2007). This study suggested a role of abnormal ECM metabolism in hypertension, and raised the interesting possibility that antihypertensive effect of EA may modulate collagen metabolism. Furthermore, EA effectively reduced oxidative stress. Thus, the reducing of oxidative stress promoted inhibition of MMPs activity and therefore decrease collagen degradation resulting in inhibited the development of myocardial and vascular fibrosis.

In conclusion, EA showed to be an antihypertensive effect by attenuated the elevation of blood pressure and heart rate, improves oxidative stress status and reverse the alterations of cardiac remodeling by decrease in interstitial and perivascular collagen accumulation in myocardium leading to decrease cardiac hypertrophy. This could be its antioxidant property that related to the alleviation of oxidative stress.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกอ. และ สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)

1) Berkban, T., Boonprom, P., Bunbupha, S., Welbat, J.U., Kukongviriyapan, U., Kukongviriyapan, V., Pakdeechote, P., **Prachaney, P.** Ellagic acid prevents L-NAME-induced hypertension via restoration of eNOS and p47phox expression in rats, Nutrients, 2015, 7(7): 5265-5280.

2) อยู่ในระหว่างการจัดทำ manuscript เพื่อตีพิมพ์ใน Annals of Anatomy ชื่อเรื่อง Ellagic acid prevents cardiovascular remodeling in nitric oxide deficiency rats.

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

สามารถนำผลการวิจัยใช้สอนในระดับบัณฑิตศึกษาได้ และพัฒนาตนเองในด้านการทำวิจัย

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

1. การเสนอผลงานทางวิชาการแบบโปสเตอร์ในงานประชุมวิชาการ FEPS 2014 ณ เมือง Budapest ประเทศ Hungary ระหว่างวันที่ 27-30 สิงหาคม 2557

Ellagic acid prevents cardiac fibrosis and attenuates high blood pressure in chronic nitric oxide-deficient hypertensive rats (ภาคผนวกหมายเลข 2)

2. การเสนอผลงานทางวิชาการแบบโปสเตอร์ในงานประชุมวิชาการ 7th Scientific Meeting of Asian Society for Vascular Biology ณ เมือง Hualien ประเทศ Taiwan ระหว่างวันที่ 27-29 ตุลาคม 2559

Ellagic acid reduces blood pressure and alleviates vascular remodeling in L-NAME hypertensive rats (ภาคผนวกหมายเลข 3)

ภาคผนวก

ภาคผนวกหมายเลข 1

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Article

Ellagic Acid Prevents L-NAME-Induced Hypertension via Restoration of eNOS and p47^{phox} Expression in Rats

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Abstract: The effect of ellagic acid on oxidative stress and hypertension induced by N$^{\omega}$-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was investigated. Male Sprague-Dawley rats were administrated with L-NAME (40 mg/kg/day) for five weeks. L-NAME induced high systolic blood pressure (SBP) and increased heart rate (HR), hindlimb vascular resistance (HVR) and oxidative stress. Concurrent treatment with ellagic acid (7.5 or 15 mg/kg) prevented these alterations. Co-treatment with ellagic acid was associated with up-regulation of endothelial nitric oxide synthase (eNOS) protein production and alleviation of oxidative stress as indicated by decreased superoxide production in the vascular tissue, reduced plasma malondialdehyde levels, reduced NADPH oxidase subunit p47^{phox} expression and increased plasma nitrate/nitrite levels. Our results indicate that ellagic acid attenuates hypertension by reducing NADPH oxidase subunit p47^{phox} expression, which prevents oxidative stress and restores NO bioavailability.

Keywords: ellagic acid; high blood pressure; nitric oxide deficiency; NADPH oxidase; antioxidant

1. Introduction

Hypertension is one of the most important factors associated with development of several diseases such as heart failure, renal failure [1], coronary heart disease, atherosclerosis, myocardial infarction [2] and stroke [3]. Endothelial dysfunction, which results from nitric oxide (NO) deficiency, is one of the causes of essential hypertension [4]. NO, derived from endothelial cells in response to physiological and pathological stimuli, mediates vasodilation, maintains vascular tone and regulates blood pressure [5,6]. Other physiological functions of NO have also been reported such as anti-inflammatory, anti-platelet aggregation, and anti-proliferative effects [7–9]. Thus, loss or decrease of NO production plays an important role in pathogenesis of diseases including hypertension, atherosclerosis, myocardial fibrosis, myocardial infarction, diabetes and stroke [10–12].

A rat model of hypertension induced by N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), a nitric oxide synthase (NOS) inhibitor, is widely used to mimic hypertension in humans [13,14]. L-NAME-treated rats have down-regulated eNOS protein expression in blood vessels [15,16] and depletion of plasma NO levels, which leads to systemic vasoconstriction, increased vascular resistance and high blood pressure. There is also an association between L-NAME-induced hypertension and oxidative stress markers. High-dose L-NAME treatment (40 mg/kg/day) has been reported to elevate levels of oxidative stress markers such as vascular superoxide ($O_2^{\bullet -}$), plasma malondialdehyde (MDA) and plasma protein carbonyl [17,18]. It has been well documented that oxidative stress contributes to the etiology of hypertension in animals [19] and humans [20,21] as characterized by the increased bioavailability of reactive oxygen species (ROS) in hypertension [22]. Elevated ROS levels stimulate vascular smooth muscle cell proliferation and increase arterial resistance due to reduced NO availability, leading to the impairment of vascular relaxation [10]. There is evidence that it is the ROS-producing enzyme, β-Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOx), that is responsible for increased vascular $O_2^{\bullet -}$ production in L-NAME hypertensive rats [23] via up-regulation of the NOx subunit $p47^{phox}$ [24].

Nowadays, there is a great deal of interest in how antioxidant substances can be used for prevention and treatment of disease. Ellagic acid is a natural polyphenolic compound present in oak species such as the North American white oak (*Quercus alba*) and European red oak (*Quercus robur*) [25]. Ellagic acid is also found in fruits, such as blackberries, cranberries, raspberries, strawberries, grapes, pomegranates, and nuts, as well as several medicinal plants [26–28]. Previous studies have revealed that ellagic acid possesses anticancer [27,29], antioxidant [30–32] and anti-inflammatory activities [33] and can aid in the prevention of degenerative diseases such as cardiovascular disease [34]. However, little is known regarding the antihypertensive effects of ellagic acid. This study aims to examine whether ellagic acid can reduce blood pressure and oxidative stress markers in L-NAME-induced hypertensive rats.

2. Materials and Methods

2.1. Reagents

Ellagic acid (purity ≥ 95%), L-NAME, ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), sulfanilamide, N-1-napthylethylethylenediamine (NED) and 1,1,3,3-tetraethoxypropane were obtained from

Sigma-Aldrich Corp. (St. Louis, MO, USA). β-Nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate dehydrogenase (G-6-PD) and Nitrate reductase were obtained from Roche Applied Sciences (Mannheim, Germany). Trichloroacetic acid (TCA) and lucigenin were obtained from Fluka Chemika Co., Ltd (Buch, Switzerland). All chemicals used in this study were obtained from standard companies and were of analytical grade quality.

2.2. Instruments

Pressure transducer equipped with AcqKnowledge data acquisition and analysis software (BIOPAC Systems Inc., Santa Barbara, CA, USA), Electromagnetic flow meter (Model FM501D, Carolina Medical Electronics, Carolina, NC, USA), tail-cuff plethysmography (IITC/Life Science Instrument model 229 and model 179 amplifiers, Woodland Hills, CA, USA), microplate reader (Tecan GmbH., Groding, Austria), UV/Visible spectrophotometer (Ultrospec 6300 Pro. Bichrom Ltd. UK), luminometer (Turner Biosystems, 23 CA, USA), Centrifuge SIGMA 3K15 (SIGMA Laborzentrifugen, Osterode am Harz, Germany) and Centrifugal concentrators (NANOSEP™, Pl Filtration, USA) were used in this study.

2.3. Animals and Experimental Protocols

2.3.1. Animals

Male Sprague-Dawley rats (240–280 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. The rats were housed at 25.1 ± 1 °C with 12 h dark–light cycle at Northeast Laboratory Animal Center, Khon Kaen. All procedures were reviewed and approved by the Institutional Animal Ethics Committee of Khon Kaen University (AEKKU 70/2555).

2.3.2. Induction of L-NAME Hypertension

The rats in the L-NAME-treated group were fed with standard chow diet (Chareon Pokapan Co., Thailand) and L-NAME (40 mg/kg/day) in their drinking water for 5 weeks to induce hypertension, whereas rats in the normal control group were fed with a standard chow diet and distilled water (DW).

2.3.3. Experimental Groups

After seven days of acclimatization, the rats were randomly divided into five experimental groups (n = 10/group) as follows.

Group 1: Control (DW)
Group 2: Control + EA 15 (Ellagic acid 15 mg/kg BW in DW)
Group 3: L-NAME (DW)
Group 4: L-NAME + EA 7.5 (Ellagic acid 7.5 mg/kg BW in DW)
Group 5: L-NAME + EA 15 (Ellagic acid 15 mg/kg BW in DW)

Ellagic acid and/or distill water vehicle were intragastrically administered daily. The doses of ellagic acid were chosen on the basis of previous studies [35]. Blood pressure was measured before entering the study and during treatment until sacrifice. Body weight was measured weekly and the dose was adjusted accordingly.

2.4. Parameter Measurements

2.4.1. Blood Pressure Measurement

Systolic blood pressure (SBP) was measured once a week using non-invasive tail-cuff plethysmography (IITC/Life Science Instrument model 229 and model 179 amplifiers, Woodland Hills, CA, USA). Briefly, conscious rats were placed in a restrainer and allowed to rest for 10–15 min prior to blood pressure measurement. The tail was placed inside the cuff, which automatically inflated and released and SBP values were obtained from the mean of three measurements.

2.4.2. Hemodynamic Assessments

On the last day of the experiment, rats were anaesthetized with an intraperitoneal injection of Pentobarbital (60 mg/kg) and placed on heating pad to maintain body temperature at 37 °C. A tracheotomy was performed for measurement of spontaneous breathing, and a polyethylene catheter was inserted into the lower abdominal aorta via the left femoral artery for continuous monitoring of blood pressure using a pressure transducer and the Acknowledge data acquisition and analysis software (BIOPAC Systems Inc., Santa Barbara, CA, USA). The catheters were filled with heparinized saline to prevent clotting. SBP, diastolic blood pressure (DBP), mean arterial blood pressure (MAP) and heart rate (HR) were continuously monitored.

Hindlimb blood flow (HBF) was continuously measured by an electromagnetic flow meter (Carolina Medical Electronics, Carolina, NC, USA) connected to an electromagnetic flow probe placed around the abdominal aorta below the kidneys. Hindlimb vascular resistance (HVR) was calculated from baseline MAP and mean HBF.

After collection of the hemodynamic measurements, rats were sacrificed with an over dosage of the anesthetic drug. Blood samples were drawn from the bifurcation of the abdominal aorta into EDTA tubes for assay of the oxidative stress markers plasma MDA and NO metabolites. The carotid arteries were rapidly excised for analysis of vascular $O_2^{\bullet -}$ production and the thoracic aorta was isolated to evaluate eNOS and NADPH oxidase subunit ($p47^{phox}$) protein expression.

2.4.3. Assay of Vascular $O_2^{\bullet -}$ Production

Vascular $O_2^{\bullet -}$ production was measured using a lucigenin-enhanced chemiluminescence method [36]. The carotid arteries (about 3–5 mm in length) were placed in ice-cold saline and adipose and connective tissues were removed. The vessel segments were incubated with Krebs-KCL buffer (pH 7.4) and allowed to equilibrate at 37 °C for 30 min. Lucigenin (100 μM) was added and the sample tube placed in a luminometer (Turner Biosystems, Sunnyvale, CA, USA). The photon counts were integrated every 30 s for 5 min and averaged. The vessels were then dried at 45 °C for 24 h for determination of dry weight. $O_2^{\bullet -}$ production in vascular tissue was expressed as relative light unit count per minute per milligram of dry tissue weight.

2.4.4. Assay of Plasma Malondialdehyde

The concentration of plasma MDA was measured by a spectrophotometric method as previously described [37]. Briefly, plasma samples (150 μL) were reacted with 10% TCA, 125 μL of 5 mM EDTA, 125 μL of 8% SDS, and 10 μL of 0.5 μg/mL BHT for 10 minutes at room temperature. Then, an equal volume of 0.6% TBA was added and the mixture was boiled in a water bath for 30 min. After cooling to room temperature, the mixture was centrifuged at 10,000 g for 5 min at 25 °C. The absorbance of the supernatant was measured at 532 nm by spectrophotometer (Amersham Bioscience, Arlington, MA, USA) and compared to a standard curve generated with 0.3 to 10 μmol/L 1,1,3,3-tetraethoxypropane.

2.4.5. Assay of Plasma Nitrate and Nitrite

The concentrations of plasma nitrate and nitrite, the end products of NO metabolism, were measured using an enzymatic conversion method [38] with some modifications [39]. Plasma samples were first deproteinized by ultrafiltration using centrifugal concentrators (Pall Corp., Ann Arbor, MI, USA). The supernatants were mixed with 1.2 μmol/L NADPH, 4 mmol/L glucose-6-phosphate disodium (G-6-P), 1.28 U/mL glucose-6-phosphate dehydrogenase (G-6-PD) and 0.2 U/mL nitrate reductase, and then incubated at 30 °C for 30 min. The mixture was reacted with a Griess solution (4% sulfanilamide in 0.3% naphthalenediamine dichloride, NED) for 15 min. The absorbance of samples was measured on a microplate reader with a filter wavelength of 540 nm (Tecan GmbH., Groding, Austria).

2.4.6. Western Blot Analysis of $p47^{phox}$ NADPH Oxidase Subunit and eNOS Protein

Expressions of the $p47^{phox}$ NADPH oxidase subunit and eNOS proteins in aortic homogenates were determined following a previously described method with some modifications [17,40]. The aortic homogenates (15 μg) were electrophoresed on an SDS polyacrylamide gel and electrotransferred onto a polyvinylidenedifluoride membrane. Membranes were blocked with 5% skimmed milk in phosphate buffered saline (PBS) with 0.1% Tween-20 at room temperature for 2 h and incubated overnight at 4 °C with primary antibody of mouse monoclonal anti-eNOS (1:2500, BD Biosciences, CA, USA) or mouse monoclonal anti-$p47^{phox}$ (1:2500, Santa Cruz Biotechnology, CA, USA). The membranes were washed with PBS 3 times for 7 min before being incubated with the secondary antibody, horseradish peroxidase goat anti-mouse IgG (1:2500, Santa Cruz Biotechnology, CA, USA), for 2 h at room temperature. The blots were developed in Amersham™ ECL™ Prime solution (Amersham Biosciences Corp., Piscataway, NJ, USA), and densitometric analysis was performed using an ImageQuant™ 400 imager (GE Healthcare Life Sciences, Piscataway, NJ, USA). β-actin (1:5000, Santa Cruz Biotechnology, CA, USA) was used as a loading control. The intensity of the specific eNOS and $p47^{phox}$ protein bands were normalized to that of β-actin, and data were expressed as a percentage of the values determined in control aorta from the same gel.

2.5. Statistical Analysis

Data were expressed as mean ± SEM. The significance of differences between means were analyzed by one-way analysis of variance (ANOVA) and followed by Student Newman–Keul's test to show specific group differences. Statistical significance was assigned at a p value of less than 0.05.

3. Results

3.1. Effect of Ellagic Acid on Systolic Blood Pressure

At the beginning of the experiment, average baseline SBP was similar among groups (Figure 1). L-NAME administration induced a rapid progressive increase in SBP. Rats receiving L-NAME with concurrent administration of ellagic acid showed increased SBP compared to controls but reduced SBP compared to L-NAME treatment alone. The L-NAME+EA 15 mg/kg/day group showed a significantly lower SBP than the L-NAME+EA 7.5 mg/kg/day group (157.8 ± 4.8 mmHg *vs.* 165.7 ± 4.3, $p < 0.05$).

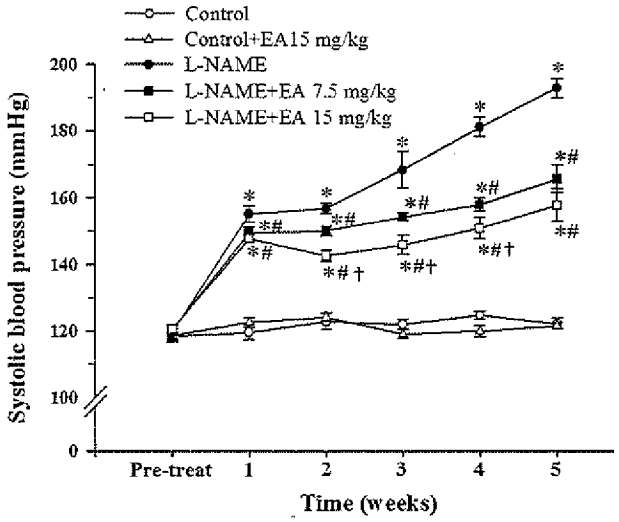

Figure 1. Effect of ellagic acid on systolic blood pressure during N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) administration for five weeks. Values are expressed as mean ± SEM, $n = 8$. * $p < 0.05$ when compared to control group, # $p < 0.05$ when compared to L-NAME group and † $p < 0.05$ when compared to L-NAME+EA 7.5 mg/kg group.

3.2. Effect of Ellagic Acid on Hemodynamic Status

Animals that received only L-NAME were hypertensive as evidenced by significant increases in SBP, DBP, MAP and HR compared to control rats (Table 1). Concurrent treatment with ellagic acid significantly attenuated the elevation of the BP parameters and HR in L-NAME hypertensive rats (Table 1). L-NAME administered rats also showed significantly lower HBF levels and significantly higher HVR levels compared with control rats (4.2 ± 0.3 *vs.* 8.3 ± 0.8, $p < 0.05$ and 39.5 ± 3.5 *vs.* 12.1 ± 1.4, $p < 0.001$, respectively). Both the 7.5 mg/kg and 15 mg/kg doses of ellagic acid ameliorated the L-NAME induced changes in HBF and HVR in hypertensive rats (Table 1).

Table 1. Effect of ellagic acid on cardiovascular parameters and body weight.

Parameters	Control		L-NAME		
	Vehicle	EA (mg/kg/day)	Vehicle		EA (mg/kg/day)
		15		7.5	15
SBP (mmHg)	121.9 ± 1.8	122.7 ± 2.3	199.4 ± 6.1 *	167.5 ± 3.0 *,#	164.6 ± 4.9 *,#
DBP (mmHg)	78.3 ± 2.0	78.8 ± 2.1	140.4 ± 4.0 *	113.6 ± 3.1 *,#	111.2 ± 3.7 *,#
MAP (mmHg)	95.4 ± 1.9	95.5 ± 2.2	164.3 ± 4.6 *	136.8 ± 3.2 *,#	133.1 ± 4.2 *,#
HR (beat/min)	357.9 ± 6.0	362.5 ± 5.9	424.8 ± 6.2 *	379.4 ± 5.5 *,#	368.0 ± 3.1 *,#
HBF (mL/100 g tissue/min)	8.3 ± 0.8	7.1 ± 0.4	4.2 ± 0.3 *	6.4 ± 0.2 *,#	6.4 ± 0.3 *,#
HVR (mmHg/min/100 g/mL)	12.1 ± 1.4	13.8 ± 0.8	39.5 ± 3.5 *	20.5 ± 1.2 *,#	20.4 ± 1.2 *,#
Body weight (g)	417.1 ± 4.6	418.0 ± 6.0	409.5 ± 2.6	414.4 ± 4.4	410.5 ± 4.3

Values are expressed as mean ± SEM, $n = 10$. * $p < 0.05$ when compared to control group and # $p < 0.05$ when compared to vehicle L-NAME group.

3.3. Effects of Ellagic on Nitrate/Nitrite Production and eNOS Protein Expression

The concentration of nitrate/nitrite NO metabolites in plasma is shown in Figure 2A and the expression of eNOS protein in isolated rat aortas is shown in Figure 2B. The levels of NO metabolites in plasma were significantly reduced in L-NAME rats when compared with normal control rats (2.9 ± 0.2 μmol/L *vs.*11.2 ± 0.6 μmol/L, $p < 0.05$) and administration of L-NAME was associated with down-regulation of eNOS protein expression compared to control rats ($p < 0.05$). There was no change in plasma nitrate/nitrite levels or eNOS protein expression in control rats treated with ellagic acid alone (Figure 2A,B). Co-treatment of L-NAME administered rats with either 7.5 mg/kg or 15 mg/kg ellagic acid for five weeks significantly restored nitrate/nitrite production (6.4 ± 0.5 μmol/L *vs.* 7.1 ± 0.4 μmol/L, $p < 0.05$, respectively) and eNOS protein expression, $p < 0.05$, respectively).

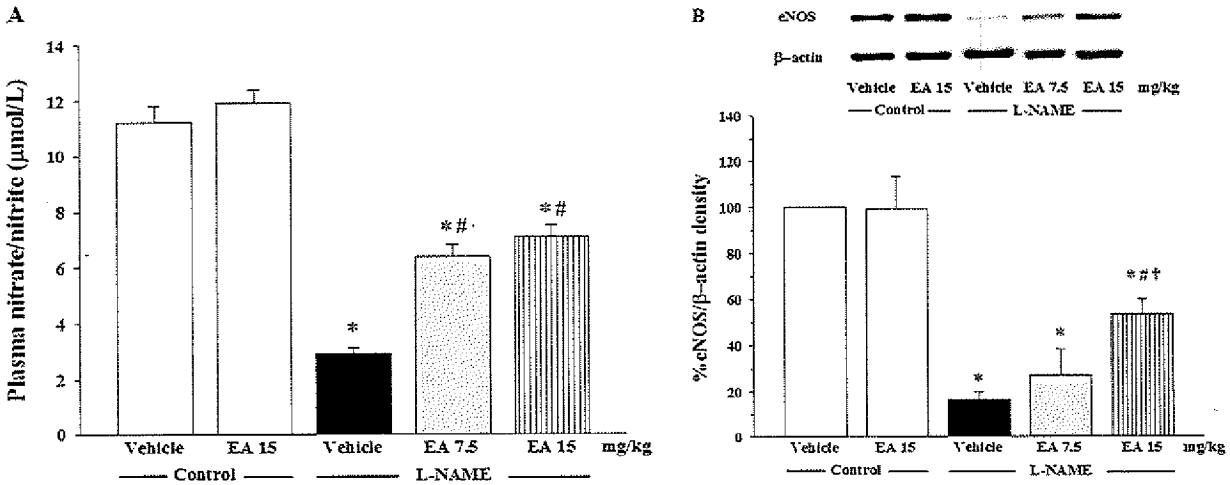

Figure 2. Effect of ellagic acid on (**A**) nitrate/nitrite production ($n = 7$) and (**B**) eNOS protein expression ($n = 5$). Values are expressed as mean ± SEM, * $p < 0.05$ when compared to control group, # $p < 0.05$ when compared to L-NAME group and † $p < 0.05$ when compared to L-NAME+EA 7.5 mg/kg group.

3.4. Effect of Ellagic Acid on Oxidative Stress

Oxidative stress was determined by measuring the concentration of plasma MDA and by assaying vascular superoxide production. The level of plasma MDA in the L-NAME group was three-fold higher than in the control group (10.9 ± 0.9 μmol/L *vs.* 3.3 ± 0.1 μmol/L, $p < 0.05$). Administration of both 7.5 and 15 mg/kg ellagic acid significantly reduced the level of plasma MDA in a dose dependent manner (6.9 ± 0.1 μmol/L and 5.0 ± 0.5 μmol/L, $p < 0.05$, respectively, Figure 3). L-NAME also induced a three-fold elevation in vascular superoxide production compared to the vehicle control (175.3 ± 11.2 count/min/mg dry weight *vs.* 59.9 ± 8.9 count/min/mg dry weight, $p < 0.05$) (Figure 4A). Again, ellagic acid at both the 7.5 and 15 mg/kg doses significantly inhibited this elevation (137.1 ± 11.8 count/min/mg dry weight and 90.4 ± 4.6 count/min/mg dry weight, $p < 0.05$, respectively). In addition, there was a strong correlation between vascular superoxide production and SBP ($R = 0.856$; Figure 4B).

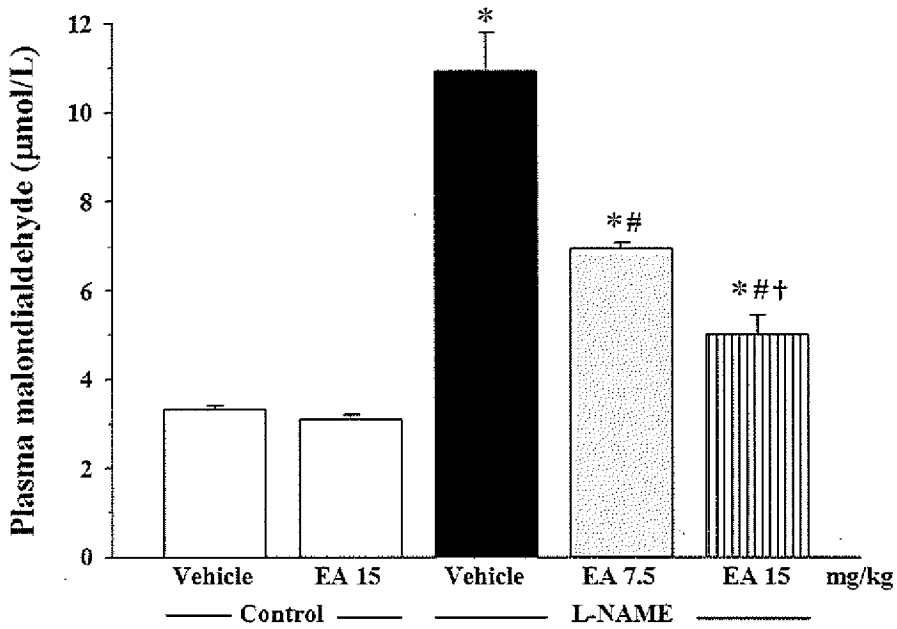

Figure 3. Effect of ellagic acid on plasma malondialdehyde levels. Values are expressed as mean ± SEM, $n = 7$. * $p < 0.05$ when compared to control group, # $p < 0.05$ when compared to L-NAME group and † $p < 0.05$ when compared to L-NAME+EA 7.5 mg/kg group.

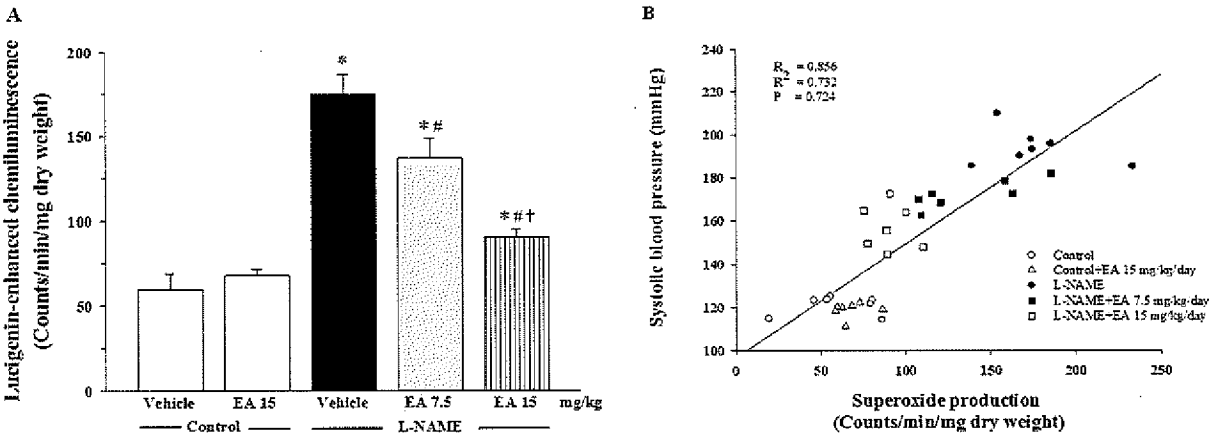

Figure 4. (**A**) Effect of ellagic acid on vascular superoxide production. Values are expressed as mean ± SEM lucigenin-enhanced chemilumiescence counts/min/mg dry weight, $n = 7$. * $p < 0.05$ when compared to control group, # $p < 0.05$ when compared to L-NAME group and † $p < 0.05$ when compared to L-NAME+EA 7.5 mg/kg group. (**B**) Correlation between SBP and vascular superoxide production.

3.5. Effect of Ellagic Acid on p47^{phox} Protein Expression in Aortic Tissues

The expression of p47^{phox} protein in L-NAME treated rats was significantly up-regulated compared to control rats ($p < 0.05$, Figure 5A). Positive correlation of p47^{phox} protein expression and the values of the vascular $O_2^{\bullet -}$ production was observed (Figure 5B). Treatment of the L-NAME group with 7.5 mg/kg ellagic acid reduced the over-expression of p47^{phox} protein ($p < 0.05$) and treatment with 15 mg/kg ellagic acid restored p47^{phox} protein levels to normal (Figure 5A).

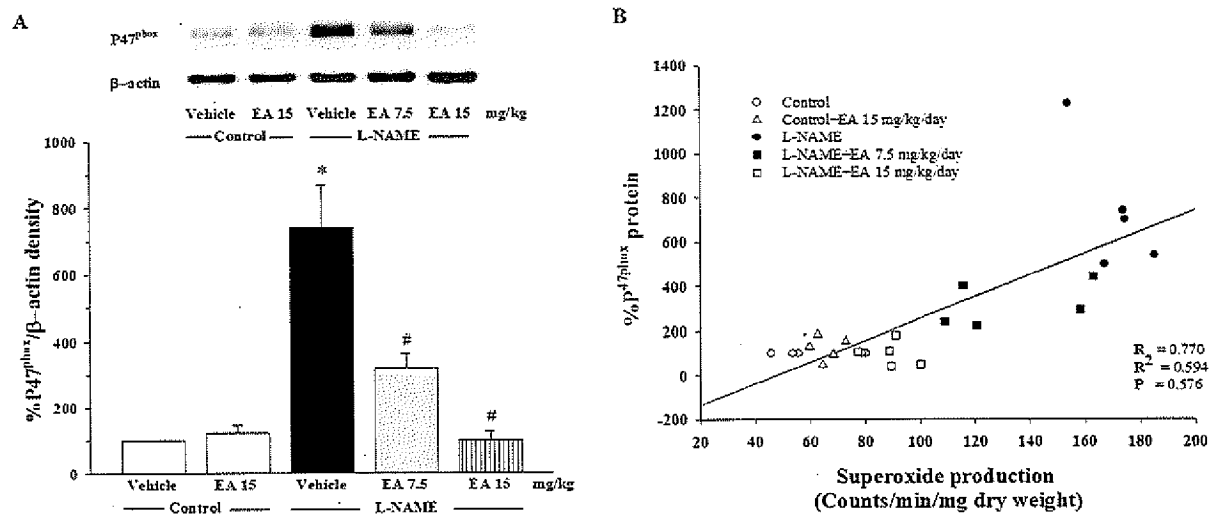

Figure 5. (**A**) Effect of EA on p47^{phox} protein expression in aortic tissues. Values are expressed as mean ± SEM, $n = 5$. * $p < 0.05$ when compared to control group and # $p < 0.05$ when compared to L-NAME group. (**B**) Correlation between p47^{phox} protein expression and vascular superoxide production.

4. Discussion

The present study examined the antihypertensive effects of ellagic acid and possible mechanisms involved in L-NAME-induced hypertensive rats. The findings demonstrate that L-NAME-induced hypertension exhibited hemodynamic alterations, including high blood pressure, high HVR, and low HBF. There was a reduction of plasma NOx that was consistent with down-regulation of eNOS expression in hypertensive rats. Oxidative stress markers were significantly increased in this animal model of experimental hypertension. The protein expression of $p47^{phox}$ NADPH oxidase subunit was markedly increased in hypertensive rats. Administration of ellagic acid reduced blood pressure, HR, HVR as well as increased HBF in L-NAME hypertensive rats. Decreased plasma NOx and eNOS expression in hypertensive rats were normalized by daily treatment with ellagic acid. Furthermore, ellagic acid also reduced plasma MDA, vascular $O_2^{\bullet -}$ production and alleviated the expression of the NADPH oxidase subunit in these rats.

It is well established that long-term administration of the L-arginine analogue L-NAME to normotensive rats can induce NO-deficient hypertension. The precise mechanism is based on the fact that NO is synthesized and released from endothelial cells to mediate vasorelaxation [6] and L-NAME reduces NO production resulting in increased total peripheral resistance and high blood pressure [41,42]. This mechanism is supported by findings that disruption of plasma NOx level and reduced protein eNOS expression have been reported in long-term L-NAME treatment [15,43,44].

Our results demonstrate that L-NAME treatment for five weeks caused a progressive increase in blood pressure resulting from increased HR and HVR. These observations were also associated with a reduction in plasma NOx levels and down-regulation of protein eNOS expression. The doses of ellagic acid (7.5 and 15 mg/kg/day) chosen for this study represent pharmacological doses. The higher dose of 15 mg/kg/day would equate to human consumption of approximately 850 mL/day of pomegranate juice [45,46]. However, this dose did not lead to any evident toxicity as indicated by the fact that the body weight of these rats was not different to that of the vehicle-treated rats. Moreover, our results showed that ellagic acid markedly improved the symptoms of hypertension in L-NAME treated rats. The protective effect of ellagic acid was characterized by reductions in HR and HVR and augmentation of HBF. While this report is the first to directly demonstrate the protective effect of ellagic acid on L-NAME-induced hypertension, several previous studies have shown beneficial effects of consumption of pomegranate juice, which contains a high concentration of ellagic acid [31,47,48]. For example, Aviram and coworkers found that consumption of pomegranate juice (50 mL/day) for a year was able to reduce SBP in atherosclerotic patients and they suggested that the pomegranate juice had a potent antioxidative capacity as one possible mechanism [47]. In another study, NOS activity was improved in diet-induced atherosclerosis in mice after ellagic acid supplementation [48]. Finally, in diabetic Wistar rats, chronic administration of pomegranate juice extract (100 and 300 mg/kg/day) for four weeks caused a reduction in MAP presumably related to its antioxidant capacity [31].

Oxidative stress is an imbalance between the production of reactive oxygen species and the antioxidant capacity, and has been found to link with the pathogenesis of hypertension [19–21]. The association between oxidative stress and hypertension has been extensively demonstrated ever since the plasma level of MDA, a biomarker of lipid peroxidation, was found to be increased in patients with hypertension [49–51]. In L-NAME hypertensive rats, we found increases in the level of plasma MDA and

vascular $O_2^{\bullet-}$. In addition, a positive correlation between SBP and vascular $O_2^{\bullet-}$ production was shown in this study, suggesting that $O_2^{\bullet-}$ rapidly reacts with NO to produce peroxynitrite ($ONOO^-$) resulting in impaired NO bioavailability [52]. These findings are supported by several studies in L-NAME hypertensive rats [44,53,54]. The increase in $O_2^{\bullet-}$ production in L-NAME hypertensive rats is probably due to the augmentation of NADPH oxidase activity, a major source of vascular $O_2^{\bullet-}$ production. It has been well documented that $O_2^{\bullet-}$ production is primarily produced from NADPH oxidase, which has several subunits [23]. In this study, we found the up-regulation of $p47^{phox}$ protein expression, an NADPH oxidase subunit, in the vascular tissues. This observation has been reported that in nitric oxide deficient hypertensive rats showed an over expression of NADPH oxidase subunit $p47^{phox}$ with lipid peroxidation and high levels of vascular $O_2^{\bullet-}$ production [24,55]. Thus, our findings suggested that increased vascular $O_2^{\bullet-}$ level observed in L-NAME hypertensive rats play an important role for decreasing NO bioavailability as it was confirmed by the low level of plasma NOx.

Here we report that ellagic acid reduced the development of L-NAME-induced hypertension in rats by significantly reducing oxidative stress markers, and NADPH oxidase subunit $p47^{phox}$ expression. There is substantial evidence to show that ellagic acid has potent antioxidative effects. Goswami and coworkers found that dietary supplementation with reduced oxidative stress and improved sexual dysfunction in type 1 diabetic rats [56]. In addition, ellagic acid was reported to prevent diet-induced atherosclerosis in WT mice and this was related to improvements in NO availability, reduced oxidative stress and activation of the Nrf2 antioxidant transcription factor [48]. It has also been reported that oxidation can alter the extracellular matrix of the arterial wall [57] and changes to the arterial wall have been implicated as a key factor in the pathogenesis of hypertension [58]. Therefore, any effects of ellagic acid on the extracellular matrix of the vascular wall of the L-NAME induced-hypertensive rat should be investigated.

5. Conclusions

This study demonstrates that ellagic acid exhibits protective effects on the development of hypertension induced by a NOS inhibitor. These protective effects are associated with improved NO bioavailability, higher eNOS protein expression and reduced oxidative stress status, possibly due to a reduction in NADPH oxidase subunit $p47^{phox}$ expression.

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Author Contributions

Conceived and designed the experiments: P.Pa. and P.P.; Performed the experiments: T.B., P.B., S.B., P.Pa. and P.P.; Analyzed the data: T.B. and P.P.; Contributed reagents/materials/analysis tools: U.K., V.K., and J.U.W.; Wrote the manuscript: T.B., P.Pa. and P.P.

Conflicts of Interest

The authors declare no conflict of interest.

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ภาคผนวกหมายเลข 2

7th Scientific Meeting of Asian Society for Vascular Biology

Poster Abstract

No.	Presenting author	Corresponding author	Title
2	Andy Po-Yi Tsai	Tony JF Lee	cGMP and potassium channels are mediating oroxylin A-induced cerebral vasodilation
3	Shingo Takatori	Hiromu Kawasaki	Promotive effect of nerve growth factor on the distribution of perivascular nerves in tumor-derived neovessels of mouse cornea
4	Wei-Chueh Wu	Chiao-Hsuan Chao	Dengue virus nonstructural protein 1 induces platelet activation
5	Li-Jing Chen	Jeng-Jiann Chiu	MicroRNA-451 Protects Against Neointima Stenosis and Atherosclerotic Plaque Formation by Directly Targeting Ras-associated Protein 5a in Vascular Smooth Muscle Cells
6	Direk Aekthammarat	Panot Tangsucharit	Moringa oleifera leaf extract decreases arterial blood pressure and vascular reactivity to adrenergic stimulation in L-NAME hypertensive rats
7	Poungrat Pakdeechote	Poungrat Pakdeechote	Asiatic acid reduces blood pressure and improves vascular function in 2K-1C hypertensive rats
8	Parichat Prachaney	Parichat Prachaney	Ellagic acid reduces blood pressure and alleviates vascular remodeling in L-NAME hypertensive rats
9	Pattanapong Boonprom	Parichat Prachaney	Garcinia mangostana pericarp extract alleviates hypertension and normalizes morphological changes of resistance artery via oxidative stress reduction in NO-deficient hypertensive rats
10	Reggie Lee	Hung Wen (Kevin) Lin	Interruption of perivascular sympathetic nerves of cerebral arteries offers neuroprotection against ischemia
11	Yoshitaka Fujimoto	Susumu Minamisawa	Pulmonary hypertension due to left atrium stenosis caused intrapulmonary venous arterialization in rats
12	Yoshito Zamami	Keisuke Ishizawa	Search for a preventative therapy for Bevacizumab-induced hypertension using the drug repositioning approach
13	Shih-Yu Lee	Shih-Yu Lee	Rhodiola crenulata extraxt attenuates high glucose induced endothelial dysfunction in human umbilical vein endothelial cells
14	Tsung-Han Hsieh	Cheng-Chou Yu	ICOS：A Crucial Immunomodulator for Development of Immunotherapy

No. 8

Ellagic acid reduces blood pressure and alleviates vascular remodeling in L-NAME hypertensive rats

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Hypertension is one of the health problems worldwide. The changes of the vascular composition from the oxidative stress results in an alteration of the vascular function and eventually causes a raise of blood pressure. Antioxidant substances have been interested for an optional treatment of hypertensive condition. Ellagic acid, a natural polyphenolic compound presented in several fruits has antioxidant and anti-inflammatory properties. The aimed of this study was to investigate the protective effect of ellagic acid on blood pressure and vascular structure in Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced hypertensive rats. Male Sprague-Dawley rats were administrated with L-NAME (40 mg/kg/day) for five weeks. During L-NAME treatment, ellagic acid 15 mg/kg/day or vehicle was given daily to hypertensive rats. L-NAME-treated rats exhibited high blood pressure and thoracic aorta alterations including an excessive collagen and VSMCs, and restrained elastin ($p < 0.05$). In addition, increases in aortic MMP-2 and MMP-9 levels were presented with the arterial wall hypertrophy in L-NAME hypertension. Vascular superoxide ($O2^{\bullet-}$) production, malondialdehyde (MDA) levels in plasma and plasma tumor necrosis factor-alpha (TNF-α) concentration were significantly increased in hypertensive rats ($p < 0.05$). Ellagic acid significantly reduced blood pressure and vascular $O2^{\bullet-}$ production, plasma MDA levels, plasma TNF-α levels ($p < 0.05$) and improved vascular remodeling ($p < 0.05$), which was consistent with reductions of aortic MMP-2 and MMP-9 levels ($p < 0.05$). Thus, the present results indicate that ellagic acid reduced blood pressure and vascular remodeling via its antioxidant and anti-inflammatory effects.

ภาคผนวกหมายเลข 3

P4.34

Ellagic acid prevents cardiac fibrosis and attenuates high blood pressure in chronic nitric oxide-deficient hypertensive rats

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Objective: We investigated antihypertensive and antifibrosis effects of ellagic acid (EA) on NG-nitro-L-arginine methyl ester (L-NAME)-induced hypertensive rats.

Methods: Male Sprague-Dawley rats were given L-NAME (40 mg/kg/day) to induce hypertension, and simultaneously treated with EA 15 mg/kg/day for 5 weeks (L-NAME+EA group), or a vehicle (L-NAME group). Age-matched rats served as a control group. After 5 weeks of treatment, rats were anaesthetized with peritoneal injection of pentobarbital-sodium (60 mg/kg) and systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) were evaluated. Rats were scarified by exsanguinations and the heart was isolated. The left ventricle was excised and weighed. The relative heart weight or left ventricular weight (LVW) per body weight (BW) was determined as ventricular hypertrophy index. The paraffin sections of left ventricle were stained by Picrosirius red. Fibrosis was quantified using polarisation microscopy and ImageJ analyses software.

Results: EA significantly reduced SBP, DBP, MAP and HR of L-NAME treated rats when compared to those of the L-NAME group (164.62±4.92 vs. 199.39±6.14 mmHg, 111.23±3.68 mmHg vs. 140.40±3.95 mmHg, 133.08±4.20 mmHg vs. 164.30±4.61 mmHg and 368.03±3.12 bpm vs. 424.78±6.19 bpm, $p < 0.05$, respectively). After 5 weeks of L-NAME administration, LVW/BW ratio was significantly increased when compare to those in the control groups (2.65±0.07 mg/g vs 2.17±0.04 mg/g, $p < 0.05$). Administration of EA caused significant decrease in this ratio when compared to the L-NAME-induced hypertensive groups (2.32±0.09 mg/g vs 2.65±0.07 mg/g, $p < 0.05$). The amount of fibrosis in the left ventricle of L-NAME group was significant increase compared to control (4.00±0.28 % vs. 1.42±0.20 %, $p < 0.05$), whereas in L-NAME+EA group, the amount of fibrosis in the left ventricle was lesser than that in L-NAME group (1.54±0.13 % vs. 4.00±0.28 %, $p < 0.05$).

Conclusion: In conclusion, these data suggest a cardio-protective role of the EA against L-NAME-induced hypertension.

P4.35

Pituitary adenylate cyclase-activating polypeptide (PACAP) induces location- and age-related relaxations of isolated arteries

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Introduction: Pituitary adenylate cyclase activating polypeptide (PACAP) is a well-known neuropeptide with widespread organ and tissue distribution, which also has vasomotor effects. However, less is known regarding the organ specific and age related vasomotor effects of PACAP, which could be important for better understanding its physiological roles.

Hypothesis: We hypothesized that the vasomotor effects of PACAP depend on the tissue origin of the vessels and aging substantially modulates its actions.

Methods: Thus carotid (CA) and basilar arteries (BA) were isolated from young (2 months old), middle age (12 months old) and old (30 months old) rats. Their vasomotor responses were measured with an isometric myograph (DMT-610M) in response to cumulative concentrations of PACAP1-38 (10-9 M - 10-6 M).

Results: PACAP1-38 induced 1) a significantly greater concentration-dependent relaxation in CA compared to that of BA of young, middle age and old rats; 2) relaxations of CA significantly decreased, whereas it did not change substantially in BA, as a function of age; 3) sodium-nitroprusside (SNP) - induced relaxation did not change after PACAP1-38 administration in any conditions.

Conclusions: These findings suggest that PACAP1-38 has greater vasomotor effect in peripheral arteries than in cerebral arteries and aging has less effect on PACAP-induced relaxation of cerebral arteries than in peripheral arteries suggesting that PACAP could be a vasoprotective substance in old age.

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Keywords: PACAP1-38, isolated arteries, aging, cerebral and peripheral circulation, dilation