

# Comparison of extracts from cooked and raw lentil in antagonizing angiotensin II-induced hypertension and cardiac hypertrophy

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**Abstract.** – AIM: The objective of this study is to examine effects of extracts from cooked lentils on angiotensin II (Ang II)-induced hypertension, cardiac hypertrophy and fibrosis in normotensive rats.

**MATERIALS AND METHODS:** Animals were divided into four groups (n=5 each group): control group, Ang II group, Ang II plus cooked lentil extract (Ang II+CLE) group, and Ang II plus raw lentil extract (Ang II+RLE) group. The telemetry blood pressure transducers were implanted into all rats. A telemetry BP probe was positioned intra-abdominally and secured to the ventral abdominal muscle with the catheter inserted into the lower abdominal aorta. Heart wall thickness, cross-sectional area of cardiomyocytes, diameter of the arterial cross-sections, and perivascular fibrosis in heart and kidney were measured. The surface area of positive-staining cardiomyocytes was analyzed using image analysis software. Reactive oxygen species (ROS) generation was determined using an oxidant-sensitive fluorogenic probe.

**RESULTS:** Rats that received cooked or raw lentil extracts (oral administration, 8 weeks) show significantly attenuated Ang II-induced elevation in blood pressure, cardiac hypertrophy, perivascular fibrosis. Results demonstrated that pretreatment of cardiomyocytes with cooked or raw lentil extract significantly attenuated the Ang II-induced increase in the size of cells ( $16.0 \pm 1.7\%$  and  $21.2 \pm 2.9\%$ , respectively,  $n=5$ ,  $p < 0.05$ ), and cooked or raw lentil extracts also attenuated the Ang II-induced increase in the reactive oxygen species levels in cardiomyocytes ( $19.8 \pm 2.2\%$  &  $26.6 \pm 3.1\%$ , respectively,  $n=5$ ,  $p < 0.05$ ).

**CONCLUSIONS:** This study showed that extracts from cooked lentils could prevent Ang II-induced elevation in blood pressure, cardiac hypertrophy, small arterial remodeling and perivascular fibrosis, and heating process does not have any significant affect on these protective effects.

**Key Words:**

Raw lentil extract; Cooked lentil extract; Cardiomyocyte hypertrophy; Reactive oxygen species.

## Introduction

Cardiac hypertrophy is recognized as a risk factor for the development of congestive heart failure and a very strong predictor of cardiovascular mortality due to its effects on increasing the risk for cardiac ischemia, left ventricular dysfunction, and sudden cardiac death<sup>1</sup>. It is also an adaptive remodeling response to increased cardiac wall stress caused by blood pressure or volume overload<sup>2</sup>. Ang II plays an important role in the pathogenesis of hypertension, which could trigger heart structural adaptations, causing cardiac hypertrophy<sup>3</sup>. In addition to the load-induced hypertrophy, Ang II also directly contributes to the development of hypertrophy by its growth factor properties and stimulation of AT1 receptors in cardiac myocytes<sup>4</sup>. This is supported by observations in transgenic mice overexpressing angiotensinogen gene or AT1 receptor, specifically in heart, develops severe cardiac hypertrophy and fibrosis<sup>5</sup>. Molecular mechanisms by which Ang II evokes cardiac hypertrophy are the activation of protein kinase cascades, initiation of a fetal-like gene program, impairment of calcium handling, and increased production of reactive oxygen species (ROS)<sup>6</sup>. ROS have emerged as important triggers of the hypertrophic responses, both *in vitro* and *in vivo*, in response to the stretch or other hypertrophy stimuli, such as Ang II<sup>7,8</sup>. Several studies have shown that ROS generation is increased by Ang II treatment

in cardiomyocytes and that inhibition of ROS generation by antioxidants abolishes Ang II-induced cardiomyocytes enlargement<sup>9</sup>.

It has been well studied that diet plays an important role in the pathogenesis and in the prevention of cardiovascular diseases<sup>10</sup>. Epidemiological studies have demonstrated that legume consumption is inversely associated with risk of cardiovascular diseases<sup>11</sup>. These beneficial effects could be mediated by their bioactive components in legumes. One type of their bioactive component has been confirmed by our previously study, phenols which has strong antioxidant activity *in vitro*<sup>12</sup>. Thus, it is believed that the cardiovascular protective effect of legumes could be mediated by those antioxidant bioactive components. Our recent studies have demonstrated that lentils, one of legume family, exhibit greater antioxidant activity as compared with other legume members, such as beans, peas, and soybeans<sup>12,13</sup>. It is even more interesting that raw lentil extract significantly attenuates Ang II-induced increases in cell size and ROS levels in cardiomyocytes of human and rat *in vitro*, and has protective effect on hypertension *in vivo*<sup>14,15</sup>.

One important issue is total phenolic content in beans would be decreased after heating process<sup>16</sup>. Despite increased awareness lentil exerts a strong antioxidant activity, the effects of lentil extract after cooking on hypertension and cardiac hypertrophy *in vivo* are still not clear. Thus, the objective of the present study were 1) to determine the chronic effect of oral administration of cooked lentil extract on the elevated blood pressure, cardiac hypertrophy, and cardiac fibrosis in rat; 2) to identify the protective action of cooked lentil extract on cardiomyocyte *in vitro*; 3) to compare the cardiovascular protective effect of lentil extracts from uncooked and cooked process. In this study, we provide the first evidence that cooked lentil extracts attenuated Ang II-induced hypertension and cardiac hypertrophy, and the antihypertensive effect of lentil is not significantly reduced after cooking.

## Materials and Methods

### Preparation of Raw Lentil Extracts and Cooked Lentil Extracts

Lentils (*L. culinaris* var. Morton) were purchased from Spokane Seed Co. (Spokane, WA, USA). Lentil extracts were prepared as described

previously<sup>13</sup>. Briefly, lentil flour was extracted twice with 70% ethanol in 1:10 (w/v) at room temperature by stirring, firstly for 3h and then over night with another fresh solvent. The combined extract was concentrated by a rotary evaporator (Labconco Co, Kansas City, MO, USA) at 40°C, freeze-dried, and stored at -20°C until use. For preparation of cooked lentil extracts, lentils were soaked for 5 hours in about 3 times tap water, and then heated and boiled for 10 minutes. Ethanol amount was calculated and added to make ethanol 70%. The mix was blended for 2 minutes with slow speed, and ready to be extracted as before.

### Animal

Male Sprague-Dawley (SD) rats (Charles River Laboratories, Wilmington, MA, USA), 140-150 g, were used. The animals were housed under controlled conditions with a 12-h light/dark cycles. Food and water were available to the animals *ad libitum*. The experimental procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee (protocol# A0919) and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Animals were divided into four groups (n=5 each group): control group, Ang II group, Ang II plus cooked lentil extract (Ang II+CLE) group, and Ang II plus raw lentil extract (Ang II+RLE) group. The telemetry blood pressure transducers were implanted into all rats. After five days recovery from surgery, basal blood pressure (BP) and heart rate (HR) were measured for 6 days. Then, CLE (50 mg/day, in 1 ml drinking water) and RLE (50 mg/day, in 1 ml drinking water) were administered by using the intragastric gavage technique in CLE and RLE groups respectively, and both of control group and Ang II group were given 1ml drinking water. Intragastric gavage process was administrated carefully to avoid hurting rats and disturb the telemetry device. Four weeks later, subcutaneous Ang II infusion (Ang II 200 ng/kg/min, using osmotic mini-pumps) was performed except for control group rats. The surgery for osmotic mini-pump implantation was performed as described in previous publication<sup>17</sup>. Rats were anesthetized by inhalation of a mixture of oxygen (1 L/min) and isoflurane (5% induction, 3% maintenance, Halocarbon, River Edge, NJ, USA) through a nose cone. A 0.5-inch incision was made between the shoulder blades and the pump was inserted subcutaneously.

### **Blood Pressure and Heart Rate Measurement**

BP and HR recording were carried out with a radio-telemetry system as previously described<sup>18</sup>. The same anesthetization procedure was selected as above. A telemetry BP probe (model TA11PA-C40, Data sciences International, St. Paul, MN, USA) was positioned intra-abdominally and secured to the ventral abdominal muscle with the catheter inserted into the lower abdominal aorta. The telemetry signals were processed and digitized as radio frequency data, which were recorded and stored using the Dataquest IV system (Data Sciences International, St. Paul, MN, USA). Mean value of BP and HR were recorded continuously for 1h per day between 10:00AM and 11:00AM in conscious state throughout the study duration.

### **Assessment of Cardiac Hypertrophy, Arterial Remodeling, and Fibrosis in Heart and Kidney**

After euthanizing with a lethal dose of sodium pentobarbital (200 mg/kg, Sigma, St. Louis, MO, USA) intraperitoneally, the heart and kidney was collected. The hearts and kidneys were measured, weighed, and fixed in 10% formalin/PBS, and embedded in paraffin. The middle segment of the hearts and kidneys were cut into five sub-serial sections with a thickness of 10  $\mu\text{m}$  at intervals of 0.3 mm. The sections were stained with hematoxylin/eosin or Sirius red for measurement of heart wall thickness, cross-sectional area of cardiomyocytes, diameter of the arterial cross-sections, and perivascular fibrosis in heart and kidney. The media/lumen ratio of small arteries which had similar diameter was calculated as described previously<sup>19</sup>. Micrographs were detected using an Olympus microscope equipped with a color digital camera (Infinity 2). The histology and morphology were examined and analyzed by the Image Analysis Software (Image Pro plus6.0, Media Cybernetics, Bethesda, MD, USA). The external diameter of the arteries measured was no more than 100 $\mu\text{m}$  and the number of arterial cross-sections measured was more than 50.

### **Cardiomyocyte Culture, Immunocytochemistry and Measurement of Cell Surface Area**

Primary cultured cardiomyocytes were prepared from 1-day-old neonatal rats after euthanizing with a lethal dose of sodium pentobarbital (200 mg/kg) intraperitoneally according to our

previous published procedures<sup>20</sup>. Cardiomyocytes were pretreated with Hank's Balanced Salt Solution (HBSS), CLE (25, 50, and 100  $\mu\text{g}/\text{mL}$ ) and RLE (25, 50, and 100  $\mu\text{g}/\text{mL}$ ) for 24h. Then Ang II (100 nmol/L) was added and cultured for another 24h. Each treatment was performed in triplicate wells. Monoclonal anti- $\alpha$ -sarcomeric actin antibody (Sigma, St. Louis, MO, USA) was used for the identification and measurement of the cardiomyocyte surface area via a fluorescent microscope as our previous publication<sup>21</sup>. The surface area of positive-staining cardiomyocytes was analyzed using image analysis software (Image J). At least 50 cells were randomly selected and analyzed for surface area for each well.

### **Measurement of Intracellular ROS Generation**

ROS generation was determined using an oxidant-sensitive fluorogenic probe, Dihydroethidium (DHE; excitation/emission wavelength: 485 nm/590 nm; Molecular probes) as our previous publication<sup>21</sup>. In brief, cultured cardiomyocytes were treated with control, CLE (50  $\mu\text{g}/\text{ml}$ ), RLE (50  $\mu\text{g}/\text{ml}$ ) for 24 hours. The cells were loaded with 100 nmol/L DHE for 30 minutes at 37°C. The cells were rinsed with Phosphate Buffered Saline (PBS), followed by addition of 100 nmol/L Ang II for 5 minutes. The fluorescence density of ethidium fluorescence within the cells was detected by using a fluorometric imaging plate reader (Spectra Max Gemini EM, Molecular Devices Corp., Sunnyvale, CA, USA), in the same cells before and after Ang II addition. Each treatment condition was run in triplicate within experiments and each set of experiments was performed using three separate culture dishes.

#### *Statistical Analysis*

Data are expressed as mean  $\pm$  SD. Statistical comparisons were made by two-way analysis of variance (ANOVA) followed by Kruskal-Wallis ANOVA on ranks for multivariate. Value of  $p < 0.05$  was considered to be statistically significant.

## **Results**

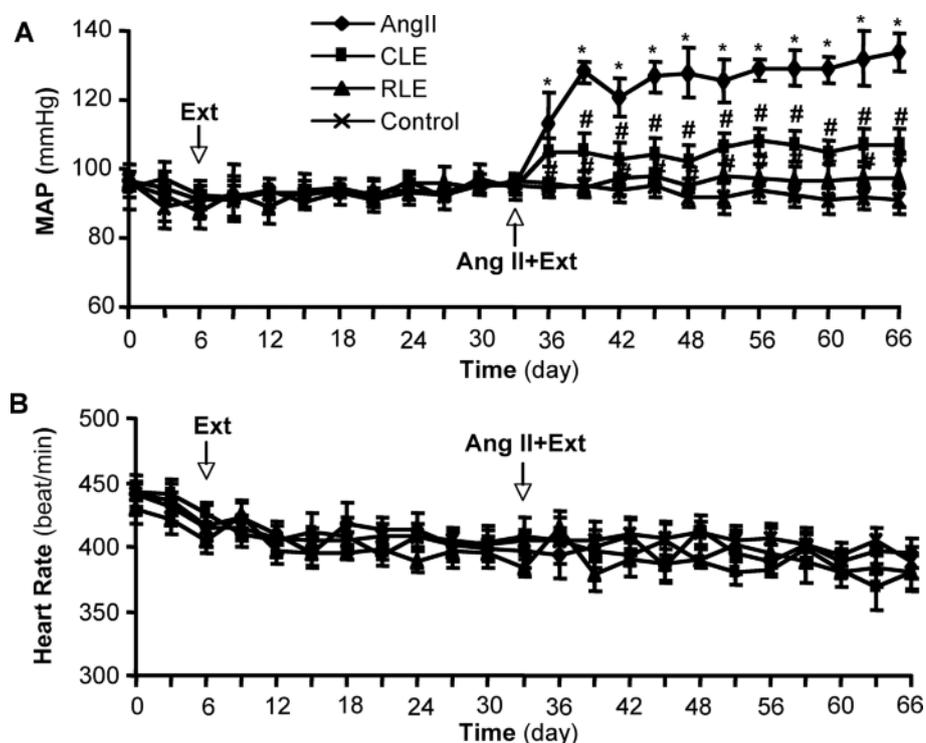
### **CLE and RLE Attenuated Ang II-induced Increase in BP**

The chronic effect of oral administration of CLE and RLE was examined in the Ang II- induced hypertension rats. The results of mean arterial pressure (MAP) and HR recorded in the rats received treatment with control, Ang II, Ang II+CLE, and Ang

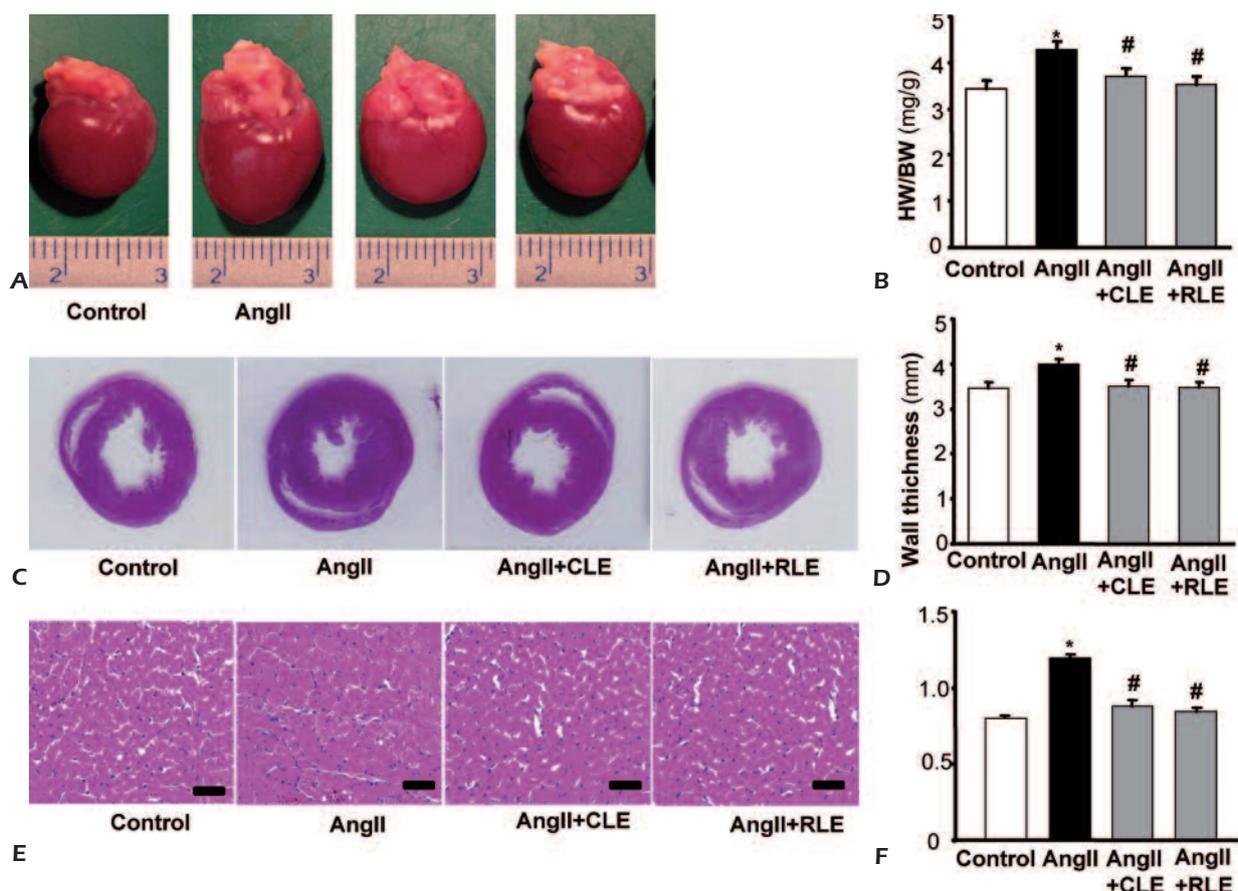
II+RLE were presented in Figure 1A and B. Oral administration of CLE (50 mg/day) or RLE (50 mg/day) for 4 weeks did not significantly alter either basal BP or basal HR as shown in Figure 1. Chronic subcutaneous infusion of Ang II (200 ng/kg/min) significantly increased MAP from  $93.4 \pm 2.0$  mmHg to  $126.7 \pm 2.1$  mmHg ( $n=5, p < 0.01$ ) in Ang II group. The time-dependent pressor effect of Ang II reached to the peak in 5 days and lasted at least 4 weeks (Figure 1A). However, this pressor effect of Ang II was significantly attenuated by pre-treatment with CLE (BP:  $126.7 \pm 2.1$  mmHg in Ang II group and  $105.2 \pm 1.8$  mmHg in Ang II+CLE group respectively,  $n=5, p < 0.05$ ) or RLE (BP:  $126.7 \pm 2.1$  mmHg in Ang II group and  $96.6 \pm 1.2$  mmHg in Ang II+RLE group respectively,  $n=5, p < 0.01$ ). The antihypertensive effect of RLE is not significant higher than CLE (BP reduction:  $31.0 \pm 1.1$  mmHg in Ang II+RLE group and  $24.2 \pm 1.5$  mmHg in Ang II+CLE group respectively,  $n=5, p > 0.05$ ). In addition, neither Ang II subcutaneous infusion nor lentil extracts oral administration altered the HR compared with control group.

### Effects of CLE and RLE on Cardiac Remodeling Induced by Ang II

Heart weight-to-body weight ratio (HW/BW), wall thickness and cross-sectional area of cardiomyocyte were examined in all groups of rats. The results demonstrated that subcutaneously Ang II infusion (200 ng/kg/min, 4 weeks) significantly increased HW/BW, left ventricular wall thickness, and the size of cardiomyocytes as compared with control rats (Figure 2). Oral administration of CLE (50 mg/day, 8 weeks) significantly attenuated Ang II-induced increases in HW/BW by  $69.4 \pm 4.6\%$  (Figures 2A and B), in left ventricular wall thickness by  $78.5 \pm 3.1\%$  (Figure 2C and D), in the size of cardiac myocytes by  $76.7 \pm 3.6\%$  (Figure 2E and F). In contrast, oral administration of RLE (50 mg/day, 8 weeks) attenuated Ang II-induced increase in HW/BW by  $75.3 \pm 5.1\%$  (Figures 2A and B), in left ventricular wall thickness by  $82.6 \pm 4.8\%$  (Figure 2C and D), in the size of cardiac myocytes by  $80 \pm 4.0\%$  (Figure 2E and F). There is no significant difference in the HW/BW, left ventricular wall thickness, and the size of cardiac myocytes between rats receiving CLE and RLE.



**Figure 1.** Effect of lentil extracts and Ang II on mean arterial pressure and heart rate. Mean arterial pressure (MAP) (A) and heart rate (HR) (B) were recorded using telemetry in rats treated with control (saline subcutaneous perfusion), Ang II (subcutaneous perfusion, 200 ng/kg/min), Ang II perfusion plus cooked lentil extract (Ang II+CLE, oral administration, 50 mg/day), or Ang II plus raw lentil extract (Ang II+RLE, oral administration, 50 mg/day). Data are presented as means  $\pm$  SD ( $n=5$ ). \* $p < 0.05$  vs control group. # $p < 0.05$  vs Ang II infusion group.



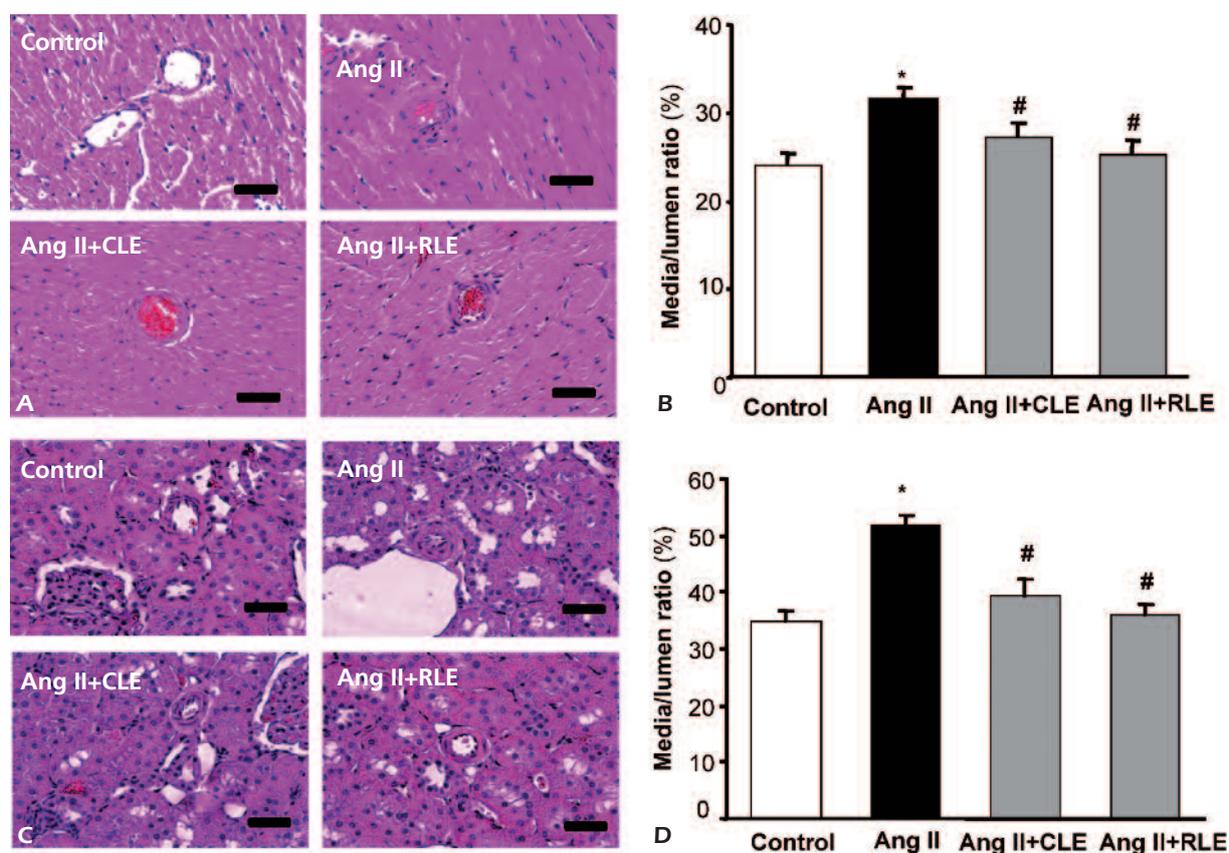
**Figure 2.** Effect of lentil extracts on Ang II-induced cardiac hypertrophy. Cardiac morphological and histological characteristics were examined in four groups of rats treated with control, Ang II, Ang II (subcutaneous perfusion, 200 ng/kg/min), Ang II perfusion plus cooked lentil extract (Ang II+CLE, oral administration, 50 mg/day), Ang II plus raw lentil extract (Ang II+RLE, oral administration, 50 mg/day). **A**, Representative images of the heart gross morphology of rats treated under the conditions indicated. The scale in the image is in centimeter. **B**, Bar graphs showing heart weight/body weight ratios of rats treated under the conditions indicated. **C**, H&E staining of a short axis cross section of hearts of rats treated with control, Ang II, Ang II+CLE, and Ang II+RLE. **D**, Bar graphs showing left ventricular wall thickness (mm) measured at the mid-point of the left ventricles in rats treated under the conditions indicated. **E**, Micrographs (40X magnification) showing representative heart sections stained with H&E in each group of rats. The scale in the photo is 50 micrometer. **F**, Average cardiomyocyte cross-sectional areas from transverse cardiac sections. Data are mean  $\pm$  SD ( $n=5$ ), \* $p < 0.05$  vs control group. # $p < 0.05$  vs Ang II group.

### Effects of CLE and RLE on Peripheral Vascular Remodeling and Perivascular Fibrosis Induced by Ang II

We observed the effects of CLE or RLE on the vascular remodeling (Figure 3) and perivascular fibrosis (Figure 4) in the heart and kidney of rats received subcutaneous infusion of Ang II. Subcutaneous Ang II infusion (200 ng/kg/min, 4 weeks) significantly increased small arterial media/lumen ratio ( $23.8 \pm 1.2\%$  and  $31.5 \pm 1.4\%$  in control and Ang II treatment rats respectively,  $n=5$ ,  $p < 0.05$ ) in the hearts, and ( $35.0 \pm 1.8\%$  and  $51.7 \pm 1.9\%$  in control and Ang II treatment rats respectively,  $n=5$ ,  $p < 0.01$ ) in kidney. Oral administration of RLE (50 mg/day, 8 weeks) significantly attenuat-

ed increases in small arterial media/lumen ratio by 20.4% (Figure 3A and B) in the heart and 30.3% in the kidney caused by Ang II (Figure 3C and D). In contrast, CLE, in the same treatment protocol, attenuated the increases in small arterial media/lumen ratio induced by Ang II by 19.2% in heart (Figure 3A and B) and 27.5% in kidney (Figure 3C and D). There are no significant differences in small arterial media/lumen ratio between CLE and RLE treatment rats.

Perivascular fibrosis in heart (Figure 4A and B) and kidney (Figure 4C and D) was examined in the four groups of rats. Subcutaneous Ang II infusion (200 ng/kg/min, 4 weeks) significantly increased perivascular fibrosis ( $27.3 \pm 1.9\%$  and  $37.1 \pm 3.0\%$



**Figure 3.** Effect of lentil extracts on Ang II-induced small arterial remodeling in heart and kidney. Upper panels are representative microscopic images of the artery cross-section of the heart (**A**) and kidney (**B**) stained with H&E in rats treated with control, Ang II, Ang II+CLE, and Ang II+RLE. The scale in the images is 50 micrometer. Lower panels are bar graphs summarizing the media/lumen ratios of small arteries in the heart (**C**) and kidney (**D**). Data are presented as the mean  $\pm$  SD (n=5). \* $p < 0.05$  vs control group. # $p < 0.05$  vs Ang II infusion group.

in control and Ang II treatment rats respectively,  $n=5$ ,  $p < 0.05$ ) in the hearts, and  $32.7 \pm 2.0\%$  and  $43.9 \pm 2.9\%$  in control and Ang II treatment rats respectively,  $n=5$ ,  $p < 0.01$ ) in kidney. Oral administration of CLE (50 mg/day, 8 weeks) significantly attenuated the increases in perivascular fibrosis caused by Ang II by 21.6% in heart (Figures 4 A and B) and 21.9% in kidney (Figures 4C and D). In contrast, RLE (oral administration, 50 mg/day, 8 weeks) attenuated increases in perivascular fibrosis caused by Ang II by 25.3% in heart (Figure 4A and B) and 24.8% in kidney (Figures 4C and D).

#### **Effects of CLE and RLE on Ang II-induced Cardiomyocyte Hypertrophy in vitro**

The direct effect of CLE and RLE on Ang II-induced cardiomyocyte hypertrophy was checked in cells cultured from normotensive rats. Exposure of cardiomyocytes to 50  $\mu\text{g/mL}$  CLE or RLE for 48h did not alter the size of myocardial cells significant-

ly, as compared to the control (HBSS) (data not shown). Ang II (100 nmol/L, 24h) treatment induced significant cardiomyocyte hypertrophy, which was attenuated by pretreatment with CLE (50  $\mu\text{g/ml}$ ) or RLE (50  $\mu\text{g/ml}$ ) (Figure 5). Measurement of cell surface area revealed an increase of  $42.1 \pm 3.7\%$  in response to Ang II as compared to the control ( $n=5$ ,  $p < 0.05$ ). Treating cardiomyocytes with CLE or RLE significantly alleviated Ang II-induced hypertrophy by  $16.0 \pm 1.7\%$  and  $21.2 \pm 2.9\%$  respectively ( $n=5$ ,  $p < 0.05$ ) (Figure 5B). The hypertrophy preventing effects of CLE and RLE were dose-dependent. The Ang II-induced cardiomyocyte hypertrophy was attenuated by  $12.6 \pm 4.5\%$ ,  $21.2 \pm 2.9\%$ , and  $28.9 \pm 1.2\%$  after treatment with RLE at doses of 25, 50 and 100  $\mu\text{g/mL}$ , respectively (data not shown). In contrast, the Ang II-induced cardiomyocyte hypertrophy was attenuated by  $11.3 \pm 2.5\%$ ,  $16.0 \pm 1.7\%$ , and  $27.2 \pm 4.9\%$  after treatment with CLE at doses of 25, 50 and 100  $\mu\text{g/mL}$ ,

respectively (data not shown). There was no significant difference between the effect of CLE and RLE on Ang II-induced cardiomyocyte hypertrophy.

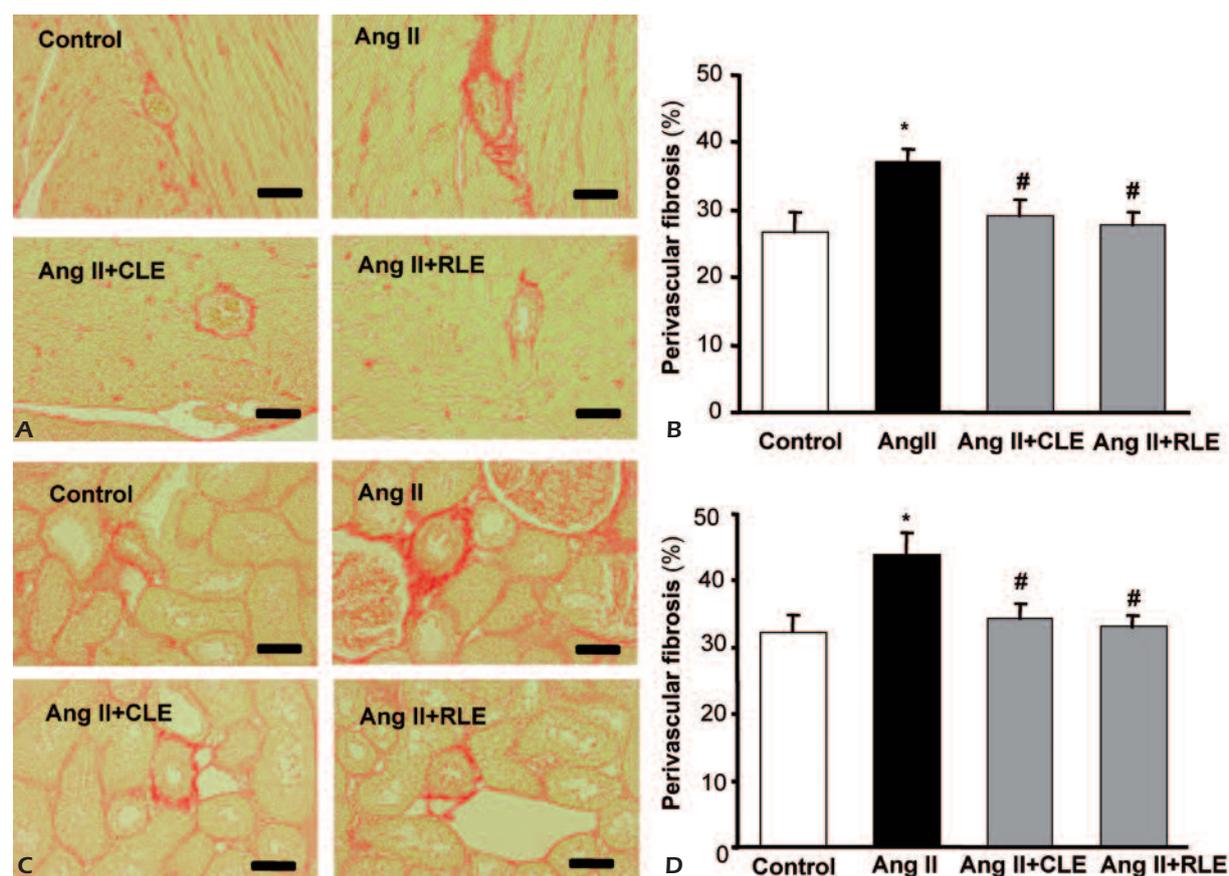
### **Effects of CLE and RLE on Ang II-induced Cellular ROS Stress in Cultured Cardiomyocytes**

The effect of CLE, RLE, and Ang II on intracellular ROS levels was measured in cultured cardiomyocytes. The results (Figure 6) demonstrated that Ang II treatment significantly increased intracellular ROS generation by  $71.5 \pm 5.8\%$  as compared to the control. Pretreatment of cardiomyocytes with CLE (50  $\mu\text{g/mL}$ , 24h) significantly attenuated Ang II-induced increase of intracellular ROS levels by  $19.8 \pm 2.2\%$  ( $n=5$ ,  $p < 0.05$  as compared to the Ang II treatment alone). Pretreatment of cardiomyocytes with RLE (50  $\mu\text{g/mL}$ , 24h) significantly attenuated Ang II-induced increases in intracellular ROS levels

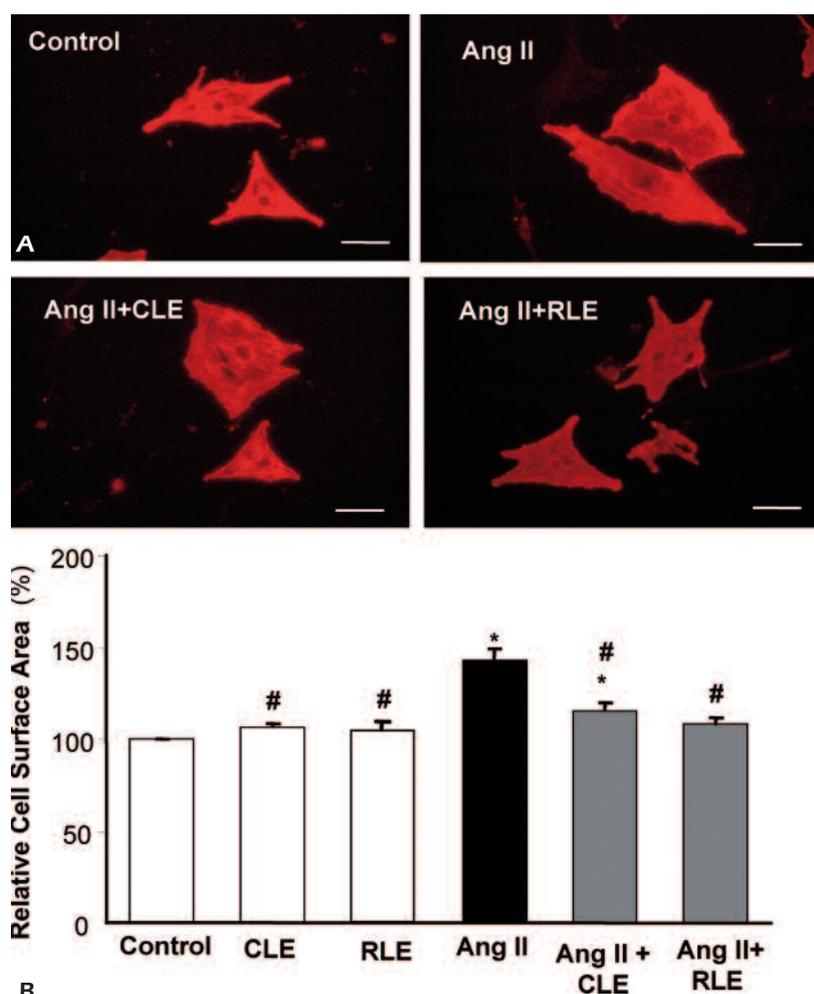
by  $26.6 \pm 3.1\%$  ( $n=5$ ,  $p < 0.05$  as compared to the Ang II treatment alone). However, treatment with CLE or RLE alone did not alter intracellular ROS levels under the same dose (data not shown).

### **Discussion**

The current study presents the first evidence that oral administration of CLE prevents hypertension induced by subcutaneous infusion of Ang II and performs antioxidative effects on Ang II-induced cardiomyocyte hypertrophy. This conclusion is supported by the following observations: 1) oral administration of CLE for 8 weeks significantly attenuated Ang II-induced increases in MAP, HW/BW ratio, left ventricular wall thickness, small arterial media/lumen ratio, perivascular fibrosis and the size of cardiomyocytes in rats;



**Figure 4.** Effect of lentil extracts on Ang II-induced perivascular fibrosis in heart and kidney. The perivascular fibrosis in heart (A,C) and in kidney (B,D) were measured in the rats treated with control, Ang II, Ang II+CLE, and Ang II+RLE. Upper panels are representative microscopic images of the arterial cross-section of the heart (A) and kidney (B) stained with Sirius Red in rats treated under the conditions indicated. The scale in the images is 50 micrometer. Lower panels are bar graphs summarizing the perivascular fibrosis in the heart (C) and kidney (D). Data are presented as the mean  $\pm$  SD ( $n=5$ ). \* $p < 0.05$  vs control group. # $p < 0.05$  vs Ang II infusion group.



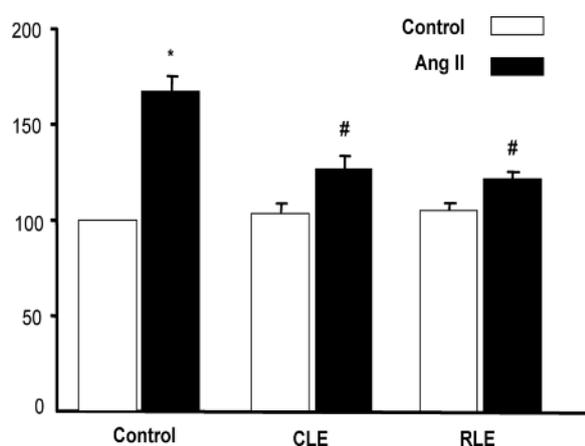
**Figure 5.** Effect of lentil extracts on Ang II-induced cardiac hypertrophy in rat cardiomyocytes. **A**, Representative fluorescence micrographs of cultured cardiomyocytes stained with  $\alpha$ -sarcomeric actin antibody after the following treatments: control (con, HBSS, 48h), Ang II (100 nmol/L, 24h) alone, Ang II (100 nmol/L, 24h) plus cooked lentil extract (CLE, 50  $\mu$ g/ml, 48h), and Ang II (100 nmol/L, 24h) plus raw lentil extract (RLE, 50  $\mu$ g/ml, 48h). **B**, Bar graphs summarizing the size of cardiomyocytes after the treatments as described above. Data are means  $\pm$  SD and derived from three experiments and at least triplicate wells in each experiment. \* $p < 0.05$  vs control (HBSS). # $p < 0.05$  vs the treatment with Ang II alone.

2) treatment with CLE significantly prevents Ang II-induced hypertrophy and ROS generation in cardiomyocytes cultured from neonatal rats; 3) The CLE has similar antihypertensive and antioxidant effect as RLE.

It is well known that Ang II plays an important role in the regulation of blood pressure and in the pathogenesis of hypertension.<sup>22-24</sup> This conclusion is also supported by the current observation that chronic subcutaneous Ang II infusion results in a significantly elevation of blood pressure, small arterial remodeling, and perivascular fibrosis. Recent studies demonstrate that ROS formation and the subsequent inflammatory processes induced by Ang II may play a critical role in the pathogenesis of hypertension<sup>25,26</sup>. Ang II promotes excess amounts of intracellular ROS production via stimulation of NADPH oxidase or mitochondrial-dependent pathway in vascular smooth muscle cells, endothelial cells, and fibroblasts, which in turn contributes to endothe-

lial dysfunction, vascular remodeling, and perivascular fibrosis. Accordingly, some of these benefits that can be derived from the reduction of ROS have been observed in the prevention of these complications of hypertension<sup>27</sup>. For example, an antioxidant-enriched diet improves hypertension and inflammatory changes in the SHR rats<sup>28</sup>. In current study, we demonstrate that CLE or RLE prevents Ang II-induced hypertension, and shows strong antioxidant action.

Cardiovascular hypertrophy is frequently associated with chronic hypertension because of a compensatory response and wall stress. In addition to this load-induced hypertrophic response, Ang II may directly contribute to the development of hypertrophy via its growth factor properties on cardiac cells. This hypothesis is strongly supported by an observation on transgenic mice overexpression of angiotensinogen gene specifically in the heart, which develops myocardial hypertrophy and enhanced local Ang II



**Figure 6.** Effect of lentil extracts on Ang II-induced increases in intracellular ROS levels in rat cardiomyocytes. ROS levels were detected using oxidant-sensitive fluorogenic probe dihydroethidium. The effects of Ang II (100 nmol/L) on intracellular ROS levels were examined on cardiomyocytes pre-treated with control (HBSS), cooked lentil extract (CLE, 50  $\mu$ g/ml, 24h), or raw lentil extract (RLE, 50  $\mu$ g/ml, 24h). The fluorescence density of ethidium was measured in the same cardiomyocytes before and after addition Ang II (100 nmol/L, 5 min). Data are mean $\pm$ SE and derived from three experiments and at least triplicate wells in each experiment. \* $p$  < 0.05 vs control (HBSS), # $p$  < 0.05 vs the treatment with Ang II alone.

production<sup>29</sup>. In this study, we demonstrate that oral administration of CLE and RLE significantly attenuated Ang II-induced myocardial hypertrophy in normotensive rats. This protective effect of CLE and RLE on cardiomyocytes may be mediated by attenuation of the blood pressure and reduction of pressure-loaded stress. Beside the indirect action through reduction of blood pressure, CLE and RLE may also attenuate Ang II action in the heart locally. This hypothesis is supported by the *in vitro* cell culture study showing that treatment of cultured cardiomyocyte with CLE or RLE significantly attenuated Ang II-induced cardiomyocyte hypertrophy.

The cellular mechanisms underlying the protective effect of CLE and RLE on Ang II-induced cardiac hypertrophy are not clear. Accumulated amount of studies demonstrate that over produced ROS and inflammation factors generated by Ang II stimulation contributes to the development of hypertrophy and remodeling in the heart<sup>8,25,26,28</sup>. Lentil extracts contains significant amount of phenolic compounds which has been shown a strong antioxidant property<sup>12</sup>. Therefore, similar antioxidative mechanisms may occur after oral administration of CLE and RLE, which prevent Ang II-induced over production of ROS. This notion is supported by our

*in vitro* observation showing that treatment of cardiomyocyte with CLE and RLE significantly attenuate Ang II-induced ROS production. However, the exact molecular and cellular mechanisms underlying CLE and RLE-induced reduction in intracellular ROS levels and whether this mechanism contributes to the protective effect on cardiac hypertrophy still need to be further investigated.

Lentils constitute an important source of diet for humans in many countries and contain abundant phenolic compounds, and prevent cardiomyocyte hypertrophy and hypertension in our previous *in vitro* and *in vivo* studies<sup>12,14,15</sup>. The heating process could significantly decrease the total phenolic contents and antioxidant activities of beans during in our previous *in vitro* study<sup>14</sup>. This study indicates that the antihypertensive effect of lentil is reduced by cooking, but it is not significantly decreased as total phenolic compounds change, which means some other antioxidant mechanism may be activated possibly during heating process. This observation means the lentil also has enough antihypertensive effect after boiling, which will further support lentil consumption is better for cardiovascular disease.

## Conclusions

The current study demonstrated that oral administration of CLE significantly prevents Ang II-induced cardiac hypertrophy, hypertension, small arterial remodeling and perivascular fibrosis. The antioxidant mechanisms may be involved in these effects. The CLE has similar antihypertensive and antioxidant effect as RLE, suggesting that different mechanisms may be involved during heating process. These observations suggest that inclusion of lentil in the diet may have benefit to a variety of cardiovascular diseases, which are characterized by an increase in oxidative stress or by the activation of renin-angiotensin system.

## Acknowledgements

This work was supported by Grants 13600 and 15053 from USDA CSREES (University of Idaho CSFL Research Program) and by the American Heart Association (Program# 10GRNT3170012). This research also was supported by Jilin Province Science and Technology Department (Program# 20110718).

## Conflict of Interest

The Authors declare that they have no conflict of interests.

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