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Antihypertensive effects of Hsian-tsao and its active compound in spontaneously hypertensive rats

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Abstract

The present study aimed to investigate the effects of Hsian-tsao (*Mesona procumbens* Hemsl.) and its active compound on blood pressure, lipid peroxidation and total antioxidant status of spontaneously hypertensive rats (SHRs). Male SHRs were orally administrated either a water extract of Hsian-tsao (WEHT) (1.0 g/kg) or caffeic acid (CA) [0.1 g/kg of body weight (BW)] on a daily basis for 6 weeks. The results indicated that both hepatic and plasmatic malondialdehyde concentration were increased and total liver glutathione (GSH) levels and antioxidant enzyme activities were decreased in SHRs when compared to the control Wistar Kyoto rats at the end of the trail. In SHRs, oral administration of WEHT or CA for 6 weeks reduced blood pressure as well as plasma and hepatic malondialdehyde levels and increased hepatic antioxidant enzyme activities when compared to SHRs control rats. Reverse transcriptase-polymerase chain reaction results indicated that the changes in hepatic antioxidant enzyme mRNA levels by WEHT or CA were similar to those noted in the enzyme activity levels. The hepatic levels obtained from WEHT or CA-administrated rats had significantly greater oxygen radical absorbance capacity values and total GSH levels than those of control rats. Following oral administration of CA, phenolic acid was detected in the plasma, and C_{max} value after 1.0 h administration was 0.92 μmol/L. These findings indicate that a supplement of Hsian-tsao may prevent development of increased blood pressure and enhance the total antioxidant status in vivo.

Keywords: Antihypertension; Mesona procumbens; Caffeic acid; Spontaneously hypertensive rats

1. Introduction

Hypertension is associated with many chronic conditions such as insulin resistance, obesity, carbohydrate tolerance, hyperuricacidemia, atherosclerosis and cardiovascular diseases [1]. Hypertension affects 30% of adults in the Western world and is the leading cause of death and morbidity worldwide [2]. Therefore, hypertension has received increasing attention by researchers.

Clinically, various antihypertensive drugs such as hypotensive diuretics, beta-blocking agents, calcium antagonists, angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists and alpha-receptor blocking agents have been used to manage hypertension and alleviate symptoms [3]. However, the efficacy of these drugs is only 40–60%, and usually, two or more antihypertensive drugs from different categories need be combined to achieve optimal results. Additionally, the side effects from these medications are an important concern.

The development of a safe and effective way to manage hypertension has challenged medical researchers for centuries. Recently, various foods with differing hypotensive mechanisms have been specified for health promotion. Several food-derived peptides can inhibit angiotensin by converting enzymes such as alpha-lactalbumin, casein, zein, gelatin and yam dioscorin, all of which are hydrolyzed by pepsin, trypsin or chymotrypsin [4–6]. Although the mechanisms leading to high blood pressure remain largely unknown, among the many factors implicated in the pathophysiology of hypertension, there is compelling evidence that reactive oxygen species may be important [7]. If oxidative stress is truly implicated in the pathophysiology of hypertension, then strategies to reduce bioavailability of

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reactive oxygen species should have important therapeutic potential in the management of hypertension. Targeted overexpression of antioxidant systems and interference with expression of oxidant systems have also been used in animal models of hypertension [8]. Some flavonoids and polyphenol derivatives from natural products have been reported to have vasodilating and hypotensive effects [9,10].

The herb *Mesona procumbens* Hemsl., called Hsian-tsao in China, is consumed as both an herbal drink and a jelly-type dessert in the Orient. It is also used as an herbal remedy in the folk medicine of China to treat heat-shock, hypertension, diabetes, liver disease and muscle and joint pains. In our previous studies, we found that phenolic compounds extracted from Hsian-tsao significantly contributed to antimutagenic antioxidant activity and free radical scavenging activity [11–13]. Yen et al. [14] indicated that the ultraviolet C (UV-C) and/or H_2O_2-induced DNA damage in human lymphocytes is significantly reduced by water extract of Hsian-tsao (WEHT). Recently, WEHT was found to exhibit efficient protective action against *tert*-butylhydroperoxide-induced hepatic and oxidative damage in rats [15]. Hsian-tsao has demonstrated strong antioxidant activity; however, there have been no studies of the antihypertensive effect of Hsian-tsao and its bioactive compound (caffeic acid) on blood pressure in animals. The spontaneously hypertensive rat (SHR) is the most common model of high blood pressure overload cardiomyopathy and heart failure [16]. These rats descend from a Wistar male with hypertension from a colony in Kyoto, Japan, and they are characterized by the fact that they suffer from prehypertension [systolic blood pressure (SBP) 100–120 mm Hg] during the first 6–8 weeks of their life and then develop sustained hypertension (SBP >150 mmHg) over the next 12–14 weeks [17]. The advantage of using SHRs is that they offer specific and uniform genetic predisposition, thus allowing the study of the causes, mechanisms, and pathology of hypertension.

In this study, an effort to determine the antihypertensive effects of Hsian-tsao on blood pressure and oxidative status was initiated using spontaneously hypertensive rats. Furthermore, the potency of caffeic acid, the major compound of phenolic acid in Hsian-tsao, was examined.

2. Materials and methods

2.1. Materials and chemicals

Dried Hsian-taso (*Mesona procumbens* Hemsl.) was purchased from a local market in Taichung, Taiwan. Caffeic acid (3,4-dihydroxycinnamic acid, 98% purity), β-nicotinamide adenine dinucleotide phosphate, sodium acetate, ammonium acetate, glutathione (GSH), sodium chloride, 1,1,3,3-tetramethoxypropane, thiobarbituric acid (TBA), 2,4-dinitrofluorobenzene, nitrobule tetrazolium (NBT) and sodium bicarbonate were purchased from Sigma Chemical (St. Louis, MO, USA). Iodoacetic acid and solvents were purchased from E. Merck (Darmstadt, Germany). A TRIzol RNA isolation kit was obtained from Life Technologies (Rockville, MD, USA), and primers for reverse transcriptase-polymerase chain reaction (RT-PCR), dNTP reverse transcriptase, and Taq polymerase were obtained from Gibco BRL (Cergy Pontoise, France). Protein assay reagent was purchased from Bio-Rad (Hercules, CA, USA). All other chemicals used were of the highest grade purity available.

2.2. Preparation of WEHT

The dried Hsian-tsao was cut into small pieces and ground into a fine powder in a mill (RT-08, Rong Tsong, Taichung, Taiwan). A Hsian-tsao sample (10 g) was extracted with boiling water (200 ml) for 2 h. The extracts were filtered through Whatman no. 1 filter paper, and the filtrate was freeze-dried into a powder form. The yield of freeze-dried residue corresponded to 15.2% of the original dry weight. The caffeic acid (CA) content in extracts isolated from Hsian-tsao was 17.2 mg/g of lyophilized powder. The deep brown extract powder was dissolved directly in 0.9% saline for further tests.

2.3. Antihypertensive activity of WEHT

The effect of orally administrated WEHT or CA on systolic blood pressure was determined according to the method described by Yamakoshi et al. [18], with minor modifications. Male 6-week-old spontaneously hypertensive rats (SHRs) and Wistar-Kyoto rats (WKYs) were obtained from the Experimental Animal Center at National Taiwan University. All rats had free access to Lab Diet (5001 Rodent Diet, PMI, St. Louis, MO, USA). The rats lived in a room kept at a temperature of 23±1°C and relative humidity of 55±10%, and received light 12 h/day. Tap water was provided for rats to consume ad libitum as drinking water. An adaptation period of two weeks was allowed for vehicle administration and blood pressure measurements before initiation of the experimental protocols. Eighteen SHRs were randomly divided into three groups (six rats per group) and assigned to receive WEHT (1.0 g/kg of BW) or CA (0.1 g/kg of BW) dissolved in a physiologic saline buffer solution. Rats were treated orally by gavage for 6 weeks. The control group received only the vehicle (physiologic saline solution). During the experimental periods, rats had free access to tap water and food. BW was measured every week. Animals showing no health abnormalities were used for the experiments. The blood pressure and heart rate were measured non-invasively with a tail-cuff method using the Softron BP system (BP-98A, Softron, Tokyo, Japan) after warming the animals at 37°C for 10 min. At least six determinations were made in every session of SBP measurements and the mean of six values within 5 mmHg was taken as the SBP level. All experimental procedures involving animals were conducted in accordance with National Institutes of Health guidelines. This experiment was approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University, Taichung, Taiwan.

2.4. Lipid peroxidation assay

Rats were killed by decapitation, and the abdomens of the animals were opened and perfused using an injection of ice-cold saline. Liver tissues were removed promptly and immediately placed on ice. Approximately 100 mg of liver was homogenized in ice-cold 20 mmol/L Tris-HCl (pH 7.4) to produce a tissue homogenate. The homogenate was centrifuged at 2500×*g* for 30 min at 4°C. Aliquots of the homogenate were collected and immediately tested for lipid peroxidation. The lipid peroxidation product, malondialdehyde (MDA), was assayed according to an improved thiobarbituric acid fluorometric method at 555 nm with excitation at 515 nm using 1,1,3,3-tetramethoxypropane as a standard [19]. The protein concentration was determined using a commercial kit (Bio-Rad Laboratory Ltd.) with bovine serum albumin (BSA) as a standard. The results were expressed as MDA formation per milligram of protein.

2.5. Assay of GSH

Liver tissues were homogenized in an ice-cold potassium phosphate buffer (pH=7.4). The homogenate was centrifuged at 2500×*g* for 30 min at 4°C. The content of reduced GSH was determined by modifying the method of Van Dam et al. [20]. Liver homogenate and a 5% trichloroacetic acid (TCA) mixture was preincubated for 5 min at 4°C and then centrifuged at 8000×*g* for 10 min at 4°C. Aliquots of the homogenate were collected and 5-dithio-bis-(2-nitrobenzoic acid) was immediately added to them and incubated for 5 min at 4°C. Absorbance was measured at 412 nm, and the concentration of GSH was calculated using the absorbance of 1 mol/L of product with E412=13 600 mol/L^{-1} cm^{-1}.

2.6. Assay for antioxidant enzymes

Total superoxide dimutase (SOD) activity was determined by the method of Spitz and Oberley [21] with slight modifications. The reaction mix was freshly prepared in 50 mmol/L potassium phosphate buffer. This solution registered at pH 7.8 and consisted of 1.33 mmol/L diethylenetriaminepentaacetic acid, 1.0 U/ml catalase (CAT), 70 μmol/L nitroblue tetrazolium, 0.2 mmol/L xanthine, 0.05 mmol/L bathocuproine sulfonate, and 0.13 g/L BSA. Next, 0.8 ml of the reaction mix was added to each cuvette, followed by addition of 100 μl of the sample. The cuvettes were prewarmed at 37°C for 3 min. The reaction was then started by adding 100 μl of xanthine oxidase (0.1 U/ml). The formation of formazan blue was monitored at 560 nm and 37°C for 5 min. The total SOD activity of sample was calculated using a concurrently run SOD (Sigma Chemical) standard curve and expressed as units per milligram of sample protein. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. Based on the procedure of Bradford [22], sample protein content was quantified with Bio-Rad protein assay dye with BSA as the standard.

The GSH peroxide (GPx) activity was measured according to Flohe and Gunzler [23]. Briefly, 1 mmol/L EDTA, and 2 mmol/L sodium azide, 100 μl of sample, 100 μl of 10 mmol/L GSH, 100 μl of GSH reductase (2.4 U/ml), and 100 μl of 1.5 mmol/L NADPH were added to an assay cuvette containing 0.5 ml of 50 mmol/L potassium phosphate (pH 7.0). The cuvette was incubated at 37°C for 3 min. After addition of 100 μl of 2 mmol/L H_2O_2, the rate of NADPH consumption was monitored at 340 nm and 37°C for 5 min. This was designated as the total rate of NADPH consumption. The non-enzyme-dependent consumption of NADPH was also measured as above except that 100 μl of sample was replaced by 100 μl of sample buffer. The rate of enzyme-dependent NADPH consumption was obtained by subtracting the non-enzyme-dependent NADPH consumption rate from the total NADPH consumption rate. GPx activity was calculated using the extinction coefficient of 6.22 mmol/L^{-1} cm^{-1}, and expressed as nanomoles of NADPH consumed per minute per milligram of sample protein.

The CAT activity was determined using the method described by Aebi [24], in which the disappearance of H_2O_2 is followed spectrophotometrically at 240 nm. The reaction medium consisted of 50 mmol/L sodium phosphate buffer, pH 7.2, and 10 mmol/L H_2O_2. The results are reported as nanomoles per milligram of protein.

2.7. Oxygen radical absorbance capacity

The oxygen radical absorbance capacity $(ORAC)_{ROO}$ assay was based on the procedure described by Cao and Prior [25]. Free radicals were produced by 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH), and the oxidation of the fluorescent indicator protein β-PE was measured. Both reagents were prepared in 75 mmol/L phosphate buffer (pH 7.0), and 50 μmol/L Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble vitamin E analog, was used as the calibration standard. The liver homogenates were deproteinized using 0.25 mol/L perchloric acid (PCA) and centrifuged at 10,000×*g* for 10 min at 4°C. The resultant supernatants were then stored at −80°C prior to analysis. The reaction was performed in 96-well microtiter plates (Costar 96-well black opaque plate, Corning Costar, Cambridge, MA, USA) and consisted of 170 μl of β-PE (80 mg/L) and 10 μl of a diluted (1:1) sample incubated at 37°C for 15 min. The reaction was initiated by the addition of 20 μl of AAPH (240 mmol/L), and the fluorescence (emission 590 nm, excitation 530 nm) was recorded every 5 min until readings had declined to less than 5% of initial value. This method of $ORAC_{ROO}$ assay has been validated by using fluorescein as the fluorescent probe. The ORAC values were calculated and expressed as Trolox equivalents (μmol/L) per milligram of wet liver weight.

2.8. RNA extraction and RT-PCR

RT-PCR was performed to determine the level of antioxidant enzyme gene expression. Total RNA from rat heart tissues were isolated using the TRIzol RNA isolation kit (Life Technologies, Rockville, MD, USA) as described in

manufacturer's manual. Samples were quantitated by spectrophotometry and diluted to a concentration of 15 μg/L. cDNA was synthesized with random primers using the Reverse Transcription system (Promega, Madison, WI, USA) according to the manufacturer's instructions and 2 μg total RNA. cDNA was amplified in 50-μl reactions containing 2 μl of the cDNA reaction mix, 1×PCR buffer (20 mmol/L Tris HCl, pH 8.4, and 50 mmol/L KCl), 1.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP and 2.5 U of Taq DNA polymerase (GIBCO Life Technologies, Gaithersburg, MD, USA). Reactions were heated to 94°C for 1 min before adding 20 pmol of each primer. Cu/Zn SOD was amplified at 94°C for 45 s, 56°C for 30 s and 72°C for 45 s for a total of 23 cycles followed by a 10-min extension at 72°C using the following primers: 5′-gaaggccgtgtgcgtgctg-3′ and 5′-ggacacattggccacaccg-3′. GAPDH was amplified at 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s for a total of 28 cycles, followed by a 10-min extension at 72°C using the following primers: 5′-tcggacgcctggttaccag-3′ and 5′-ccagccttctccatggtgg-3′. GPx and CAT were amplified at 94°C for 30 s, 58°C for 30 s and 72°C for 1 min for a total of 30 cycles followed by a 10-min extension at 72°C using the following primers: GPx, 5′-tgcaaccagttcgggaggc-3′ and 5′-gagatagcacg- gcaggtcc-3′; CAT, 5′-cccgatgtcctgaccaccg-3′ and 5′-ctctccag- cgactgtggag-3′. Amplification products were resolved by electrophoresis on a 1.8% agarose gel containing 0.06 mg/L ethidium bromide. The gel was then photographed under ultraviolet transillumination. For quantification, the PCR bands on the photograph of the gel were scanned using a densitometer linked to a computer analysis system. Net band intensity (background subtracted intensity) was normalized to values for GAPDH and plotted as arbitrary units. Water samples or RNA samples containing no RT were amplified in parallel to ensure that no contaminating DNA was present during PCR.

2.9. Determination of phenolic compounds in rat plasma

CA was dissolved in physiologic saline buffer solution, and oral administrated at 0.1 g/kg BW to SHRs (n=12, 6 weeks old) by gastric intubation. Physiologic saline was orally administrated to the control group (n=12). Three rats per group were killed at 0.5, 1.0, 2.0 and 4.0 h post administration by using a heparinized needle and syringe under anesthesia with diethyl ether in the inferior vena cava. Plasma was immediately prepared by centrifugation at 1000g for 15 min at 4°C and stored at −80°C until use. The identification and quantification of the phenolic acids were analyzed by high-performance liquid chromatography (HPLC) (Hitachi, Tokyo, Japan) using the LiChrosphere RP-18 column (150×4 mm, 5 μm) and photodiode array detector (measured at 280 nm). Next, 25 μl of 0.1 mol/L sodium acetate buffer (pH 5.0) and 450 μl of 0.83 mol/L acetic acid in methanol were added to 25 μl of plasma. The mixture was vortexed for 30 s, sonicated for 30 s, and finally centrifuged at 8500×g for 5 min at 4°C. Elution was carried out at room temperature and utilized 2% (v/v) acetic acid in water as Solvent A and 0.5% acetic acid in water and acetonitrile (50:50, v/v) as Solvent B. The elution gradient program was as follows: 10% B to 55% B (50 min), 55% B to 100% B (10 min), and 100% B to 10% B (5 min) at a flow rate of 1 ml/min. The injection volume for standard sample extracts was 20 μl. CA was quantified using the external standard method. Quantification was based on peak area. Calibration curves of the standards were made by diluting stock standards in methanol to yield 0–300 mg/L (CA). Linear regression was fitted to the data to obtain regression coefficients >0.99 for CA standard curves.

2.10. Statistical analysis

All data are expressed as means±SEM and analyzed differences between groups using analysis of variance. If significant difference at a level of P<05 was observed between groups, Dunnett's test was used to compare the means of two specific groups.

3. Results

3.1. Effect of WEHT and CA on the relative tissue weight of spontaneously hypertensive rats

Table 1 shows the relative liver and kidney tissue weights of rats in each group. The initial BWs were 189±3, 191±4, 190±5 and 195±4 g, and final BWs were 309±2, 310±1, 296±4 and 302±5 for WKY rats, SHRs control, SHRs plus WEHT and SHRs plus CA groups, respectively. The BWs of rats in the four groups did not differ significantly at the beginning or at the end of the study. Moreover, there are no differences among the relative liver and kidney weights of SHRs with or without gavage of WEHT (1.0 g/kg BW) or CA (0.1 g/kg of BW) for 6 weeks when compared with WKY rats.

3.2. Effect of WEHT or CA supplementation on systolic blood pressure in spontaneously hypertensive rats

Before treatment, the mean values of systolic blood pressure at 10 weeks of age were significantly higher in SHRs compared to WKY rats of the same age. Long-term WEHT or CA administration induced a progressive reduction in systolic blood pressure in SHRs. This effect reached

Table 1
Relative liver and kidney weights of WKY rats and SHRs with or without gavage with WEHT or CA for 6 weeks

Groups	BW (g)	Relative organ weight[a] (g/100 g BW)	
		Liver	Kidney
WKY control	319±2	2.75±0.10	0.82±0.02
SHRs control	310±1	2.98±0.08	0.79±0.09
SHRs+WEHT[b] (1.0 g/kg)	296±4	2.81±0.09	0.76±0.03
SHRs+CA (0.1 g/kg)	302±5	2.80±0.06	0.76±0.02

[a] The data represented the means±S.E.M. from six rats.
[b] WEHT was dissolved in 0.9% NaCl solution.

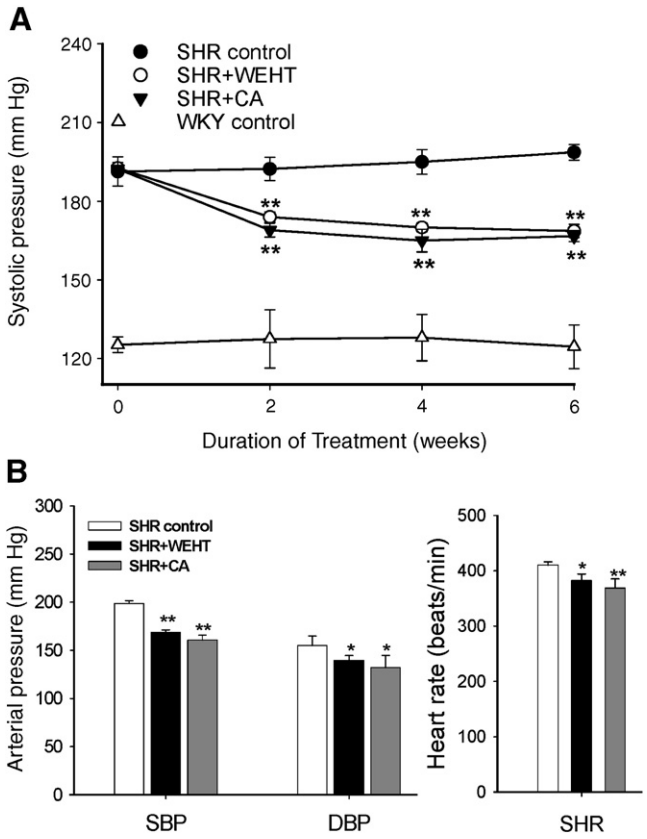

Fig. 1. Effects of WEHT and CA on arterial blood pressure and heart rate. (A) Systolic arterial pressure over time as measured by tail-cuff plethysmography in the SHRs control (n=6), SHRs plus WEHT (WEHT, n=6), SHRs plus CA (CA, n=6) and WKY vehicle (n=6) groups. (B) SBP, DBP, and heart rate in the SHRs control (white bars, n=6), SHRs plus WEHT (black bar, n=6) and SHRs plus CA (gray bar, n=6) groups. Values are expressed as means±S.E.M.

statistical significance after the first week of treatment but systolic blood pressure was still significantly higher than WKY rats (Fig. 1A). At the end of 6 weeks of treatment, direct measurement of blood pressure in conscious rats showed that WEHT or CA induced a significant reduction in systolic (17.7% and 23.4%, respectively) and diastolic (11% and 15%, respectively) arterial blood pressure in SHRs of the same age (Fig. 1B). Heart rate was also significantly reduced by WEHT or CA in SHRs of the same age (7.3% and 11.2%, respectively) (Fig. 1B).

3.3. Effect of WEHT or CA on hepatic and plasmatic MDA levels

Lipid peroxidation has been recognized as a potential mechanism for cell injury. To evaluate the effect of treatment of WEHT or CA on liver and plasma lipid peroxidation in SHRs, we monitored the levels of MDA, an indicator of oxidative damage and one of the principle products of lipid peroxidation. SHRs treated with the vehicle showed higher concentration of MDA in liver homogenates than WKY rats (P<.05, Fig. 2A). WEHT or CA at a dosage of 1.0 g/kg or 0.1 g/kg of BW significantly inhibited lipid peroxidation in liver homogenate of SHRs by 55.6 % and 27%, respectively. Accordingly, MDA levels of plasma were also elevated in SHRs (P<.05 vs. WKY rats, Fig. 2B). Chronic WEHT or CA treatment also significantly reduced plasma MDA levels in SHRs by 54% and 30%, respectively.

3.4. Effect of WEHT or CA on hepatic GSH content and total antioxidant status

As the oxidative stress of tissue generally involves the GSH system, we therefore measured the level of GSH and the total antioxidant status of each group. Table 2 shows that GSH content was significantly lower (24%) in SHRs than in WKY rats. WEHT or CA treatment significantly increased hepatic GSH content in hypertensive rats. Similarly, a 39% decrease was observed in the total antioxidant status of liver in SHRs when compared with WKY rats. The treatment with WEHT or CA significantly elevated the $ORAC_{ROO}$ value in the liver 55 and 61%, respectively, as compared to the control SHRs.

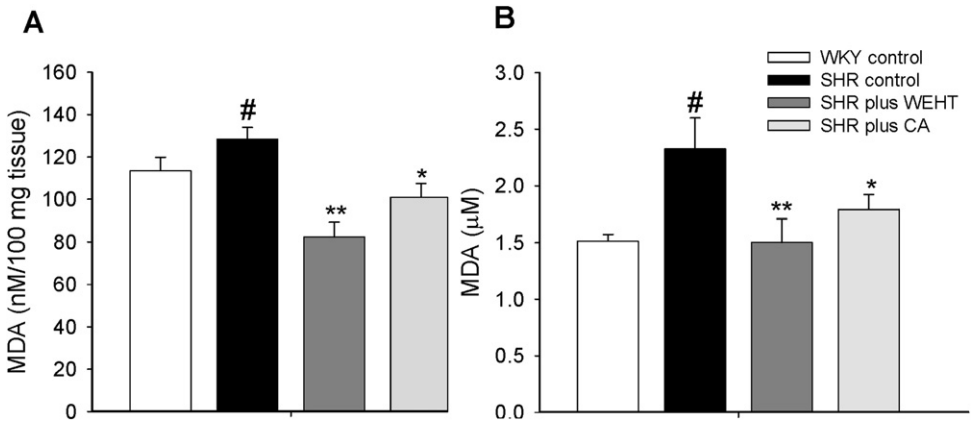

Fig. 2. Total liver (A) and plasma (B) MDA content in the WKY control (n=6), SHRs control (n=6), SHRs plus WEHT (WEHT, n=6) and SHRs plus CA (CA, n=6) groups. Data are expressed as mean±S.E.M. of at least five animals per group: $^{\#}P$<.05 vs. WKY control group; *P<.05 vs. SHRs control group; **P<.01 vs. SHRs control group.

Table 2
Effect of WEHT or CA on reduced GSH and ORAC in livers of SHRs

	WKY control	SHRs control	SHRs plus WEHT	SHRs plus CA
GSH (μmol/mg protein)	10.4±0.8	8.4±1.3#	12.6±0.3*	13.2±0.8**
ORAC (μmol trolox equivalents/mg protein)	5.1±0.2	3.7±0.1#	8.4±0.3**	9.5±0.8**

Values are expressed as mean±S.E.M. of at least five animals per group.
* $P<05$ vs. SHRs control group.
** $P<01$ vs. SHRs control group.
# $P<05$ vs. WKY control group.

3.5. Effects of WEHT or CA on tissue antioxidant enzyme activities

Total superoxide dimutase activities in the liver and kidney were significantly lower (61.6 and 46.5%, respectively) in SHRs than in WKY rats (Table 3). WEHT or CA treatment significantly increased the activity of total SOD in the liver and kidneys of SHRs. A significant decrease in GPx activities was also observed in the liver and kidneys of SHRs when compared with WKY rats (62% and 26%, respectively). WEHT or CA significantly modified this enzyme activity in SHRs. Livers of SHRs showed lower CAT activities than that from WKY rats (31%). No significant changes in CAT activities were observed in WEHT or CA treatment SHRs. However, CAT activities in the kidney were similar between SHRs and WKY rats, and these activities were modified by chronic WEHT or CA administration.

3.6. mRNA antioxidant gene expression in SHR livers following WEHT or CA treatment

To further examine whether antioxidant gene mRNA expression was modulated by WEHT or CA, an RT-PCR analysis was performed. As shown in Fig. 3, expression of hepatic CuZnSOD mRNA in SHRs was significantly lower than WKY rats after normalizing to GAPDH mRNA levels, and the treatment with WEHT or CA could modify this expression in SHR groups. On the other hand, a decrease in the expression of GPx and CAT mRNA was also observed in SHRs when compared with WKY rats. This effect was enhanced significantly by treatment with WEHT or CA.

3.7. Plasma phenolic compounds after oral administration of CA in SHR

Because the antihypertensive effect of CA was confirmed, the blood metabolites after a single oral administration of CA (0.1 g/kg of BW) were analyzed by HPLC. CA was detected in the plasma as intact forms (data not shown). Fig. 4 shows the time course of changes in the concentration of CA in SHR plasma after the administration of CA. No CA was detected in rat plasma before administration. Following oral administration of CA, there was a rapid increase in the plasma concentration to 0.55±0.09 μmol/L at 0.5 h post administration. The plasma concentration reached a maximum of 0.91±0.08 μmol/L at 1.0 h post administration in the case of CA, and then it decreased.

4. Discussion

Medicinal herbs have been used in traditional medicine in Asia for centuries [26]. Most herbs are relatively inexpensive and easily available; also, they have few adverse effects. Accordingly, there is growing interest in the use of herbs and their bioactive compounds. Phenolic compounds, which are commonly found in plants, have been considered to play an important role as dietary antioxidants in preventing oxidative damage in living systems [27,28]. In our previous study, it was shown that phenolic acids may be important antioxidant components in Hsian-tsao; among these, CA has the highest antioxidant activity and the content and is considered the most important [13]. However, no pharmacological or clinical study had been carried out to test the antihypertensive properties of this plant. Thus, the antihypertensive effect of an aqueous extract of Hsian-tsao and its active compound (CA) in spontaneously hypertensive rats was investigated in this study. According to Sato et al. [29] and our preliminary tests, long-term treatment was administered over a period of 6 weeks. Our experimental results showed that a single oral

Table 3
Effects of WEHT and CA administration on tissue antioxidant enzyme activities in SHRs

Treatments[a]	Liver			Kidney		
	Total SOD (Unit/mg protein)	GSH Peroxidase (nmol/min/mg protein)	Catalase (μmol/min/mg protein)	Total SOD (Unit/mg protein)	GSH Peroxidase (nmol/min/ mg protein)	Catalase (μmol/min/ mg protein)
WKY Control	10.7±1.1	138±9	11.2±1.1	11.4±0.4	112±1	12.9±0.3
SHRs Control	4.1±0.4#	52±2.1#	7.7±0.9#	6.1±0.6#	82±9#	12.1±4.5
SHRs plus WEHT (1.0 g/kg)	8.1±1.0*	88±2.6*	8.7±1.5	8.6±0.7*	96±2*	17.9±0.7*
SHRs plus CA (0.1 g/kg)	13.3±2.3**	95±1.5**	8.2±0.4	10.9±2.8**	107±2*	15.7±1.9*

[a] Rats were orally administered with 1.0g/kg BW of WEHT and 0.1g/kg BW of CA for 5 weeks. SHRs and WKY control rats received vehicle solution. Values represent means±S.E.M. of at least five animals per group.
* $P<05$.
** $P<01$ compared with SHRs control group.
# $P<05$ compared with WKY control group.

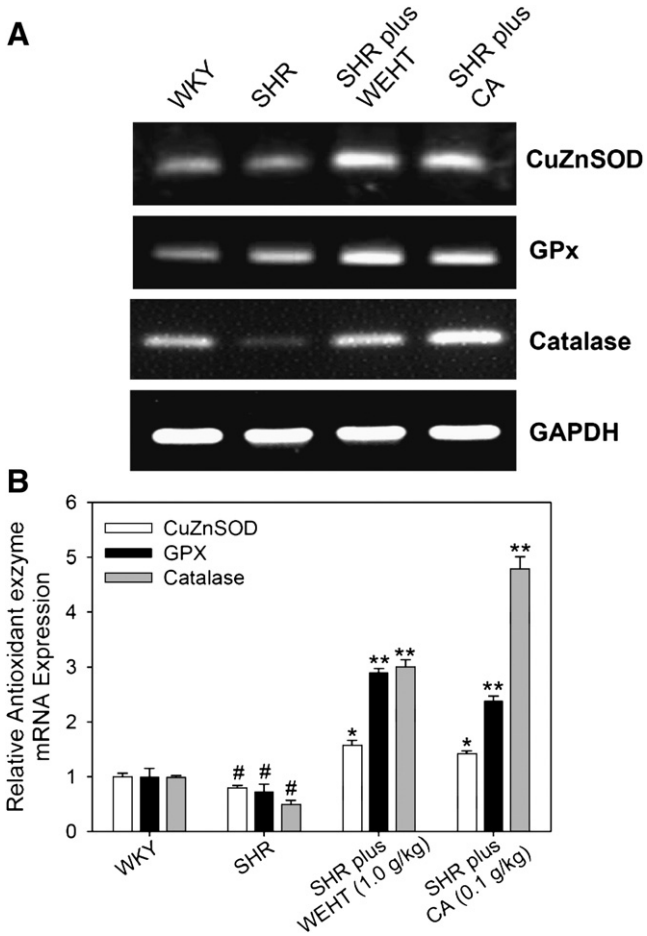

Fig. 3. mRNA expression of CuZnSOD, GPx and catalase in liver from SHRs treated and untreated with WEHT or CA. (A) Agarose gel (1.8%) represents electrophoresis of the PCR products. (B) The ratio of CuZnSOD, GPx and catalase to GAPDH mRNA in WKY, SHR, SHR plus WEHT and SHRs plus CA groups. Values represent means±S.E.M. of at least five animals per group: $^{\#}P<.05$ compared with WKY control group; $^{*}P<.05$ compared with SHRs control group; $^{**}P<.01$ compared with SHRs control group.

daily dose of WEHT (1.0 g/kg) or CA (0.1 g/kg of BW) significantly reduced SBP, diastolic blood pressure (DBP), and heart rate in SHRs, which served as a genetic rat model of hypertension (Fig. 1).

The SHR model is characterized by increased oxidative stress [30]. One important consequence of excessive free radical production is lipid peroxidation. Thiobarbituric acid reactive substances (TBARS) levels, expressed in MDA equivalents, reflect the severity of the lipid peroxidation. To evaluate the protective role of WEHT or CA against hypertension-associated oxidative stress, TBARS levels in plasma and liver tissue were examined in all experimental groups of rats. In the present study, both hepatic and plasma levels of MDA were enhanced in SHRs as compared to normotensive WKY rats. In SHRs, treatment with WEHT or CA for 6 weeks significantly reduced both plasma and hepatic MDA levels. However, none of these effects were observed in WKY rats. To our knowledge, this is the first report showing that chronic treatment with WEHT and its active compound, CA, exhibits antioxidant properties in an animal model of hypertension.

Reactive oxygen species (ROS) can oxidize bimolecular leading to alterations of cell function and, thus, cells have evolved several antioxidant strategies aimed at the detoxification of ROS. To defend against oxidative stress caused by oxygen toxicity, many endogenous nonprotein small antioxidant molecules and antioxidant enzymes are essential for alleviating oxidative stress by acting as a chain breaker of the oxygen radical cascade and lipid peroxidation chain reaction [31]. The primary antioxidant enzymes that minimize the oxygen radical cascade and remove cytotoxic peroxides are those such as SOD, GPx and CAT. These antioxidant enzymes prevent the formation of oxygen radicals and alleviate lipid peroxidation [32]. In the present study, our results showed that the activities of SOD, GPx and CAT were reduced in the liver and kidney of SHRs when compared to normotensive WKY rats. When WEHT or CA was chronically administered, the reduction in antioxidant enzyme activities found in SHRs was eliminated, obtaining values closer to those found in untreated, normotensive rats. This demonstrates close agreement with the work by Duarte et al. [33], which showed that GSH peroxidase and GSH reductase activities were induced by chronic quercetin treatment in SHR liver. Both liver and heart GSH peroxidase activity were induced after treatment with propionyl-L-carnitine [34]. Whether the change in the antioxidant enzymes activities was correlated with their respective mRNA expression in the WEHT or CA-treated rats was also considered. The mRNA expression of hepatic CuZnSOD, GPx and CAT in liver tissues of WEHT or CA-treated rats was higher than the control group (Fig. 3). Thus, the mRNA expression of hepatic CuZnSOD, GPx and CAT exhibited a similar trend to the level of the respective enzymes activity (Table 3). Since the mRNA expression of CuZnSOD, GPx and CAT was altered by the WEHT or CA

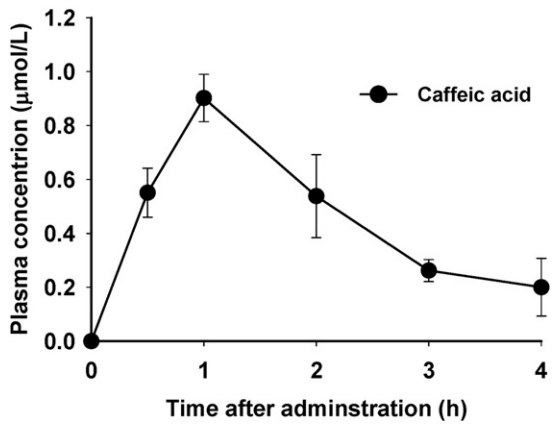

Fig. 4. Changes in concentration of CA in SHRs plasma after oral administration of a single dose of CA (●) over time. Values represent means ±S.E.M. of at least five animals per group.

supplements, these enzymes would seem to be regulated on a transcriptional level.

Antioxidant activity has been demonstrated for Hsian-tsao [13] in various in vitro systems including scavenging of peroxyl radical (ROO) and $ABTS^{+}$ radical. The $ORAC_{ROO}$ assay is one of the methods used to evaluate the antioxidant activity of biology substrates, ranging from pure compounds such as melatonin and flavonoids to complex matrices such as vegetables and animal tissues [35]. However, there is little information on the in vivo total antioxidant activity of WEHT or CA. Our results clearly demonstrated, for the first time, that the decrease in $ORAC_{ROO}$ values in the liver of SHRs compared with normal WKY rats and orally administered WEHT or CA significantly ($P<.05$) increases the antioxidant defense in rat liver (Table 2). In general, phenolic acids that induced antioxidant enzyme activities were found to have higher antioxidant capacities. GSH plays an important role in the tissue antioxidation and drug metabolism. The higher intracellular GSH content promotes reduced damage and better survival under oxidative stress [36]. Furthermore, induction of the GSH antioxidant system by chemopreventive agents has been reported in several studies [37]. The results of the present study demonstrated that the level of GSH was markedly higher on the liver of the WEHT, as opposed to CA-treated SHRs. Gómez-Amores et al. [38] showed that the reduced GSH concentration in liver and heart supernatants were only increased significantly in L-carnitine-treated SHRs compared to WKY rats, whereas the concentration of oxidized GSH was significantly decreased. The significant increase in the GSH level in the liver of the WEHT or CA-treated SHRs may be indicative of a reduced oxidative stress or an increased antioxidant capacity in the cell, thereby lowering the susceptibility to oxidative damage.

The marked increase obtained with the CA in the present study suggested that the phenolic components in Hsian-tsao were more effective to increase the antioxidant status in the liver. In vivo studies showed that the antioxidant potential in humans responds to the oral ingestion of polyphenols [39]. Thus, some substances that exist in the gut are absorbed and may be responsible for the in vivo antioxidant properties of red wine. The physiological importance of ferulic acid and notably its antioxidant property depends upon its availability for absorption and subsequent interaction with target tissues [40]. Piskula and Terao [41] reported that when (−)-epicatechin was ingested by rats, it was absorbed and present in the blood circulation as various conjugated metabolites. Moreover, (−)-epicatechin metabolites possessed an effective antioxidant activity in blood plasma [42]. In order to establish whether CA makes a contribution in the reduction of SBP and antioxidant activity in SHR, an additional study was conducted by HPLC. CA was orally administered to SHRs at the same dosage (0.1 g/kg of BW), and it was absorbed and distributed to the blood as the intact form. Comparison of the time course of changes in plasma concentrations and components showed that CA was directly absorbed and distributed to the blood, and the plasma concentrations increased in the period up to 1.0 h post administration and then gradually decreased (Fig. 4). This shows a close agreement with prior work by Azuma et al. [43], showing that the absorbed fraction of chlorogenic acid and its metabolites might induce biological effects in the blood circulation. CA inhibits oxidation of low-density lipoprotein in vitro [44] and might therefore protect against cardiovascular disease. There are no in vivo data available that show that CA is present in the blood circulation after ingestion. Our results showed that the antioxidant ability of SHRs plasma was remarkably increased after oral administration of CA and their metabolites appeared in plasma. These results indicated that CA was absorbed and their plasma metabolites may act as antioxidants in blood circulation. Yen et al. [12] reported that WEHT inhibited the peroxidation of linoleic acid and had antioxidant activity equal to that of Trolox and butylated hydroxyanisole (BHA). In addition, WEHT also had a scavenging activity on free radicals and ROS, such as hydroxyl radicals or peroxyl/hydroperoxy radicals. Thus, the antioxidant activity and scavenging effect of WEHT might be mainly related to its antihypertensive effect on blood pressure in spontaneously hypertensive rats.

CA is the major representative of hydroxycinnamic acid, with high concentrations in certain foods and beverages. For instance, red wine may contain 25 mg/L of CA [45]. CA has been reported to have antiinflammatory, antimutagenic and anticarcinogenic activities [46]. Furthermore, 5-caffeoylquinic acid at an oral dose of 200 mg/kg exhibited a significant hypotensive effect on blood pressure in a dose-dependent manner in SHRs [47]. Therefore, WEHT contains many important antioxidant components; thus, the greatest content is most important [13]. The CA content in extracts prepared form Hsian-tsao (*M. procumbens* Hemsl.) was 17.2 mg/g of lyophilized powder. Phenolic compounds have a variety of biological effects in numerous mammalian cell systems in vitro as well as in vivo. The CA in WEHT might play an important role in decreasing of blood pressure and oxidative stress in SHR rats. The effect of antioxidants in decreasing oxidative damage is believed to contribute to the low cardiovascular disease incidence. Therefore, we suggest that daily consumption of WEHT might be effective in lowering possible oxidative damage in hypertensive rats, indicating it has potentially hypotensive effects.

In conclusion, our study suggests that WEHT or its active component CA have potential blood pressure-lowering effects in hypertensive rats. It might be due to the possibly different mechanisms associated with different antioxidants, especially CA, existing in the extracts. The exact mechanisms and constituents need further investigation.

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