Moderate Effect of Flavonoids on Vascular and Renal Function In Spontaneously Hypertensive Rats

María D. Paredes[1], Paola Romecín[1], Noemí M. Atucha[1], Francisco O'Valle[2], Julián Castillo[3], M. Clara Ortiz[1], Joaquín García-Estañ[1*]

1, Departamento de Fisiología, Facultad de Medicina & Instituto Murciano de Investigaciones Biosanitarias, Universidad de Murcia, Murcia, Spain

2, Departamento de Anatomía Patológica, Facultad de Medicina, IBIMER (CIBM) & Ibs.GRANADA, Universidad de Granada, Granada, Spain

3, Instituto Universitario de Envejecimiento & Research and Development Department, Nutrafur SA-FRUTAROM Group, Alcantarilla (Murcia), Spain

* Corresponding author: jgestan@um.es (JG-E)

ABSTRACT

We have evaluated the antihypertensive effect of several flavonoid extracts in the spontaneous hypertensive rat model (SHR). Treatments were carried out for 6 and 12 weeks in two groups of SHR rats which received Apigenin, Lemon Extract, Grapefruit + Bitter Orange (GBO) extracts and Cocoa extract. Captopril was used as a positive control in the SHR group treated for 6 weeks (SHR6) and Diosmin was used as the industry reference in the SHR group treated for 12 weeks (SHR12). Captopril and GBO extracts significantly reduced the elevated blood pressure of the SHR6 animals, but none of the

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extracts was effective in the SHR12 group. Apigenin, LE, GBO and captopril also ameliorated nitric oxide-dependent and independent aortic vascular relaxation and elevated plasma and urinary excretion of nitrites, only in the SHR6 group. Kidney and urinary TBARS were also significantly reduced by GBO in the SHR6 rats. Apigenin also improved vascular relaxation in the SHR12 group and all the flavonoids studied reduced urinary TBARS excretion and proteinuria. Vascular abnormalities such as lumen/wall ratio in coronary arteries and thoracic aorta were moderately improved by these treatments in the SHR6 group. In conclusion, the flavonoids included in this study, especially apigenin, LE and GBO improved vascular vasodilatory function of young adult SHRs but only the GBO-treated rats benefited from a reduction in BP. These extracts may be used as functional food ingredients with a moderate therapeutic benefit, especially in the early phases of arterial hypertension.

Keywords: flavonoids, nitric oxide, heart, kidney, sodium balance, phenylephrine, acetylcholine

Introduction

Animal studies using flavonoid-rich foods are a valid alternative to advance on the comprehension of the mechanisms underlying their blood pressure lowering effects during arterial hypertension [1]. The intake of polyphenols has been inversely related with the reduced risk of this disease [2]. In fact, epidemiological studies associate an increased consumption of foods and beverages rich in flavonoids with a reduced risk of cardiovascular death [3-4]. Moreover, the use of products with a natural origin that may

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cause scarce side-effects is an attractive possibility to be considered when treating several pathologies [5]. Several studies have correlated the consumption of flavonoid-rich food, as well as isolated compounds, with the beneficial effects on several cardiovascular parameters such as flow-mediated dilation, blood pressure, and cardiovascular risk biomarkers [6-7]. Additionally, many flavonoids induce the release of endothelium derived vasodilator factors such as nitric oxide (NO) or endothelium-derived hyperpolarizing factor (EDHF) and decrease the release of pro-inflammatory substances with a consequent improvement of endothelial function [8-9].

In a previous study in the L-NAME model of arterial hypertension (10), we showed that some flavonoids, especially apigenin, were effective to reduce the elevated blood pressure associated with the chronic deficiency of nitric oxide (NO). An important feature of that study (10) is that we used a low dose, far from those usually applied in human therapy or experimental animals (11-12), a dose that responded to an objective criterion of a possible later commercial use in humans. As in other studies (13-14), the blood pressure-lowering effect of these flavonoids were related to a combination of vasodilator and antioxidant actions (10).

In the present study we have carried out a similar study in another model of arterial hypertension, the spontaneously hypertensive rat (SHR), thought to be the most comparable with the human form of hypertension (15-16). Thus, SHR rats have increased activity of fluid retention mechanisms, sodium reabsorption and increased vascular resistance. This increase in vascular resistance being produced first by neurogenic mechanisms and later structural vascular changes (17). Therefore, the aim of the present study was

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to evaluate the vascular and renal effects of several flavonoid extracts in SHR rats. We have also examined some of the possible mechanisms involved in their beneficial effects such as an improvement in NO bioavalaibility and endothelial and vascular function, the reduction in oxidative stress markers and the effects on cardiovascular morphological changes.

Material and methods

Animals

All the experiments were performed in male SHR and WKY rats (Harlan Lab, Barcelona, Spain) housed in a temperature controlled environment, with 12:12-h light-dark cycle in the Animal Care Facility of the University of Murcia (REGAES300305440012). The animals were kept and treated according to the guidelines established by the European Union for the protection of animals used in experiments (86/609/EEC). All procedures were approved by the Animal Care and Use Committee of the University of Murcia (C1310050303).

Experimental groups

There were two treatment groups of male SHR, one treated for 6 weeks and another group treated for 12 weeks, along with their respective controls (WKY) rats.

1. SHR6. The first treatment group (6 weeks) was composed of 56 SHR (8-9 week old, initial weight, 185-271 g) and 6 WKY rats (initial weight, 213-220 g). This group was composed of the following experimental groups:

1) Control (n = 6), WKY rats without any treatment;

2) SHR (n = 7), SHR rats without any treatment;

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3) Apigenin (A, n = 6), SHR rats treated with A (1.44 mg/Kg/day);

4) Lemon Extract (LE, n = 7), SHR rats treated with LBC (2.84 mg/Kg/day);

5) Grapefruit + Bitter Orange Extracts (GBO, n = 7), SHR rats treated with GBO extract (9.28 mg/Kg/day);

6) Cocoa Extract (COE, n = 6), SHR rats treated with COE (2.52 mg/Kg/day);

7) Captopril or CPT (n = 6), SHR rats treated with CPT (100 mg/Kg/day). This angiotensin converting enzyme inhibitor was used as a positive control, since it has been shown to be very effective for lowering blood pressure of the SHR model (18-19).

2. SHR12. The second treatment group (12 weeks) was composed of 38 SHR (8-9 week old male, initial weight, 210-267 g) and normotensive Wistar-Kyoto (WKY) rats (initial weight, 212-230 g). This was composed of 7 different experimental groups:

1) Control (n = 6), WKY rats without any treatment;

2) SHR (n = 9), SHR rats without any treatment;

3) Apigenin (A, n = 6), SHR rats treated with A (1.44 mg/Kg/day);

4) Lemon Extract (LE, n = 6), SHR rats treated with LBC (2.84 mg/Kg/day);

5) Grapefruit + Bitter Orange Extracts (GBO, n = 6), SHR rats treated with GBO extract (9.28 mg/Kg/day);

6) Cocoa Extract (COE, n = 6), SHR rats treated with COE (2.52 mg/Kg/day);

7) Diosmin (D, n = 6), rats treated with D (7,16 mg/Kg/day).

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The doses and composition of the different extracts have been previously published (10). Briefly, the doses were selected as those being economically competitive in case of a future commercial use. All treatments were administered during the required time, 6 or 12 weeks, in the drinking water, except for Diosmin that was given mixed with the powdered food, in powder feeders (Tecniplast, USA). All animals had free access to a standard rat diet with a 0.5% of sodium content (104 mEq/Kg) and tap water, with or without treatments. The concentrations of the drugs were adjusted daily according to the body weight and water and food intake. All products, except L-NAME and captopril (Sigma), were kindly provided by Nutrafur SA-FRUTAROM Group.

Experimental procedures

Rats were maintained in their cages and they were progressively accustomed to individual metabolic cages (Tecniplast, USA) three days a week. Then, in the treatment week 6th or 12th, after two days of adaptation, we measured food and water intake and urinary volume (diuresis) in 24 hours. The urine samples were collected and centrifuged (1000 g, 10 min) to remove solid matter and then kept at -80º C for posterior analysis. The urinary sodium concentration was determined using a sodium electrode (Thermo Scientific Orion, USA). Sodium balance (mEq/day/100 g) was calculated as the difference between sodium intake and excretion, and factored by body weight. Sodium intake (mEq/day) was obtained by multiplying the consumption of food per day (g/day) by sodium content of the diet (0.104 mEq/g). Urinary sodium excretion (mEq/day) was determined as the product of sodium concentration and 24-h urinary volume (ml/day).

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Measurement of blood pressure and samples extraction

After the metabolic study was completed, the animals were anesthetized with sodium pentobarbital (5 mg/Kg, ip) and placed on a heated table to maintain body temperature at 37º C. A polyethylene catheter (PE-50) was placed in the right femoral artery to measure mean arterial pressure (MAP; Hewlett Packard 1280 pressure transducer and amplifier 8805D, Andover, MA) and to collect blood samples (20-21). Then, blood was collected into heparinized tubes and plasma was obtained by centrifugation (1,000 g, 10 min, 4º C). Thereafter, the animal was euthanized by opening the thorax. We extracted the descending thoracic aorta and placed it in a Petri dish containing oxygenated and pre-warmed Krebs solution for the vascular reactivity study. Finally, kidneys, heart and abdominal aorta were also removed. All samples were frozen (- 80º C) and a small portion was also fixed with a 10%-formalin solution for pathology studies.

Vascular reactivity study

The thoracic aorta was cleaned of adhering fat and connective tissue; care was taken not to disrupt vascular endothelium, as previously described [21]. Then, the aorta was cut into four rings (3-4 mm) and mounted in 10 ml organ baths (organ bath system LE 01004, Panlab, Barcelona, Spain) containing a physiological Krebs solution with the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; edetate calcium disodium, 0.026; and glucose, 5.6. The Krebs solution was maintained at 37º C and continuously bubbled with a mixture of 95% O2 and 5% CO2. The rings are connected to isometric force transducers (TRI202P,

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Panlab) to detect tension changes that were acquired and analyzed with a data acquisition system (AD Instrument, Oxford, UK) consisting of a bridge amplifier (FE228), a data acquisition hardware (PowerLab 8/30) and a software (LabChart 6.0). Aortic rings were equilibrated for at least 45 min at a resting tension of 2 g before any specific experimental protocol was initiated. During this period, the bathing solution was replaced every 15 min and, if needed, the basal tone readjusted to 2 g.

After the stabilization period, the aortic rings were constricted using a cumulative dose–response curve to phenylephrine (PHE, 10^{-9}-10^{-4} M), administered in 0.1 ml bolus. Then, the rings were frequently washed until the resting tension was reached again and a second stabilization period of 30 min was allowed. To evaluate the vasodilator responses to acetylcholine (ACH), the aortic rings were pre-contracted with a maximal dose of PHE (10^{-4} mol/l). Once a stable plateau was reached, a cumulative dose–response curve to the ACH (10^{-9}–10^{-4} mol/l) was performed to assess the endothelium-dependent vasodilatation. Thereafter, the rings were frequently washed once again and a third stabilization period of 30 min was permitted and followed by an incubation period of 30 min with the NOS-inhibitor L-NAME (10^{-4} M) to inhibit NO synthesis. Next, a cumulative concentration-response curve to ACH was again performed, to evaluate the role of NO in the endothelium-dependent vasodilatation. Finally, we added a single dose of SNP (10^{-4} M) to test the independent vasodilator responses and the functionality of the smooth muscle. The responses to PHE are expressed in grams and the relaxation to ACH and SNP as percentage of the maximal PHE effect. Stock solutions of these drugs were prepared in distilled water and maintained frozen at -20º C. Working

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solutions were prepared daily in Krebs solution. Drug concentrations are expressed as final bath concentrations. All reagents and vasoactive compounds were purchased from Sigma-Aldrich and Panreac (Spain).

Analytical procedures

TBARS (thiobarbituric acid reactive substances) in plasma and kidney tissue were determined as a measure of lipid peroxidation by using a colorimetric method, as described previously [22]. Briefly, 0.5 ml of potassium phosphate buffer (0.1 M, pH 7.4) was added to 100 µl of plasma sample mixed or 50 µl of kidney tissue lysate. After mixing, 1 ml of reagent solution [1 mmol/l deferoxamine mesylate, 7.5% (w/v) trichloroacetic acid, 0.25 mol/lHCl and 0.37%thiobarbituric acid] was added and the mixture was vortex-mixed, covered with aluminium foil to avoid evaporation and heated at 90º C for 15 minutes in a dry block heater (Heatblock II, VWR, Thorofare, NJ USA). After the mixture had returned to room temperature, TBARS from standards (prepared from 1,1,3,3-tetraethoxypropane) and samples were extracted into 1 ml of butanol. After a vigorous vortex-mixing and a brief centrifugation (1000 g for 5 min), the absorbance of the butanol layer was read at 532 nm in a spectrophotometer (Eppendorf Biophotometer Plus, Hamburgo, Germany), and the value was expressed as nmol/mL of plasma or nmol/mg of kidney protein.

The *protein concentration* was measured in the urine and lysates using the bicinchoninic acid based-method (Sigma).

The *plasma and urinary excretion of nitrite* was determined by using the Griess reaction. Briefly, sample volumes of 100 µL were mixed with 50 µL of

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1% Sulfanilamide in 5% Potassium Phosphate. Then 50 µL of 0.1% N-(1-Naphthyl) Ethyl-Enediamine dihydrochloride was added and incubated for 15 minutes. The nitrite concentration was quantified in a spectrophotometer at 540 nm against the standards and subtracting a blank from each individual sample. The final concentration was expressed in µg/mL for plasma or µg/day for urine samples.

Histological Analysis

Aortic, cardiac and renal tissue samples were fixed in 10% buffered formaldehyde, and then processed, embedded in paraffin and sectioned (4 µm) as previously reported [10, 20]. Transversal kidney, ventricular heart and thoracic and abdominal aorta sections were stained with hematoxylin-eosin and periodic acid-Schiff stain. The morphological study was done by a pathologist in blinded randomized sections of the tissues, with light microscopy and using the most appropriate stain for each lesion. The histo-morphometric measurements were performed with the software ImageJ 1.47 (NIH, http://rsb.info.nih.gov/ij/).

In the aorta, wall thickness was measured in three different, randomly selected regions, and also three times in each region. In the heart, we analyzed the inter-ventricular septum thickness, assessed in the middle central region of the cardiac cavities as well as the relation between luminal diameter and wall thickness in main and intramural coronary arteries, obtained from 5 measurements of each artery.

In the kidney, we evaluated the main alterations observed in transversal sections that included cortex and medulla. These were: 1) the absence or

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presence of hyaline arteriopathy in all the arteries seen in the whole section; 2) the relation between luminal diameter and wall thickness in the main renal artery or principal branches (if the first is missing); and 3) the absence (0) or presence (1) of tubular cast/cylinders in the cortical and medullary region from the entire kidney section.

Statistical Methods

Data are presented as the mean ± standard error. Differences between groups were compared mainly by one-way analysis of variance (ANOVA). In the vascular reactivity experiments, the values of EC_{50} were calculated from the individual dose-response curves and expressed as the negative logarithm (pEC_{50}). Differences were considered statistically significant at a *p* level lower than 0.05.

3. Results

Blood pressure and urinary variables

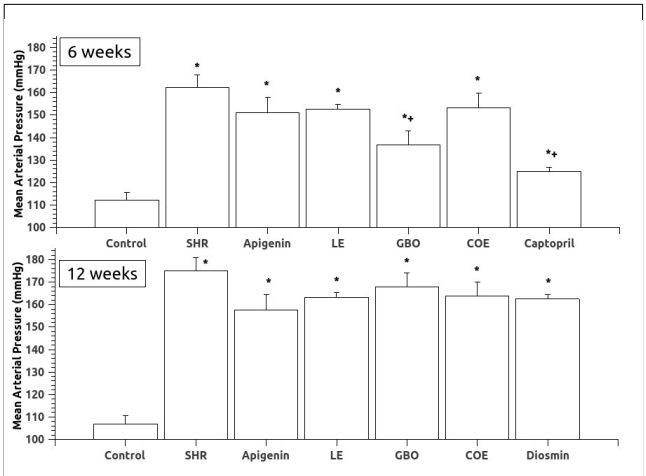

Figure 1. Mean arterial pressure in the experimental groups.

Mean arterial pressure (MAP) data are shown in figure 1 and table 1. In the SHR6 group, most of the treated groups showed slightly lower MAP values than the group with untreated hypertension (SHR), but a significant difference was observed only in the case of the

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treatment with GBO and CPT. In the SHR12 group, no statistically significant differences were observed in the treatment groups as compared to the SHR-untreated rats. Heart rate was significantly decreased by captopril in the SHR6 group and GBO, COE and D-treated groups showed lower heart rate values in the SHR 12 group.

Table 1. Final blood pressure and heart rate in the experimental groups.

SHR6	MAP (mmHg)	Heart rate (bpm)		SHR12	MAP (mmHg)	Heart rate (bpm)
WKY	112,0 ± 3,7	363,6 ± 7,5		WKY	97,0 ± 7,1	313,6 ± 17,1
SHR	162,3 ± 5,7*	384,9 ± 12,8		SHR	175,1 ± 5,4 *	395,3 ± 10,1*
A	151,0 ± 6,9*	379,2 ± 16,7		A	157,5 ± 9,2 *	399,8 ± 8,3*
LE	152,7 ± 2,1*	358,9 ± 10,8		LE	163,3 ± 5,9 *	369,2 ± 11,8*
GBO	136,7 ± 6,3*†	392,4 ± 12,9		GBO	167,8 ± 11,3 *	367,0 ± 5,6 *†
COE	153,3 ± 6,4*	378,6 ± 12,9		COE	163,7 ± 9,4 *	363,8 ± 12,9 †
CPT	125,0 ± 1,8 *†	314,3 ± 9,1*†		D	162,5 ± 5,8 *	356,4 ± 12,6 †

Body weight and hematocrit of all the experimental groups are listed in table 2. In the SHR6 group, there were no significant difference in body weight among the experimental groups. In the SHR12 group, the group that received GBO showed a significantly lower body weight compared with the untreated rats. Diuresis was not statistically different in the experimental group SHR6, except for the group treated with captopril. In the SHR12 group, diuresis was significantly lower only in COE and D-treated groups, as compared with the untreated SHR group (table 2). Regarding sodium balance, there were no differences between untreated WKY and SHR rats, but the SHR6 group treated with LE and captopril had lower sodium balances than the untreated groups. In

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the SHR12 groups, a greater sodium balance was observed in the GBO and COE-treated groups (table 2).

Table 2. Body weight and hematocrit values in the experimental groups.

SHR6	Body weight (g)	Hematocrit (%)	Food intake (g/24h)	Water intake (ml/24h)	Diuresis (ml/24h)	Natriuresis (mEq/24h)	Sodium balance (mEq/24h/100 g)
WKY	317,2 ± 12,4	47,5 ± 0,6	20,9 ± 1,1	41,6 ± 4,4	11,2 ± 1,9	0,36 ± 0,03	0,57 ± 0,03
SHR	305,5 ± 4,7	55 ± 1,06*	19,4 ± 0,4	28,4 ± 0,8*	11,7 ± 0,9	0,41 ± 0,03	0,53 ± 0,02
A	304,8 ± 4,4	53 ± 0,5*	22,4 ± 0 7†	30,0 ± 1,7	10,6 ± 1,8	0,51 ± 0,06	0,6 ± 0,04
LE	309,2 ± 4,3	54,1 ± 0,8*	18,2 ± 0,4*†	28,8 ± 1,8*	13,3 ± 1,3	0,71 ± 0,09*†	0,38 ± 0,03*†
GBO	302,4 ± 4,6	54,7 ± 1,3*	19,8 ± 1,4	26,9 ± 0,9*	9,7 ± 0,5	0,46 ± 0,05	0,53 ± 0,06
COE	302,9 ± 6,9	51,5 ± 0,7*†	19,9 ± 0,7	33,1 ± 1,8	14,8 ± 1,7	0,57 ± 0,04	0,5 ± 0,03
CPT	322,2 ± 9,2	49,7 ± 1,9†	18,5 ± 0,9	39,8 ± 4,2	20,5 ± 3,4*	0,7 ± 0,14*†	0,38 ± 0,04*†
SHR12							
WKY	354,5 ± 7,7	45,5 ± 0,6	17,7 ± 0,8	33,9 ± 1,8	9,0± 1,2	0,15 ± 0,02	0,48 ± 0,02
SHR	359,1 ± 5,9	50,5 ± 0,9*	18,2 ± 0,8	29,0 ± 1,6	11,8 ± 1,1	0,34 ± 0,04	0,41 ± 0,02
A	351,2 ± 8,7	52,2 ± 1,1*	19,4 ± 0,8	28,3 ± 1,5	11,7 ± 0,9	0,53 ± 0,08	0,42 ± 0,03
LE	330,8 ± 11,8	49,5 ± 0,4*	18,7 ± 0,6	28,9 ± 4,5	13,2 ± 3,4	0,35 ± 0,04	0,49 ± 0,04
GBO	342,4 ± 4,6†	48,4 ± 0,4*	20,1 ± 0,6*	33,7 ± 2,0*	12,6 ± 1,1	0,21 ± 0,04	0,55 ± 0,02*†
COE	320,7 ± 16,3	50,4 ± 1,0*	18,6 ± 0,7	25,7 ± 1,1	7,2 ± 1,0†	0,19 ± 0,03	0,54 ± 0,02*†
D	351,8 ± 8,0	50,6 ± 0,8*	17,4 ± 0,5	20,6 ± 1,0	8,2 ± 0,8†	0,36 ± 0,05	0,41 ± 0,02

Abbreviations: WKY (wistar kyoto rats), SHR (spontaneously hypertensive rats), A (Apigenin), LE (Lemon extract), GBO (Grapefruit + bitter orange extract), COE (Cocoa extract), D (Diosmin),

Vascular function

Dose-response curve to PHE was significantly shifted downwards in the arteries from all treated and untreated groups (figure 2), reducing the maximum contractile responses in all the SHR6 groups, as compared to the controls. The pEC_{50} was significantly enhanced also in all SHR6 groups and

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none of the treatments changed them (table 3). In the SHR12 groups, the maximum contractile responses were also reduced compared with the controls response, but the pEC50 did not change significantly (table 3).

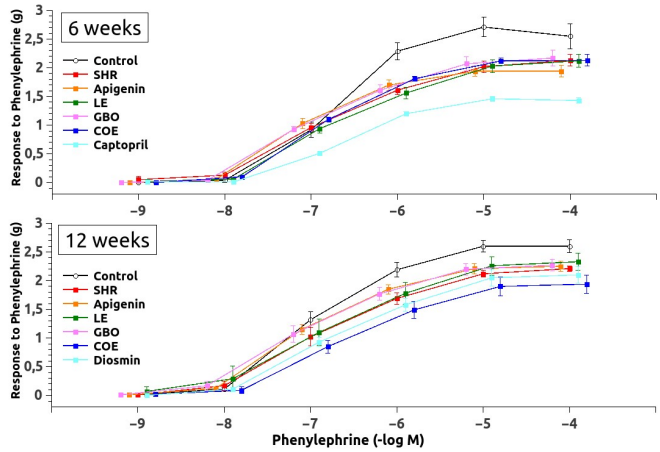

Figure 2. Pressor response to phenylephrine in aortic rings.

Maximal ACH-induced vasodilatation (figure 3 and table 3) was significantly reduced in aortic rings from the SHR6 rats compared to control rats. The relaxation to ACH improved significantly in the aorta from the SHR6 rats treated with A, LE and GBO, although the relaxation remained lower than in the control rats, except for the captopril-treated group, which was completely normalized. After administration of acute L-NAME (10-4 mol/l) to these aortic rings, the relaxant responses were further reduced, and almost abolished, but there were some residual and valuable responses only in the captopril-treated group. Regarding the SHR12 group, a similar decrease was observed in all the groups, and only the captopril-treated group showed a significantly greater relaxation.

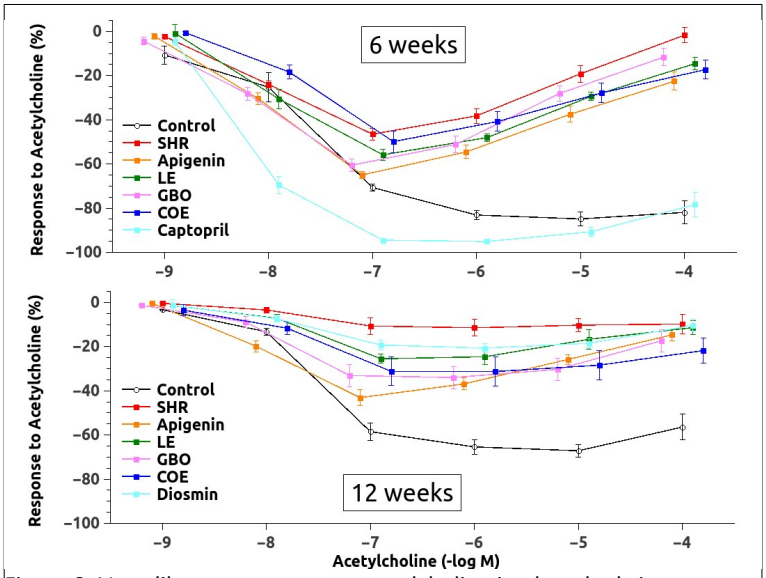

Figure 3. Vasodilatory response to acetylcholine in phenylephrine-preconstricted aortic rings.

Vasorelaxation in response to SNP was significantly reduced in the flavonoid-untreated SHR6 and SHR12 groups when compared with the control

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rats. SNP-induced vasorelaxation improved significantly in all flavonoid-treated groups (table 3).

Effect of flavonoid extracts on Oxidative Stress Status

Values of TBARS, nitrite and urinary protein excretion are shown in table 4. Regarding TBARS, a significant increase was found only in the plasma and urine of the untreated animals as compared with controls. In the SHR6 group, only captopril reduced plasma TBARS, whereas captopril, LE, GBO and COE reduced renal levels compared to the untreated SHR group. In the SHR12, only apigenin reduced renal TBARS values although the urinary values were significantly lower in all the flavonoid-treated SHR12 rats.

Regarding nitrite urinary excretion, only treatment with captopril in the SHR6 and GBO in the SHR12 groups showed a significantly greater value as compared to the untreated group. Also, urinary protein excretion was significantly higher in the untreated group but proteinuria was reduced in the LE-treated group (in SHR6 and SHR12) and in the groups treated with GBO, COE, D (in SHR12).

Table 3. Contractile response to phenylephrine and maximal relaxation to acetylcholine and sodium nitroprusside in the experimental groups.

	Phenylephrine		Acetylcholine		SNP
SHR6	**pEC_{50} (mol/L)**	**Maximal contraction (g)**	**Maximal relaxation (%)**	**After acute L-NAME**	**Maximal relaxation (%)**
WKY	-6,07 ± 0,05	2,71 ± 0,17	85,87 ± 2,67	34,17±5,45	99,58 ± 0,99
SHR	-6,69 ± 0,11*	2,13 ± 0,10*	46,90 ± 3,72*	3,70±1,46*	77,22 ± 3,19*
A	-6,8 ± 0,07*	1,94 ± 0,10*	64,97 ± 1,63*†	1,39±2,76*	88,43 ± 1,47*†

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	Phenylephrine		Acetylcholine		SNP
SHR6	**pEC_{50} (mol/L)**	**Maximal contraction (g)**	**Maximal relaxation (%)**	**After acute L-NAME**	**Maximal relaxation (%)**
LE	-6,6 ± 0,05*	2,13 ± 0,11*	55,97 ± 2,38*†	3,47±1,43*	88,54 ± 1,86*†
GBO	-6,6 ± 0,06*	2,17 ± 0,13*	60,56 ± 2,72*†	1,56±0,65*	90,09 ± 2,18*†
COE	-6,77 ± 0,03*	2,13 ± 0,13*	49,86 ± 4,86*	0,86±0,61*†	91,07 ± 1,49*†
CPT	-6,7 ± 0,07*	1,46 ± 0,04*†	95,57 ± 0,77*†	19,43±4,47†	101,89 ± 1,34†
SHR12					
WKY	-6,84 ± 0,06	2,62 ± 0,11	68,41 ± 2,80	11,26±2,14	93,94±2,21
SHR	-6,66 ± 0,05	2,03 ± 0,08*	24,00 ± 2,68*	1,47±0,94*	64,66±3,04*
A	-6,74 ± 0,05	2,25 ± 0,09*	43,13 ± 3,45*†	0,22±0,23*	80,28±2,80*†
LE	-6,75 ± 0,13	2,33 ± 0,15	26,50 ± 2,58*	2,49±1,07*	70,64±2,60*†
GBO	-6,73 ± 0,1	2,27 ± 0,10*	35,00 ± 8,44*	2,45±0,65*	80,63±5,48*†
COE	-6,6 ± 0,11	1,94 ± 0,16*	32,74 ± 6,39*	3,07±1,32*	77,69±5,82*†
D	-6,68 ± 0,09	2,11 ± 0,14*	21,38 ± 2,02*	6,22±2,24*†	79,44±3,78*†

Data are mean ± S.E.M. Abbreviations as in Table 1. pEC50 is the negative logarithm of the half maximal effective concentration (EC50). *, $p < 0.05$ vs. WKY; †, $p < 0.05$ vs. SHR.

Histopathology results

The analysis of the heart revealed that both SHR untreated and treated groups (both SHR6 and SHR20) showed no infarct zones, hyaline arteriopathy or fibrinoid necrosis (data not shown), features that were observed in the study performed in the L-NAME hypertension (10). Wall-lumen ratio of coronary arteries was decreased in the SHR6 when compared with control and treatments with A and COE increased them. In the SHR12 group, there were no changes. Interventricular heart septum thickness was significantly higher in the SHR6 untreated group compared to the control rats and only captopril lowered to a normal level. In the SHR12 group, the values of all experimental

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groups were very similar without significant differences between them. With respect to the thickness of the abdominal and thoracic aorta (table 5), there were significant reductions in the thickness of the abdominal aorta in all the SHR6-treated groups only. In the SHR12 group, the tendency of the flavonoid-treated groups was to show an increase in the thickness in both vessels. Regarding the kidney (table 5), significant increases were found in the LWR parameter in the LE and GBO-treated SHR6 groups. Also, the absence of HA and tubular cylinders was evident in all the treated SHR6 groups, except in the A group. In the SHR12 groups, HA and TC were also found in the untreated SHR and in the groups treated with A and COE.

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Table 4. Measurements of TBARS, nitrite and proteinuria in the experimental groups.

SHR6	Plasma TBARS (nmol/mL)	Kidney TBARS (nmol/mg prot)	Urine TBARS (nmol/mg prot 24h)	Plasma Nitrite (µg/ml)	Urinary excretion of nitrite (µg/ 24 h)	Urinary protein excretion (mg/24 h/ Kg bw)
WKY	6,3 ± 0,2	6,4 ± 0,4	252 ± 42	0,91 ± 0,02	11,59 ± 2,69	168,32 ± 50,37
SHR	7,6 ± 0,3*	7,5 ± 0,4	559 ± 45 *	0,82 ± 0,02 *	13,97 ± 2,76	614,88 ± 28,80 *
A	11,8 ± 1,1*†	6,7 ± 0,8	474 ± 35 *	0,95 ± 0,02 †	8,73 ± 1,78 †	563,62 ± 53,66 *
LE	10,2 ± 0,7*†	5,8 ± 0,4 †	491 ± 37 *	0,89 ± 0,01 †	23,25 ± 5,34	540,72 ± 16,48 *†
GBO	7,2 ± 0,5	6,0 ± 0,3 †	335 ± 34†	0,90 ± 0,03 †	15,74 ± 1,77 *	544,91 ± 26,16 *
COE	9,6 ± 1,5*	4,4 ± 0,3*†	473 ± 9*	0,88 ± 0,03	23,25 ± 7,83	606,61 ± 50,70 *
CPT	6,0 ± 0,4†	5,3 ± 0,4 †	615 ± 81*	0,92 ± 0,03 †	23,68 ± 1,67 *†	551,57 ± 86,25 *
SHR12						
WKY	3,9 ± 0,4	8,3 ± 1,0	207 ± 22	1,13 ± 0,09	54,86 ± 11,15	213,27 ± 13,65
SHR	7,3 ± 0,4*	11,1 ± 1,4	723 ± 32 *	1,01 ± 0,12	44,59 ± 3,27	547,52 ± 27,04 *
A	8,0 ± 0,4*	6,9 ± 0,8 †	587 ± 17 *†	0,85 ± 0,02*	37,58 ± 3,24	499,68 ± 25,21*
LE	6,8 ± 1,2	7,3 ± 1,2	359 ± 27 *†	1,16 ± 0,02	44,69 ± 1,65	314,64 ± 47,10 †
GBO	6,7 ± 2,1	8,1 ± 0,4	336 ± 21 *†	1,28 ± 0,07	73,03 ± 10,59 †	306,42 ± 40,65 †
COE	4,6 ± 0,6†	10,8 ± 2,5	331 ± 41 *†	1,28 ± 0,02	33,05 ± 4,29	353,08 ± 20,19 *†
D	3,1 ± 0,2†	8,0 ± 0,6	339 ± 20 *†	1,36 ± 0,17	31,90 ± 2,82 †	451,01 ± 20,34 *†

Data are mean ± S.E.M. *, p<0.05 vs WKY; +, p<0,05 vs SHR.

Discussion

The results of the present study show that some of the flavonoids studied have a very modest effect on blood pressure and renal and vascular function in the SHR model of arterial hypertension. Moreover, these effects can be observed only in the younger animals, treated for 6 weeks. Doubling the treatment time (12 weeks) almost eliminated these beneficial effects.

In the SHR6 groups, only GBO had a significant effect on blood pressure and this was accompanied by a significant improvement of the vascular vasodilator response, likely related to an increased production of vascular NO. A reduction in renal oxidative status with beneficial changes in the histopathological parameters in heart and kidney was also observed in this group. Although BP was not significantly modified in the apigenin and lemon

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extract-treated groups, similar vascular and renal changes were observed in these animals when compared to the GBO-treated group.

As we explained in our previous article in nitric oxide-deficient hypertensive animals (10), the dose chosen of each of the treatments responds to an objective criterion of potential subsequent application in humans. Therefore, the doses ingested daily by our animals are very low compared to those used in other studies with similar compounds, since generally, in animal studies, doses much higher are used, proportionally, than those applied in a human therapy (5, 14).

It is clear that both types of arterial hypertension models are very different. It seems that the flavonoids that we have used are more useful in the nitric oxide-deficient hypertensive animals. In fact, one of the most universal effects of flavonoids is through the augmentation of NO synthesis and action (23-24). In this way, they can be of interest in diseases of endothelial dysfunction (25). However, the SHR model is a completely different model, with an important genetic component (19). Hypertension in the SHR model develops as a result of increased peripheral resistance, first produced by neurogenic and renal factors and later structural vascular changes associated with increased vascular protein synthesis because of the chronic elevation in blood pressure. As observed, our results clearly confirm that the inhibition of the renin-angiotensin system is one of the treatments of choice for this model and this has been shown previously (19).

Table 5. Histopathological results of the heart, aorta and kidney.

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SHR6	Coronary LWR	Heart IVS	Thoracic AT	Abdominal AT	Renal LWR	Renal HA	Renal TC
WKY	2,27 ± 0,21	2,36 ± 0,14	107,2 ± 5,9	110,3 ± 7,5	1,69 ± 0,10	0,0	0,0
SHR	1,59 ± 0,03*	2,95 ± 0,18*	131,0 ± 5,6*	138,8 ± 3,4*	1,18 ± 0,08*	0,0	0,0
A	2,84 ± 0,22†	2,91 ± 0,13*	125,3 ± 2,9*	100,5 ± 2,1†	1,32 ± 0,31	0,0	0,25
LE	1,80 ± 0,21	2,73 ± 0,05*	150,4 ± 4,5*	127,0 ± 3,6 †	1,51 ± 0,04†	0,0	0,0
GBO	1,48 ± 0,13*	2,50 ± 0,07	134,6 ± 2,3*	117,5 ± 5,2 †	1,69 ± 0,10†	0,0	0,0
COE	2,80 ± 0,58†	2,83 ± 0,07*	134,5 ± 3,8*	87,1 ± 4,6†	1,10 ± 0,24	0,0	0,0
CPT	1,78 ± 0,19	2,39 ± 0,12†	133,3 ± 7,3*	101,7 ± 4,0†	1,26 ± 0,10*	0,0	0,0
SHR12							
WKY	3,08 ± 0,13	3,22 ± 0,15	104,8 ± 4,4	86,7 ± 5,8	1,76 ± 0,19	0,0	0,0
SHR	3,00 ± 0,80	3,43 ± 0,09	116,3 ± 2,2	90,5 ± 5,3	1,27 ± 0,26	0,25 ± 0,25	0,25 ± 0,25
A	1,97 ± 0,41	2,84 ± 0,23	126,8 ± 3,2*†	94,6 ± 2,7	1,61 ± 0,30	0,0	0,75 ± 0,25
LE	3,43 ± 0,28	3,04 ± 0,29	137,5 ± 3,5*†	114,8 ± 3,7*†	1,67 ± 0,44	0,0	0,0
GBO	2,20 ± 0,44	3,01 ± 0,09	131,4 ± 3,6*†	107,6 ± 4,0*†	1,79 ± 0,32	0,0	0,0
COE	3,06 ± 0,40	3,40 ± 0,15	142,2 ± 3,0*†	118,8 ± 3,7*†	1,39 ± 0,11	0,0	0,25 ± 0,25
D	3,15 ± 0,79	3,61 ± 0,09	130,8 ± 2,9*†	104,6 ± 3,3*†	1,43 ± 0,20	0,0	0,0

Data are mean ± S.E.M. Abbreviations as in table 2. LWR, lumen to wall ratio; IVS, Interventricular septum width (mm); AT, Aorta Thickness (mm); HA, Hyaline Arteriopathy; TC, tubular cylinders. *, p<0.05 vs WKY; +, p<0,05 vs SHR.

Many studies have reported a reduction in blood pressure following the consumption of flavonoid-rich products. In vitro studies have reported that flavonoids such as genistein, quercetin, and (-)-epicatechin regulated (directly or indirectly) NO production in isolated vessels or cultured endothelial cells [8, 26-29]. In our data, A, LE, GBO and of course, captopril, improved the acetylcholine relaxation in the SHR6 rats, the dependent on NO production (after acetylcholine administration). Moreover, in these SHR6 animals, the administration of sodium nitroprusside also improved the reduced relaxation in the animals receiving all the flavonoids, indicative of an action close to the NO effector in the smooth muscle cell, at the level of cGMP. These data also speak

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in favor of a reduced production of vascular NO in the SHR model, as suggested previously (19).

Other mechanisms have been suggested to explain the increased endothelial NO bioavailability promoted by flavonoids. Several studies have shown that a regular consumption of flavonoids or flavonoid-rich foods can significantly improve the oxidative status as well the endothelial function [8, 10, 24-25, 30]. In the present study, we detected a significant increase in ROS levels, as measured as TBARS, in plasma and urine in the SHR untreated animals. It is likely that the reduction in kidney TBARS, observed in some of the flavonoid-treated groups (Table 4), is also contributing to the normalization of BP. It may be interesting to consider that the treatments with a greater specific antioxidant efficacy are those having flavonoids with B-ring catechol structure (3', 4'-dihydroxy), LE (eriocitrin) and COE (catechin compounds).

It is known that the chronic elevation of systemic blood pressure is associated with the presence of proteinuria and the development of glomerulosclerosis [32], as our data show (table 4). The flavonoids treatments showed a reduction in proteinuria, but it only reached a significant difference in the LE-treated SHR6 group. Interestingly, proteinuria was also reduced significantly in the SHR 12 groups, and this is a result that merits further study.

The metabolic and hemodynamic changes of hypertension are also associated with the development of structural abnormalities, such as left ventricular hypertrophy, cardiac fibrosis, necrosis and protein remodeling, as well as with vascular wall hypertrophy [33-34], some of them shown in the present results. Maybe because of NO deficiency and probably also because of

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the hypertensive load on vascular tissues, increased monocyte and platelet adhesion with the release of growth factors would contribute to the thickening of the vascular wall. The proliferation was limited to the media, which is in agreement with the findings of others [30-32]. All the flavonoids treatments were effective to reduce the abdominal aortic thickness in the SHR 6 group, but only captopril reduced cardiac hypertrophy. Regarding the renal structural changes, only in the SHR 12 group, flavonoids seem to exert some beneficial effect, since hyaline arteriopathy was present only in the SHR untreated group, and tubule cylinders were also not observed in the LE, GBO and diosmin-treated groups.

Our results suggest that the flavonoids included in this study, and already present in the market as nutritional supplements, may be used as a functional food ingredients but the beneficial effect on hypertension is moderate, specially in the younger SHR group. Further studies are necessary to elucidate the mechanisms involved in their effects, including an evaluation of the dose-activity relationship in order to determine the molecular structures most active. In any case, our results suggest that the effects of these flavonoids may be related to a combination of vasodilator and antioxidant actions.

Clinical Perspectives.

- Flavonoids are important substances with biological actions of interest in arterial hypertension. We aimed to analyze the role of some flavonoids in a model of arterial hypertension, the spontaneously hypertensive rat (SHR), thought to be the most comparable with the human form of

hypertension.

- Grapefruit extract significantly reduced the elevated blood pressure of the younger SHR animals (12 weeks), but none of the extracts was effective in the older SHR group (18 weeks). Vascular reactivity was also ameliorated with some of these treatments.
- These extracts may be used as functional food ingredients with a moderate therapeutic benefit, especially in the early phases of arterial hypertension.

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