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Decrease in oxidative stress through supplementation of vitamins C and E is associated with a reduction in blood pressure in patients with essential hypertension

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ABSTRACT

Oxidative stress has been associated with mechanisms of EH (essential hypertension). The aim of the present study was to test the hypothesis that the antioxidant properties of vitamins C and E are associated with a decrease in BP (blood pressure) in patients with EH. A randomized double-blind placebo-controlled clinical trial was conducted in 110 men with grade 1 EH (35–60 years of age without obesity, dyslipidaemia and diabetes mellitus, non-smokers, not undergoing vigorous physical exercise, without the use of any medication and/or high consumption of fruit and vegetables). Participants were randomly assigned to receive either vitamins C + E [vitamin C (1 g/day) plus vitamin E (400 international units/day)] or placebo for 8 weeks. Measurements included 24 h ambulatory BP and blood analysis of oxidative-stress-related parameters in erythrocytes (GSH/GSSH ratio, antioxidant enzymes and malondialdehyde) and plasma [FRAP (ferric reducing ability of plasma)], and levels of 8-isoprostane, vitamins C and E were measured at baseline and after treatment. Following administration of vitamins C + E, patients with EH had significantly lower systolic BP, diastolic BP and mean arterial BP and higher erythrocyte and serum antioxidant capacity compared with either placebo-treated patients with EH or the patients with EH at baseline prior to treatment. BP correlated positively with plasma 8-isoprostane levels and negatively with plasma FRAP levels in the vitamins C + E- and placebo-treated groups. In conclusion, the present study supports the view that oxidative stress is involved in the pathogenesis of EH, and that enhancement of antioxidant status by supplementation with vitamins C and E in patients with EH is associated with lower BP. This suggests intervention with antioxidants as an adjunct therapy for hypertension.

INTRODUCTION

An increasing body of evidence suggests that oxidative stress is involved in the pathophysiological mechanism of hypertension [1–3], which is considered to be the most important cardiovascular risk factor worldwide [4]. Oxidative stress results from an imbalance between the generation of ROS (reactive oxygen species) and the

Key words: antioxidant status, antioxidant vitamin, blood pressure, essential hypertension, lipid peroxidation, oxidative stress.

Abbreviations: AAMI, Association for the Advancement of Medical Instrumentation; BMI, body mass index; BP, blood pressure; ABPM, ambulatory BP monitoring; CAT, catalase; CV, coefficients of variation; DBP, diastolic BP; EH, essential hypertension; eNOS, endothelial NO synthase; ET-1, endothelin-1; FRAP, ferric reducing ability of plasma; FRASC, ferric reducing/antioxidant and ascorbate concentration; GSH-Px, glutathione peroxidase; IU, international unit; MAP, mean arterial BP; MDA, malondialdehyde; ROS, reactive oxygen species; SBP, systolic BP; SOD, superoxide dismutase.

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antioxidant defence systems [5,6]. ROS may increase due to a decrease in the activity of antioxidant enzymes in patients with EH (essential hypertension) [7]. In turn, an increase in BP (blood pressure) may enhance vascular production of superoxide through a mechanism that is independent of the activation of the RAS (renin–angiotensin system) [8]. Although it is presently unclear whether endothelial dysfunction is a primary abnormality or a consequence of increased BP, it is known that superoxide rapidly inactivates the endothelium-derived vasodilator NO, thereby promoting vasoconstriction [9]. Consequently, attempts to counteract the hypertensive effects of ROS have led to the use of exogenous antioxidants to improve vascular function and reduce BP in animal models [10] and human hypertension [11,12]. The role of the antioxidants vitamins C and E has emerged as a possible therapy for decreasing oxidative stress and thereby lowering BP. Additionally vitamins C and E down-regulate NADPH oxidase, a major source of ROS in the vascular wall, and up-regulate eNOS (endothelial NO synthase) [13], both of these effects lower BP. Despite these biological effects, long-term clinical trials have failed to consistently support the antihypertensive effects of vitamins C and E in patients at high cardiovascular risk. However, results of studies showing that supplemental antioxidant vitamin intake lowers BP [14–16] in short-term trials are inconsistent [17]. Most of such studies have looked at all-cause or cardiovascular mortality, rarely focusing on BP as a primary end point [18]. None of the large clinical trials examined the effects of antioxidants specifically on BP [19]. Moreover, the majority of clinical trials that did not find any antihypertensive effects of antioxidant vitamins lack rigorous exclusion criteria in the selection of subjects to avoid the influence of confounders [20]. Antihypertensive drug therapy, in addition, may also have beneficial effects on both oxidative stress and endothelial function [18]. On the basis of this paradigm, the aim of the present study was to specifically address the role of oxidative stress in the development of EH. In addition, we studied the association of the antioxidant properties of vitamins C and E with its possible antihypertensive effects. A randomized double-blind placebo-controlled study was conducted to test the hypothesis that oral administration of vitamins C and E together, by improving the antioxidant status, causes a decrease in BP in patients with mild-to-moderate EH.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma–Aldrich, Merck or Riedel-de Häen, and were of the highest commercial grade available.

Study population and the diagnosis of EH

The study included 110 never-treated male patients with EH [mean age, 46 years (range, 35–60 years)]. Patients were recruited from newly diagnosed cases at the Cardiovascular Center, University of Chile Clinical Hospital, Santiago, Chile between March 2004 and December 2006. During each patient visit, BP was measured with a standard mercury sphygmomanometer. Secondary hypertension was excluded by clinical history, physical examination and appropriate tests. Inclusion criteria of the patient were: men, 35–60 years of age, hypertension [mean day time SBP (systolic BP) between 135 and 154 mmHg or DBP (diastolic BP) between 85 and 94 mmHg by ABPM (ambulatory BP monitoring)] [21], no diabetes or impaired glucose tolerance [22], the absence of proteinuria, a BMI (body mass index) between 23 and 29.9 kg/m^2, total serum cholesterol < 5.4 mmol/l, and serum triacyglycerol (triglyceride) < 2.3 mmol/l. Exclusion criteria included smoking, consumption of alcoholic beverages > 40 g/week, concomitant diseases, currently performing vigorous physical exercise, use of medication (including mineral or vitamin supplements, antihypertensive drugs or statins) and/or the high consumption of fruits and vegetables. Grade I EH was diagnosed according to the European Society of Hypertension and Cardiology criteria [23]. Dietary food and nutrient intake was assessed using a 3-day food record, and physical activity levels were controlled with a modified Baecke questionnaire [24] at baseline and at the end of the study. Recruited patients were asked not to change their physical activity habits and dietary intake during the study.

The study was conducted according to the Helsinki Declaration of the World Medical Association (2000), and informed written consent was obtained from all subjects before participation in the trial.

Normotensive control group

A total of 110 normotensive subjects were recruited from the same clinic as the subjects with hypertension. This group served as a normotensive reference to determine normal values of the biochemical parameters studied. The remaining inclusion and exclusion criteria for this group were the same as the patients with EH.

ABPM

BP was measured by ABPM on a normal workday (over 24 h from 08:30 hours) using an oscillometric monitor (SpaceLabs 90207) checked previously for accuracy against simultaneous measurements by mercury sphygmomanometer. This device fulfils the validation criteria of the British Hypertension Society protocol [25] and satisfies the criteria of the AAMI (Association for the Advancement of Medical Instrumentation) for studies in ambulatory conditions [26]. The oscillometric accuracy, assessed by Spacelabs intra-arterial average

differences for SBP and DBP (-0.6 ± 5.9 and 0.9 ± 6.4 mmHg respectively), were within the AAMI accuracy standard. The estimated oscillometric reproducibility was -0.3 ± 3.2 and 0.1 ± 3.5 mmHg (values are means $\pm$ S.D.) for SBP and DBP respectively. The adult cuff and the large adult cuff were used for arm circumferences of 24–31 cm and 32–42 cm respectively.

Study design

A randomized double-blind placebo-controlled study was conducted. Screened patients were randomly allocated to daily treatments of either placebo ($n = 55$) or vitamins C + E [$n = 55$; 1 g of vitamin C, as L-ascorbic acid, plus 400 IU (international unit) as vitamin E, as *R*,*R*,*R*,-α-tocopherol acetate] once a day for 8 weeks. Both medications were given in encapsulated forms to ensure that the placebo and vitamins were indistinguishable from each other, and were provided by Procaps and Gynopharm CFR Laboratories. All patients were asked to take the assigned medication 30 min before breakfast once a day. At baseline and at the end of the study, subjects underwent 24-h BP monitoring (ABPM) to check for BP changes, and blood samples were collected for the determination of lipid, glucose profile, plasma levels of BP modulators [renin, aldosterone, ET-1 (endothelin-1), homocysteine, folic acid and vitamin B_{12}], plasma and erythrocyte oxidative-stress-related parameters and antioxidant status. All blood samples were collected at the same time of day to minimize circadian variations. Adverse events, clinic BP measurements and compliance with the study medication were recorded during each follow-up visit and through weekly direct communications with the patients (by telephone or email). The methods of measuring BP followed the Joint National Committee guidelines [27]. All study personnel and participants remained blinded to the treatment assignment for the duration of the study.

Blood sampling

Blood samples from the antecubital vein were collected in chilled Vacutainers containing EDTA (1.5 mg/ml) and were centrifuged at 3000 *g* for 10 min to obtain plasma. Erythrocytes were subjected to hypotonic haemolysis by dilution with distilled water. Plasma samples and erythrocyte lysates were stored at $-70\,^{\circ}$C. For the measurement of 8-isoprostane, blood was collected in plastic tubes treated with the antioxidant butylated hydroxytoluene (final concentration, 1 mmol/l).

Assays of oxidative-stress-related parameters

FRAP (ferric reducing ability of plasma)

Plasma antioxidant status was assessed by measuring its ability to reduce ferric to ferrous iron, with a detection limit 10 μmol/l [28]. The inter-assay and intra-assay CVs (coefficients of variation) for FRAP were 3.0 and 1.0 % respectively.

Glutathione

GSH and GSSG levels were assayed by fluorimetry in erythrocytes and the GSH/GSSG ratio was calculated as a parameter of intracellular redox status [29]. The inter- and intra-assay CVs for GSH and GSSG were 3.1 and 4.2 % and 2.7 and 3.5 % respectively

MDA (malondialdehyde)

Erythrocyte lipid peroxidation was assayed spectrophotometrically at 532 nm by the thiobarbituric acid reaction at pH 3.5, followed by solvent extraction with a mixture of *n*-butanol/pyridine (15:1, v/v) [30]. Tetramethoxypropane was used as the external standard, and the levels of lipid peroxides are expressed as nmol of MDA/g of Hb. The inter-assay and intra-assay CVs for MDA were 10.5 and 4.8 % respectively.

8-Isoprostane

Plasma 8-isoprostane concentration (in pmol/ml), recognized as a reliable biomarker of lipid peroxidation *in vivo* [31], was determined by using an ELISA kit (Cayman) [32]. The inter- and intra-assay CVs were 9.5 and 10.7 % respectively.

Assessment of plasma vitamin C and E concentrations

Plasma vitamin C levels were assessed no later than 2 h after blood samples collection by a colorimetric FRASC (ferric reducing/antioxidant and ascorbate concentration) assay. FRASC is a fast and reliable alternative to HPLC, with a detection limit of 3.0 μmol/l [33]. The intra- and inter-assay CVs were 2.7 and 4.9 % respectively.

Plasma vitamin E concentration was measured by HPLC, and inter-assay and intra-assay CVs were 6.4 and 4.5 % respectively [34]. Blood samples were centrifuged for 5 min, the supernatant was extracted with hexane and evaporated to dryness. The dry residue was dissolved in methanol and used for reverse-phase HPLC using a NovaPack 15033.9 mm column (4 mm particle size) and a UV detector at 292 nm (Lambda max 480; Millipore-Waters).

Antioxidant enzymes

Erythrocyte lysates were homogenized in 0.25 mol/l sucrose for the determination of Cu-Zn SOD (superoxide dismutase) activity or in 1.15 % KCl/10 mmol/l Tris/HCl buffer (pH 7.4) for CAT (catalase) and GSH-Px (glutathione peroxidase) activity. The Cu-Zn SOD assay is based on the SOD-mediated increase in the rate of auto-oxidation of catechols in an aqueous alkaline solution to yield a chromophore with a maximum absorbance at 525 nm [35]. One Cu-Zn SOD unit is defined as the activity that doubles the auto-oxidation background, and results are expressed as units/g of Hb. CAT activity was assayed by the kinetic of breakdown of H_2O_2 at 240 nm by an aliquot of the 2400 *g* supernatant and are expressed as the first-order reaction rate constant (k)/g of

Hb [36]. Soluble GSH-Px activity was measured spectrophotometrically in the cytosolic fraction (100 000 *g* supernatant) by the reduction of glutathione disulfide coupled to NADPH oxidation by glutathione reductase [37]. One GSH-Px unit is defined as the activity that oxidizes 1 μmol of NADPH/min and is expressed as units/g of Hb.

Modulators of BP

Plasma levels of other BP modulators were measured, including renin and aldosterone by RIA and ET-1, homocysteine, folic acid and vitamin B_{12} by ELISA.

Statistical analysis

The required sample size was calculated by using BP as the primary endpoint; 45 patients in each group provided us with a $\geqslant 80\%$ power to detect a 5 mmHg difference in the mean BP of both treatment groups. In addition, this calculation was also applied to the biochemical parameters of the study. Descriptive statistics of variables are presented as means $\pm$ S.E.M. Baseline differences between the normotensive control group and patients with EH and between the clinical characteristics of the groups receiving vitamins C + E and placebo were assessed using Student's *t* test. Biochemical parameters and SBP, DBP and MAP (mean arterial BP) of patients with EH at baseline and following placebo and vitamin treatment were compared by one-way ANOVA, followed by Bonferroni's multiple comparison test as the post-hoc test. $P < 0.05$ was considered statistically significant. The association of variables was studied by Pearson correlation test due to their Gaussian distribution. All statistical analyses were calculated using GraphPad Prism™ version 2.0.

RESULTS

Clinical characteristics

Compliance and monitoring of side effects

Two recruited patients were unable to complete the 8 week treatment period. One patient abandoned the therapy by week 5, due to a geographical change in his work location. The second patient abandoned the treatment by the week 3 as he required an elective cholecystectomy. Both were replaced with new patients with EH following the same inclusion and exclusion criteria in order to ensure the sample size of 55 patients in each group.

No side effects were either observed or reported throughout the trial.

Baseline

Dietary and physical activity habits and body weight did not change during the study. Baseline characteristics of the normotensive control group ($n = 110$) and study patients with EH ($n = 110$) are shown in Table 1. Mean daytime SBP, DBP and MAP in the group with hypertension were significantly higher ($P < 0.001$), whereas all other parameters were within the normal range and were not significantly different between the two groups. In addition, levels of the BP modulators renin, aldosterone, ET-1, homocysteine, folic acid and vitamin B_{12} were not significantly different between groups.

Table 1 Clinical characteristic of patients with EH and healthy normotensive subjects

Values are means $\pm$ S.E.M. HDL, high-density lipoprotein; LDL, low-density lipoprotein. *P value was determined using an unpaired Student's t test.

Characteristic	Normotensive subjects	Patients with EH	P value*
n	110	110	
Age (years)	44.1 $\pm$ 0.9	45.6 $\pm$ 1.1	0.29
BMI (kg/m^2)	25.1 $\pm$ 0.3	25.5 $\pm$ 0.2	0.26
Heart rate (beats/min)	72.2 $\pm$ 1.1	73.6 $\pm$ 1.4	0.43
Serum glucose (mmol/l)	4.96 $\pm$ 0.04	5.07 $\pm$ 0.08	0.22
Creatinine (μmol/l)	77.4 $\pm$ 1.3	79.2 $\pm$ 1.9	0.43
Total cholesterol (mmol/l)	4.62 $\pm$ 0.17	4.72 $\pm$ 0.22	0.71
HDL-cholesterol (mmol/l)	1.26 $\pm$ 0.02	1.20 $\pm$ 0.05	0.26
LDL-cholesterol (mmol/l)	2.62 $\pm$ 0.20	2.82 $\pm$ 0.14	0.41
Serum triacylglycerols (mmol/l)	1.44 $\pm$ 0.06	1.50 $\pm$ 0.02	0.34
Renin (pmol $\cdot$ l^{-1} $\cdot$ h^{-1})	24.1 $\pm$ 2.1	21.1 $\pm$ 1.9	0.29
Aldosterone (nmol/l)	0.22 $\pm$ 0.02	0.24 $\pm$ 0.01	0.37
ET-1 (pmol/l)	2.67 $\pm$ 0.18	2.71 $\pm$ 0.14	0.86
Homocysteine (μmol/l)	8.62 $\pm$ 0.21	9.11 $\pm$ 0.33	0.21
Folic acid (nmol/l)	44.9 $\pm$ 1.2	46.1 $\pm$ 1.2	0.47
Vitamin B_{12} (pmol/l)	226 $\pm$ 6	226 $\pm$ 6	1.00
Daytime			
SBP (mmHg)	119 $\pm$ 0	139 $\pm$ 0	< 0.001
DBP (mmHg)	77.4 $\pm$ 0.3	96.3 $\pm$ 0.7	< 0.001
MAP (mmHg)	92 $\pm$ 1	107 $\pm$ 1	< 0.001

After treatment with vitamins C + E or placebo

After treatment, the clinical characteristics of the patients with EH treated with either vitamins C + E or placebo were within the normal ranges and no significant differences were observed (Table 2). Patients with EH treated with vitamins C + E had significantly lower SBP (Figure 1A), DBP (Figure 1B) and MAP (results not shown) compared with either the placebo-treated patients ($n = 55$) or the patients with EH at baseline prior to treatment ($n = 110$).

Antioxidant status and oxidative-stress-related parameters

Baseline

As described in the Materials and methods section, normotensive subjects were recruited from a group who had participated in a previous related study to serve as a reference group to determine the normal values of the biochemical parameters studied. Plasma and erythrocyte

Table 2 Effects of treatment with vitamins C + E or placebo on clinical characteristics in patients with EH

Values are means ± S.E.M. HDL, high-density lipoprotein; LDL, low-density lipoprotein. *P value was determined using an unpaired Student's t test.

Characteristic	Patients with EH treated with		
	Placebo	Vitamins C + E	P value*
n	55	55	
Age (years)	45.6 ± 1.5	45.4 ± 1.1	0.91
BMI (kg/m^2)	25.3 ± 0.3	25.6 ± 0.7	0.69
Heart rate (beats/min)	72.1 ± 1.1	74.4 ± 1.7	0.25
Serum glucose (mmol/l)	4.96 ± 0.12	5.16 ± 0.16	0.31
Creatinine (μmol/l)	80.1 ± 1.2	79.1 ± 2.1	0.68
Total cholesterol (mmol/l)	4.64 ± 0.18	4.76 ± 0.23	0.49
HDL-cholesterol (mmol/l)	1.21 ± 0.03	1.18 ± 0.02	0.40
LDL-cholesterol (mmol/l)	2.79 ± 0.09	2.83 ± 0.10	0.76
Serum triacylglycerols (mmol/l)	1.49 ± 0.11	1.52 ± 0.09	0.83
Renin (pmol/l/ h)	19.1 ± 2.0	22.2 ± 2.2	0.30
Aldosterone (nmol/l)	0.25 ± 0.01	0.24 ± 0.03	0.75
ET-1 (pmol/l)	2.73 ± 0.15	2.70 ± 0.23	0.91
Homocysteine (μmol/l)	9.30 ± 0.39	9.11 ± 0.41	0.73
Folic acid (nmol/l)	47.3 ± 1.3	45.6 ± 1.1	0.32
Vitamin B_{12} (pmol/l)	221 ± 7	231 ± 7	0.31

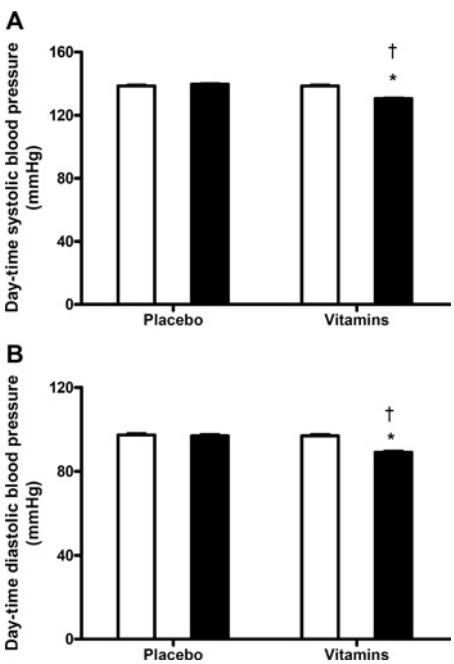

Figure 1 Daytime SBP (A) and DBP (B) in patients with EH before (open bars) and after (closed bars) treatment with either vitamins C + E or placebo

Values are means ± S.E.M., $n = 55$. Sources of variation were assessed by one-way ANOVA, followed by Bonferroni's multiple comparison test as the post-hoc test. *$P < 0.001$ compared with basal; †$P < 0.001$ compared with placebo-treated patients with EH.

Table 3 Plasma and erythrocyte oxidative-stress-related parameters in patients with EH and healthy normotensive subjects

Values are expressed as means ± S.E.M. *P value was determined using an unpaired Student's t test.

Parameter	Normotensive subjects	Patients with EH	P value*
n	110	110	
Plasma			
FRAP (μmol/l)	426 ± 14	311 ± 8	< 0.01
Vitamin C (μmol/l)	44.2 ± 1.7	37.5 ± 1.8	< 0.01
Vitamin E (μmol/l)	16.1 ± 1.2	16.4 ± 1.1	0.84
8-Isoprostane (pmol/l)	76.7 ± 3.6	105 ± 3	< 0.01
Erythrocytes			
CAT (k/g of Hb)	290 ± 6	220 ± 8	< 0.01
SOD (units/g of Hb)	1472 ± 21	1157 ± 26	< 0.01
GSH-Px (units/g of Hb)	6.11 ± 0.11	5.48 ± 0.14	< 0.01
GSH/GSSG ratio	5.98 ± 0.14	5.20 ± 0.13	< 0.01
MDA (nmol/g of Hb)	311 ± 8	383 ± 11	< 0.01

oxidative-stress- and antioxidant-status-related parameters are shown in Table 3. Patients with EH had a lower antioxidant status, as plasma FRAP, vitamin C levels and erythrocyte GSH/GSSG ratios were decreased significantly ($P < 0.01$) by 27, 15 and 15 % respectively (Table 3). In addition, the activity of CAT, Cu-Zn SOD and GSH-Px in erythrocytes of patients with hypertension were 24, 21 and 10 % lower respectively, than the values in normotensive subjects ($P < 0.01$). Furthermore, patients with EH had increased plasma 8-isoprostane levels (37 %) and erythrocyte MDA or lipid peroxidation levels (23 %) compared with the normotensive subjects ($P < 0.001$).

After treatment with vitamins C + E or placebo

Patients treated with vitamins C + E compared with either the placebo-treated patients with EH or the patients with EH at baseline prior to treatment had significantly lower plasma and erythrocytes lipid peroxidation, as assessed by 8-isoprostane and MDA levels respectively (Figure 2A). Plasma and erythrocyte antioxidant capacity, as assessed by FRAP, GSH/GSSH ratio and plasma vitamin C levels, were significantly higher in patients with EH treated with vitamins C + E compared with either the placebo-treated patients with EH (Figure 2B) or the patients with EH at treatment prior to treatment. However, no significant differences were observed in plasma vitamin E concentrations between the two study groups (Figure 2C). The activity of the antioxidant enzymes Cu-Zn SOD, CAT and GSH-Px were higher in the patients with EH treated with vitamins C + E compared with either the placebo-treated patients (Figure 3) or the patients with EH at baseline prior to treatment.

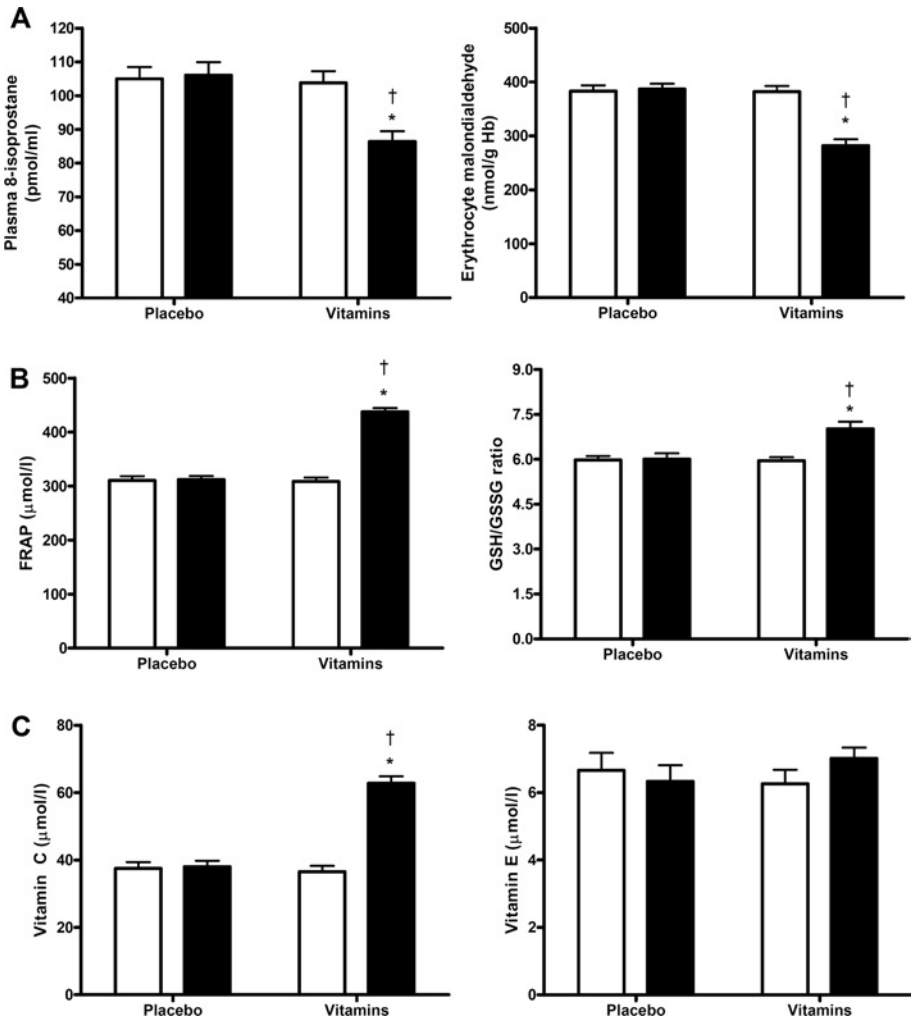

Figure 2 Lipid peroxidation (A) and antioxidant-capacity-related parameters (B and C) in patients with EH before (open bars) and after (closed bars) treatment with either vitamins C + E or placebo

Values are means ± S.E.M., $n = 55$. Lipid peroxidation was assessed by plasma 8-isoprostane and erythrocyte malondialdehyde levels (A). Antioxidant-capacity-related parameters were assessed by (B) the antioxidant capacity of plasma (FRAP) and the erythrocyte GSH/GSSG ratio, and (C) plasma vitamin C and E concentrations. Sources of variation were assessed by one-way ANOVA, followed by Bonferroni's multiple comparison test as the post-hoc test. $*P < 0.01$ compared with basal; $\dagger P < 0.01$ compared with placebo-treated patients wit EH.

Finally, a negative correlation between the total plasma antioxidant capacity (i.e. FRAP) and SBP and DBP was observed in both the placebo-treated and vitamins C + E-treated groups (Figures 4A and 4C respectively). Plasma lipid peroxidation levels correlated positively with SBP and DBP in placebo-treated and vitamins C + E-treated groups (Figures 4B and 4D respectively).

DISCUSSION

The present study provides evidence showing that oral administration of vitamins C + E to patients with mild-to-moderate EH results in a significant decrease in BP by enhancing their blood antioxidant status.

Previous studies have reported decreased [16], no change [38–40] or increased [41,42] BP after oral administration of antioxidant vitamins. The results of the present study show a decrease in BP following antioxidant vitamin administration (Figure 1), and is in agreement with others using a similar protocol design and vitamin treatment [16]. This controversy may be due to the fact that these studies had a lack of consideration of inclusion criteria for participant selection and the pharmacokinetic properties of antioxidant vitamins, difficulties in comparison due to various associations of antioxidants, the stage of the disease and incomplete information on the actual oxidative stress status, which should be considered as a necessary component of the clinical history of the patients enrolled [20]. Moreover, most previous

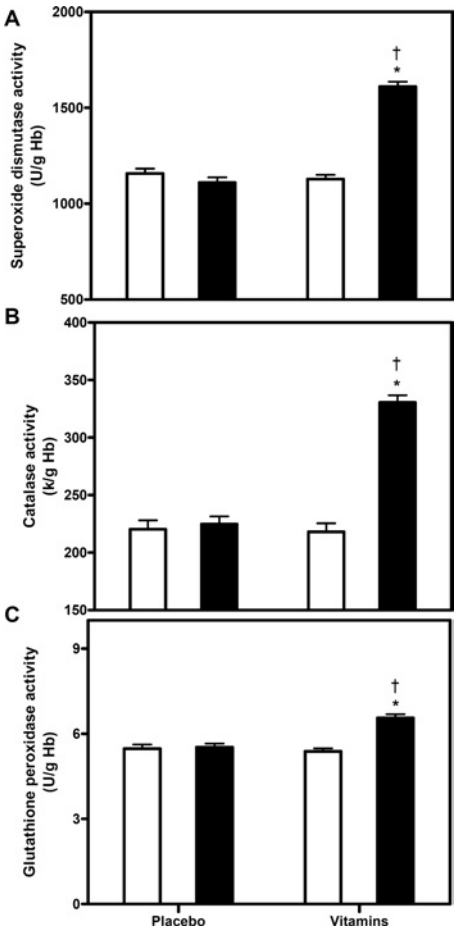

Figure 3 **Activity of erythrocyte SOD (A), CAT (B) and GSH-Px (C) in patients with EH before (open bars) and after (closed bars) treatment with either vitamins C + E or placebo**

Values are means ± S.E.M., $n = 55$. Sources of variation were assessed by one-way ANOVA, followed by Bonferroni's multiple comparison test as the post-hoc test. *$P < 0.001$ compared with basal; †$P < 0.001$ compared with placebo-treated patients with EH.

studies on the administration of antioxidant vitamins have focused on the prevention of cardiovascular events as the end point [43–46], and only a few have examined their potential antihypertensive effects [40]. In the present study, patient inclusion criteria were more rigorous and the confounding effects of other causes likely to interfere with oxidative-stress/antioxidant-status-related parameters were avoided.

In contrast, the lack of antihypertensive efficacy observed in studies using supplementation with vitamin C alone could be due to the pharmacokinetics of vitamin C and/or the decreased bioavailability of NO under conditions of oxidative stress. The antihypertensive effect of vitamin C is expected to occur at 10 mmol/l, a plasma concentration unobtainable in humans through oral administration [47]. However, this concentration is

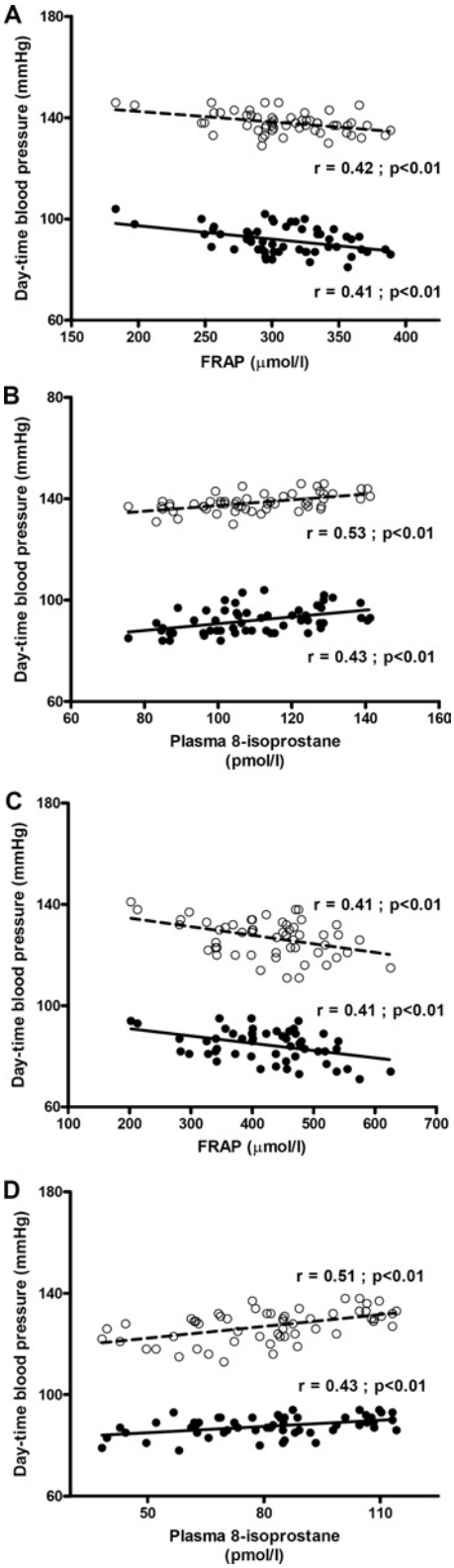

Figure 4 **Pearson correlation of SBP (○) and DBP (●) with plasma antioxidant capacity (FRAP) and plasma 8-isoprostane levels in patients with EH following treatment with either placebo (A and B) or vitamins C + E (C and D)**

required to compete efficiently with the reaction of NO with superoxide, due to their high reaction rate constant, which is even higher than the reaction between SOD and NO [48]. The lack of a therapeutic antihypertensive plasma vitamin C concentration via oral administration may be due to its renal threshold at doses between 60 and 100 mg/day. The steady-state concentration of vitamin C is attained at approx. 80 μmol/l, and plasma is completely saturated at daily doses of $\geqslant$400 mg [49]. Indeed, in our present study, plasma vitamin C concentrations did not reach values higher than 70 μmol/l (Figure 2C). Recent pharmacokinetic modelling indicates that, with oral administration, even at very large and frequent doses of vitamin C, plasma concentrations will only be increased modestly, from 70 μmol/l to a maximum of 220 μmol/l, whereas intravenous administration increases it as high as 14 mmol/l [50]. Thus the antihypertensive effect may only occur in plasma following infusion of high vitamin C doses. Intra-arterial administration of vitamin C has been shown to cause a decrease in BP in subjects with EH [51]. The dosage of vitamin E supplementation used in the present study was based on previous trials to assess its clinical effect on the risk of cardiovascular events. These studies demonstrated that doses higher than 400 IU/day may increase all-cause mortality and should be avoided [46]. Because of vitamin E distribution in various lipophilic environments, its plasma levels are not necessarily associated with its oral dosage [52].

The underlying molecular mechanisms of the antioxidant effects of vitamin C on BP modulation are as yet unclear. These effects have been shown to be mediated, in part, by the ability of vitamin C to protect BH4 (tetrahydrobiopterin), a cofactor necessary for the increase in the activity of eNOS and thus NO generation by oxidation [53]. It should be noted that, if eNOS becomes uncoupled, it results in a form now recognized as an important source of superoxide rather than NO [54].

The administration of vitamin E alone failed to exert an antihypertensive effect, but instead it caused an increase in BP when administered to individuals with Type 2 diabetes [41]. This may be associated with vitamin-E-induced oxidative stress and the oxidation of α-tocopherol to α-tocopheroxyl in a lipophilic environment, particularly in plasma membranes. Our present results have shown that there are no significant differences in plasma vitamin E levels between patients with EH treated with vitamins C + E compared with either placebo-treated patients with EH (Figure 2C) or patients with EH at baseline prior to treatment. This result could be explained by the distribution of the administered vitamin E, thus indicating that plasma vitamin E levels are a poor index of its status [52]. The abundance of PUFAs (polyunsaturated fatty acids) in biological membranes, which are particularly sensitive to damage by oxidative stress, account for the increase in lipid peroxidation, probably resulting from either ROS and/or α-tocopheroxyl. Vitamin C may reduce the α-tocopheroxyl radical, thereby abrogating lipid peroxidation [53]. The association of vitamins C and E used in the present study, as well as in others [16], has an antihypertensive effect, probably due to the fact that this combined therapy is expected to provide a reinforcement of their individual properties through a complementary effect in improving endothelial dysfunction [55]. Both vitamins C and E not only behave as scavengers of ROS, but also are able to induce the down-regulation of NADPH oxidase and the up-regulation of eNOS [56], thereby supporting further their antihypertensive effect.

The increased oxidative stress and decreased antioxidant capacity found in patients with EH at baseline compared with normotensive subjects is in agreement with previous studies [57,58]. However, Cracowski et al. [59] found no significant differences in urinary 8-isoprostane levels between normotensives and patients with never-treated mild-to-moderate EH, at least in the early stages of the disease. This controversy may be explained by the patient inclusion criteria, which did not consider the influence of factors such as physical activity, previous and/or current antioxidant supplementation, high consumption of fruits and vegetables and/or intake of non-antihypertensive (e.g. statins) medication, all potential confounders of oxidative stress status. The present study did consider these factors in order to obtain a rigorously selected sample of patients to specifically examine the effects of antioxidant vitamins on BP. The results of the present study have shown a significant decrease in SBP, DBP and MAP following supplementation with vitamins C + E in patients with EH compared with either placebo-treated patients with EH (Figure 1) or the patients with EH at baseline, which is in agreement with other studies [16,60]. The slight decrease in BP induced by antioxidant vitamin administration was expected due to the mild-to-moderate baseline BP levels included in the protocol. These changes are associated with a decrease in lipid peroxidation parameters, plasma 8-isoprostane and erythrocyte MDA (Figure 2A), but not with changes in plasma concentration of BP modulators, including renin, aldosterone, ET-1 and homocysteine (Table 2). In addition, an improvement in antioxidant status is shown by the increase in FRAP levels and the GSH/GSSG ratio (Figure 2B), and increased activity of antioxidant enzymes in erythrocytes (Figure 3). Moreover, the strong association of plasma 8-isoprostane and FRAP levels with SBP and DBP (Figure 4), together with the results mentioned above, is consistent with the involvement of oxidative stress in the mechanism of EH. To our knowledge, the present study is the first to report this association following antioxidant vitamin supplementation to patients with EH and is in agreement with previous studies in non-treated subjects with hypertension [58,61,62]. In contrast, a lack of an association between oxidative stress and BP was reported by Plantinga et al. [39], even though they used the same doses and time

of exposure to supplementation with vitamins C + E; however, these authors used a cross-over study design. In addition, these authors did not consider the possible influence of other BP modulators that may contribute to the regulation of BP, in contrast with the present study.

In support of the working hypothesis of the present study, it has been shown recently that antioxidant supplementation, including 10 mmol/l L-ascorbate, attenuated reflex cutaneous vasodilation in subjects with hypertension, but not in normotensive subjects [63]. Although that study did not assess the effects of supplementation with vitamins C + E on BP or oxidative stress in normotensive subjects, it should be noted that previous studies using similar doses of vitamin C and/or vitamin E have demonstrated that this supplementation did not reduce either BP [16,64] or oxidative stress [65] in healthy subjects.

In summary, the results reported in the present study show for the first time a specific association between oxidative-stress-related parameters and BP, thus suggesting the role of oxidative stress in the pathogenesis of EH. Moreover, the concomitant decrease in BP and oxidative stress raises the possibility that oxidative stress may be considered as a therapeutic target against the development of hypertension. Therefore oral administration of vitamins C + E in patients with EH is suggested as an adjunct therapy for hypertension.

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REFERENCES

1 Paravicini, T. M. and Touyz, R. M. (2006) Redox signaling in hypertension. Cardiovasc. Res. **71**, 247–258
2 Lassègue, B. and Griendling, K. (2004) Reactive oxygen species in hypertension. An update. Am. J. Hypertens. **17**, 852–860
3 Rodrigo, R., Passalacqua, W., Araya, J., Orellana, M. and Rivera, G. (2003) Implications of oxidative stress and homocysteine in the pathophysiology of essential hypertension. J. Cardiovasc. Pharmacol. **42**, 453–461
4 Yusuf, S., Hawken, S., Ounpuu, S. et al. (2004) Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART Study): case control study. Lancet **364**, 937–952
5 Becker, L. B. (2004) New concepts in reactive oxygen species and cardiovascular reperfusion physiology. Cardiovasc. Res. **61**, 461–470
6 Juránek, I. and Bezek, Š. (2005) Controversy of free radical hypothesis: reactive oxygen species: cause or consequence of tissue injury? Gen. Physiol. Biophys. **24**, 263–278
7 Pedro-Botet, J., Covas, M. I., Martin, S. and Rubies-Prat, J. (2000) Decreased endogenous antioxidant enzymatic status in essential hypertension. J. Hum. Hypertens. **14**, 343–345
8 Beswick, R. A., Dorrance, A. M., Leite, R. and Webb, R. C. (2001) NADH/NADPH oxidase and enhanced superoxide production in the mineralocorticoid hypertensive rat. Hypertension **38**, 1107–1111
9 Zicha, J., Dobesova, Z. and Kunes, J. (2001) Relative deficiency of nitric oxide-dependent vasodilation in salt-hypertensive Dahl rats: the possible role of superoxide anions. J. Hypertens. **19**, 247–254
10 Chen, X., Touyz, R. M., Bae Park, J. and Schiffrin, E. L. (2001) Antioxidant effects of vitamins C and E are associated with altered activation of vascular NADPH oxidase and superoxide dismutase in stroke-prone SHR. Hypertension **38**, 606–611
11 Duffy, S. J., Gokce, N., Holbrook, M. et al. (2001) Effect of ascorbic acid treatment on conduit vessel endothelial dysfunction in patients with hypertension. Am. J. Physiol. Heart Circ. Physiol. **280**, H528–H534
12 Boshtam, M., Rafiei, M., Sadeghi, K. and Sarraf-Zadegan, N. (2002) Vitamin E can reduce blood pressure in mild hypertensives. Int. J. Vitam. Nutr. Res. **72**, 309–314
13 Ulker, S., McKeown, P. P. and Bayraktutan, U. (2003) Vitamins reverse endothelial dysfunction through regulation of eNOS and NAD(P)H oxidase activities. Hypertension **41**, 534–539
14 Fotherby, M. D., Williams, J. C., Forster, L. A., Craner, P. and Ferm, G. A. (2000) Effect of vitamin C on ambulatory blood pressure and plasma lipids on older persons. J. Hypertens. **18**, 411–415
15 Mullan, B. A., Young, I. S., Fee, H. and McCance, D. R. (2002) Ascorbic acid reduces blood pressure and arterial stiffness in type 2 diabetes. Hypertension **40**, 797–803
16 Galley, H. F., Thornton, J., Howdle, P. D., Walker, B. E. and Webster, N. R. (1997) Combination oral antioxidant supplementation reduces blood pressure. Clin. Sci. **92**, 361–365
17 Ness, A. R., Chee, D. and Elliott, P. (1997) Vitamin C and blood pressure: an overview. J. Hum. Hypertens. **11**, 343–350
18 Ward, N. C and Croft, K. D. (2006) Hypertension and oxidative stress. Clin. Exp. Pharmacol. Physiol. **33**, 872–876
19 Touyz, R. M. (2004) Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? Hypertension **44**, 248–252
20 Rodrigo, R., Guichard, C. and Charles, R. (2007) Clinical pharmacology and therapeutic use of antioxidant vitamins. Fundam. Clin. Pharmacol. **21**, 111–127
21 Myers, M. G., Tobe, S. W., McKay, D. W. and Bolli, P. (2005) New algorithm for the diagnosis of hypertension. Am. J. Hypertens. **18**, 1369–1374
22 American Diabetes Association (2004) Diagnosis and classification of diabetes mellitus. Diabetes Care **27**, S5–S10
23 European Society of Hypertension-European Society of Cardiology Guidelines Committee (2003) European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension. J. Hypertens. **21**, 1011–1053
24 Baecke, J. A., Burema, J. and Frijters, J. E. (1982) A short questionnaire for the measurement of habitual physical activity in epidemiological studies. Am. J. Clin. Nutr. **36**, 936–942
25 O'Brien, E., Mee, F., Atkins, N. and O'Malley, K. (1991) Accuracy of the Spacelabs 90207 determined by the British Hypertension Society Protocol. J. Hypertens. **9**, 573–574
26 Groppelli, A., Omboni, S., Parati, G. and Mancia, G. (1992) Evaluation of noninvasive blood pressure monitoring devices Spacelabs 90202 and 90207 versus resting and ambulatory 24-hour intra-arterial blood pressure. Hypertension **20**, 227–232
27 Chobanian, A. V., Bakris, G. L., Black, H. R. et al. (2003) Seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure. Hypertension **289**, 2560–2572
28 Benzie, I. F. and Strain, J. J. (1996) The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. Anal. Biochem. **239**, 70–76

29 Hissin, P. J. and Hilf, R. (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. **74**, 214–226
30 Ohkawa, H., Ohishi, N. and Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. **95**, 351–358
31 Milne, G. L., Musiek, E. S. and Morrow, J. D. (2005) F_2-isoprostanes as markers of oxidative stress *in vivo*: an overview. Biomarkers **10**, (Suppl. 1), 10–23
32 Pradelles, P., Grassi, J. and Maclouf, J. (1985) Enzyme immunoassays of eicosanoids using AchE as label: an alternative to radioimmunoassay. Anal. Chem. **57**, 1170–1173
33 Chung, W. Y., Chung, J. K., Szeto, Y. T., Tomlinson, B. and Benzie, I. F. (2001) Plasma ascorbic acid: measurement, stability and clinical utility revisited. Clin. Biochem. **34**, 623–627
34 Morena, M., Cristol, J. P., Bosc, J. Y. et al. (2002) Convective and diffusive losses of vitamin C during haemodiafiltration session: a contributive factor to oxidative stress in haemodialysis patients. Nephrol. Dial. Transplant. **17**, 422–427
35 Nebot, C., Moutet, M., Huet, P., Xu, J. Z., Yadan, J. C. and Chaudiere, J. (1993) Spectrophotometric assay of superoxide dismutase activity based on the activated autoxidation of a tetracyclic catechol. Anal. Biochem. **14**, 442–451
36 Aebi, H. (1974) Catalase, Methods in Enzymatic Analysis (Bergmeyer, H.U, ed.), pp. 673–677, Academic Press, New York
37 Flohé, L. and Günzler, W. A. (1984) Assays of glutathione peroxidase. Methods Enzymol. **105**, 114–121
38 Kim, M. K., Sasaki, S., Sasazuki, S., Okubo, S., Hayashi, M. and Tsugane, S. (2002) Lack of long-term effect of vitamin C supplementation on blood pressure. Hypertension **40**, 797–803
39 Plantinga, Y., Ghiadoni, L., Magagna, A. et al. (2007) Supplementation with vitamins C and E improves arterial stiffness and endothelial function in essential hypertensive patients. Am. J. Hypertens. **20**, 392–397
40 Hercberg, S., Galan, P., Preziosi, P. et al. (2004) A randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. Arch. Intern. Med. **164**, 2335–2342
41 Ward, N. C., Wu, J. H., Clarke, M. W. et al. (2007) The effect of vitamin E on blood pressure in individuals with type 2 diabetes: a randomized, double-blind, placebo-controlled trial. J. Hypertens. **25**, 227–234
42 Ward, N. C., Hodgson, J. M., Croft, K. D., Burke, V., Beilin, L. J. and Puddey, I. B. (2005) The combination of vitamin C and grape-seed polyphenols increases blood pressure: a randomized, double-blind, placebo-controlled trial. J. Hypertens. **23**, 427–434
43 The Heart Outcomes Prevention Evaluation Study Investigators (2005) Vitamin E supplementation and cardiovascular events in high-risk patients. N. Engl. J. Med. **342**, 154–160
44 The HOPE, HOPE-TOO Trial Investigators (2005) Effects of long-term vitamin E supplementation on cardiovascular events and cancer. A randomized controlled trial. JAMA, J. Am. Med. Assoc. **293**, 1338–1347
45 Salonen, R. M., Nyyssonen, K., Kaikkonen, J. et al. (2003) Six-year effect of combined vitamin C and E supplementation on atherosclerotic progression: the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) Study. Circulation **107**, 947–953
46 Miller, III, E. R., Poster-Barriuso, R., Dalal, D., Reimersma, R. A. and Appel, L. J. (2005) Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. Ann. Intern. Med. **142**, 1–11
47 Jackson, T. S., Xu, A., Vita, J. A. and Keaney, Jr, J. F. (1998) Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. Circ. Res. **83**, 916–922
48 Pryor, W. A. and Squadrito, G. L. (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am. J. Physiol. **268**, L699–L722
49 Padayatty, S. J., Katz, A., Wang, Y. et al. (2003) Vitamin C as an antioxidant: evaluation of its role in disease prevention. J. Am. Coll. Nutr. **22**, 18–35
50 Padayatty, S. J., Sun, H., Wang, Y. et al. (2004) Vitamin C pharmacokinetics: implications for oral and intravenous use. Ann. Intern. Med. **140**, 533–537
51 Schneider, M. P., Delles, C., Schmidt, B. M. W. et al. (2005) Superoxide scavenging effects of *N*-acetylcysteine and vitamin C in subjects with essential hypertension. Am. J. Hypertens. **18**, 1111–1117
52 Rodrigo, R., Parra, M., Bosco, C. et al. (2005) Pathophysiological basis for the prophylaxis of preeclampsia through early supplementation with antioxidant vitamins. Pharmacol. Ther. **107**, 177–197
53 Heller, R., Werner-Felmayer, G. and Werner, E. R. (2006) Antioxidants and endothelial nitric oxide synthesis. Eur. J. Clin. Pharmacol. **62**, (Suppl. 13), 21–28
54 Förstermann, U. and Münzel, T. (2006) Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation **113**, 1708- 1714
55 Bilodeau, J. F. and Hubel, C. A. (2003) Current concepts in the use of antioxidants for the treatment of preeclampsia. J. Obstet. Gynaecol. Can. **25**, 742–750
56 Attia, D. M., Verhagen, A. M., Stroes, E. S. et al. (2001) Vitamin E alleviates renal injury, but not hypertension, during chronic nitric oxide synthase inhibition in rats. J. Am. Soc. Nephrol. **12**, 2585–2593
57 Russo, C., Olivieri, O., Girelli, D. et al. (1998) Anti-oxidant status and lipid peroxidation in patients with essential hypertension. J. Hypertens. **16**, 1267–1271
58 Rodrigo, R., Bachler, J. P., Araya, J., Prat, H. and Passalacqua, W. (2007) Relationship between (Na + K)-ATPase activity, lipid peroxidation and fatty acid profile in erythrocytes of hypertensive and normotensive subjects. Mol. Cell. Biochem. **303**, 73–81
59 Cracowski, J. L., Baguet, J. P., Ormezzano, O. et al. (2003) Lipid peroxidation is not increased in patients with untreated mild-to-moderate hypertension. Hypertension **41**, 286–288
60 Duffy, S. J., Gokce, N., Holbrook, M., Huang, A., Frei, B. and Keany, J. F. (1999) Treatment of hypertension with ascorbic acid. Lancet **354**, 2048–2049
61 Simic, D. V., Mimic-Oka, J., Pljesa-Ercegovac, M. et al. (2006) Byproducts of oxidative protein damage and antioxidant enzyme activities in plasma of patients with different degrees of essential hypertension. J. Hum. Hypertens. **20**, 149–155
62 Laffer, C. L., Bolterman, R. J., Romero, J. C. and Elijovich, F. (2006) Effect of salt on isoprostanes in salt-sensitive essential hypertension. Hypertension **47**, 434–440
63 Holowatz, L. A. and Kenney, W. L. (2007) Local ascorbate administration augments NO- and non-NO-dependent reflex cutaneous vasodilation in hypertensive humans. Am. J. Physiol. Heart Circ. Physiol. **293**, H1090–H1096
64 Sato, K., Dohi, Y., Kojima, M. et al. (2006) Effects of ascorbic acid on ambulatory blood pressure in elderly patients with refractory hypertension. Arzneimittelforschung **56**, 535–540
65 Meagher, E. A., Barry, O. P., Lawson, J. A., Rokach, J. and FitzGerald, G. A. (2001) Effects of vitamin E on lipid peroxidation in healthy persons. JAMA, J. Am. Med. Assoc. **285**, 1178–1182

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