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ORIGINAL ARTICLE

# Folic acid restores endothelial function in ACTH-induced hypertension

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**KEYWORDS**

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Folic acid;
Antioxidant

**Abstract** Hypertension is associated with increased oxidative stress and vascular endothelium dysfunction. The aim was to study the effect of folic acid (FA) on hypertension, blood nitric oxide (NO), homocysteine (HCY), malondialdehyde (MDA) and reduced glutathione (GSH); aortic tissue glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD); and vascular endothelial function in adrenocorticotrophic hormone (ACTH)-induced hypertension rats. Rats were treated with saline or FA alone (0.04 g/L/day orally, control), or subcutaneous ACTH-induced hypertension (0.2 mg/kg/day, ACTH) groups. Treated FA groups were started before (Folic–ACTH, prevention) and during (ACTH–Folic, reversal) ACTH administrations. Systolic blood pressure (SBP), thymus/-body weight ratio, blood urea, creatinine, NO, HCY, MDA and GSH; aortic endothelium-dependent vasodilator (EDD) in response to acetylcholine (ACh), aortic tissue extract for CAT, GPx, and SOD activity; and histopathological changes of aorta and kidney were assessed. Saline or FA alone did not change SBP ($P > 0.05$). FA, in prevention study, significantly decreased SBP, increased serum NO and GSH, enhanced relaxation response (EDD%) to $1 \times 10^{-4}$ M ACh; increased aortic tissue GPx, CAT and SOD activity, also revealed nearly normal endothelial cell layer and moderately positive cytoplasmic staining for $CD34^{+}$ expression versus ACTH-treated rats ($P < 0.05$). In contrast, FA, in reversal study, did not show significant changes in most of the measured parameters as ACTH-treated group ($P > 0.05$). FA can be used as an adjuvant therapy for prevention and treatment of ACTH-induced hypertension. The protective role of FA in ACTH-induced hypertension could be attributed *via* decreasing HCY, MDA (oxidative stress); increasing NO, GSH, GPx, CAT, SOD activity (antioxidants); and restoring endothelial dysfunction.
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## 1. Introduction

Hypertension is one common disease and can lead to many complications such as stroke, ischemic heart diseases, heart

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failure, renal insufficiency and dissecting aortic aneurysm.[1] One common type of hypertension appeared as a side effect for long term use of glucocorticoids in many chronic diseases such as rheumatoid arthritis, systemic lupus erythematosus, bronchial asthma, malignancies, and organ transplant recipients.[2] Adrenocortical steroids such as adrenocorticotrophic hormone (ACTH) or dexamethasone (Dex), both naturally occurring and synthetic, have long been recognized as a cause of hypertension, and expressed in terms of ACTH- or Dex-induced hypertension.[3,4]

ACTH-induced hypertension was associated with reduced plasma reactive nitrogen intermediate (NOx) concentration.[5] L-Arginine was the precursor of nitric oxide (NO, a potent vasodilator) produced by nitric oxide synthase (NOS). L-Arginine prevented and partially reversed ACTH-induced hypertension,[5] indicating that L-arginine is likely to be working through NO generation. Long treatment with BH4 can reduce the development of hypertension, but not in ACTH-induced hypertension.[6] Endothelial NOS required the cofactor tetrahydrobiopterin (BH4),[7] which participated in the stabilization of the eNOS dimer, and was abolished by the NOS inhibitor.[5] Reactive oxidant species (ROS) is one of the biochemical changes in hypertension that leads to structural alteration in the vascular wall by promoting vascular smooth muscle hypertrophy and impairment in endothelial dependent vasodilatation (endothelial dysfunction).[8] Many forms of hypertension are associated with oxidative stress and vascular endothelial dysfunction with NO deficiency.[2] It has been recently proposed that the NO/ROS balance permanently affects blood pressure control. When this shift is toward more ROS and less NO, adult blood pressure is increased.

Lipid peroxidation (mainly malondialdehyde, MDA) was one of the best known biochemical consequences of oxidative cell injury (ROS).[7] Elevated homocysteine (HCY) (hyperhomocysteinemia, HHCY) has been associated with cardiovascular diseases such as hypertension, coronary heart disease and diabetes[9] and stroke.[10] Patients with active Cushing's syndrome have accompanied higher serum HCY levels and lower folate levels than control.[11] Therefore, folic acid (FA) was postulated to have cardiovascular protective effects.[12]

The role of FA administration in hypertension provided some benefits in reducing the cardiovascular risk and maintaining the flexibility of arteries.[13] In contrast, other studies showed FA has no clinical benefit in reducing HCY,[14] and also, FA in combination with vitamin $B_{12}$ may even increase some cardiovascular risks.[15] The exact role of FA in hypertension remains unclear and is still an issue of debate.

The aim was to study the effect of FA (prevention and reversal effect) on hypertension, serum of NO, HCY, MDA and reduced glutathione (GSH); aortic tissue glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD) levels; and vascular endothelial function in adrenocorticotrophic hormone (ACTH)-induced hypertension in rats.

## 2. Materials and methods

### 2.1. Animals

Male albino rats weighing 150–200 gm were used and had acclimatized for one week prior to the experiment, animals were housed in plastic cage, free access to water and semisynthetic balanced diet under controlled temperature (21–23 °C) and lighting (12 h light/dark cycles). This study was approved by the Animal Experimentation Ethics Committee of Egyptian National University.

### 2.2. Treatments

Induction of hypertension in rats was done by injection subcutaneously ACTH (0.2 mg/kg/day, Synacthen Depot, Novartis Pharmaceuticals) diluted in sterile saline (0.9% NaCl, El Nasr, Egypt).[3] Treatment of hypertension with FA (0.04 g/L in drinking water, Sigma), this is equal to 20 mg/kg/day or 5 mg/-day/rat according to previous protocols.[3,4] Fifty rats were divided into 5 groups each of 10 rats:

- Group I (Saline group, Control): Rats received sterile saline, vehicle for ACTH (1 ml/kg/day, subcutaneously (S.C) injection) for 13 days (treatment day T0–T12) and tap water to drink throughout the study.
- Group II (FA group, Control): Rats were treated with FA (0.04 g/L in drinking water)[3] and saline (1 ml/kg/day, S.C.) for 13 days (T0–T12) and tap water to drink throughout the study.
- Group III (ACTH-hypertension group): Rats were treated with ACTH (0.2 mg/kg/day, S.C.) for 13 days from T0 – T12, and saline was given daily and orally 4 days before (pretreatment day P0 –P3) followed by saline injection cotreatment from T0–T12.
- Group IV (FA + ACTH group, Prevention study): Rats were treated with ACTH (0.2 mg/kg/day, S.C.) from T0 – T12. FA was given daily (0.04 g/L in drinking water) 4 days before (P0–P3) followed by ACTH cotreatment from T0–T10.
- Group V (ACTH + FA group, Reversal study): Rats were treated with ACTH (0.2 mg/kg/day, S.C.) for 13 days (T0–T12), tap water to drink until T7, and FA was given daily (0.04 g/L in drinking water) from day 8 to day 12 (T8 – T12).

### 2.3. Systolic blood pressure (SBP) measurements

SBP was monitored using rat-tail sphygmomanometer and pneumatic transducer (Harvard, UK). Briefly, rats should be placed in the restrainers on a heated plate (40 °C) for 15–30 min prior to taking reading. The pneumatic cuff fits over the rat's tail, then inflated to occlude the pulse and allowed to deflate slowly until the pulse pressure is observed on the pulse channel of the recorder. A 4-channel recorder (Harvard, UK) is used to obtain a written record of both blood flow and cuff pressure. The pulse sign should be monitored to see when the pulse signal begins to become detectable and reach the maximum pulse height. The start of pulsation was viewed on the tracing and referenced to the pressure curve signal at that point this reading is analogous to SBP. Rats were trained for 5 consecutive days (each cession consisting of 10 unrecorded measurements) to familiarize the animal with rat tail cuff. Rats of systolic blood pressure of 140 mmHg or more were considered hypertensive.

*2.4. Thymus/body weight ratio*

Body weight of rats was measured in pretreatment and also at the end of treatment. Estimation of thymus/body weight ratio: At the end of the experiment, the rats were killed by cervical decapitation, midline incision from the xiphisternum to the neck is made, thymus is extracted and weighted to estimate thymus/body weight ratio as a marker of glucocorticoid activity. OHAUS electronic scale was used for estimation of rat weight and thymus weight. Data were expressed as mg/100 g body weight.

*2.5. Aortic endothelial function assay*

Assessment of endothelial dependent dilation (EDD%): At the end of the experiment, the thorax of the rat was opened and the thoracic aorta was cut through as near the heart as possible and dissected free as far as the diaphragm. The aorta was cleaned of fat and connective tissue, and cut into rings (3–4 mm in length). Aortic rings were mounted in 10 ml tissue baths filled with Krebs–Henseleit buffered solution (KHS) pH 7.4 (El-Nasr company, Egypt). The composition of KHS was (in mM) 120 NaCl, 4.8 KCl, 1.25, $CaCl_2$, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 25.0 $NaHCO_3$, and 11.0 glucose. The medium was maintained at 37 °C, pH 7.4, and exposed continuously with 95% $O_2$ and 5% $CO_2$. The preparation was connected to a strain gauge isometric tension transducer that was connected to the pin chart recorder of the physiographic MK (Bio-system U.S.A). After 90 min of stabilization under an optimal resting tension of 1 g, ring segments were contracted with KCl (30 mM) to test their viability and thereafter rinsed three times with KHS. After resting tension stabilization, $1 \times 10^{-6}$ M phenylephrine was injected to induce a rapid increase in vascular tone which was followed by a stable vasocontraction. The aortic rings were then allowed to equilibrate for another 30 min and then, vasorelaxation responses to different concentrations of acetylcholine (ACh $1 \times 10^{-8}$ M–$1 \times 10^{-4}$ M; El-Gomhoria company, Egypt) were recorded to determine the endothelium-dependant vasorelaxation in precontracted phenylephrine preparation. Vasorelaxation responses to different concentrations of sodium nitroprusside (SNP $1 \times 10^{-8}$ M1 $\times 10^{-4}$ M; SD Fine Chemicals, Ltd., India) were also recorded to determine endothelium-independent vasorelaxation in pre-contracted phenylephrine preparation. At the end of the experiments, each segment was maximally relaxed by $1 \times 10^{-4}$ M papaverine hydrochloride to confirm the sufficient relaxant ability of the preparation. Cumulative relaxation data were expressed as a percentage of phenylephrine-induced contraction. Otherwise, successful endothelial damage was confirmed by the absence of relaxation to acetylcholine.[16,17]

*2.6. Laboratory analysis*

Blood samples were collected from retro-orbital veins after last 24 h post-treatment. The supernatant serum was collected in a dry tube for further investigation. Blood urea and serum creatinine, serum nitric oxide (NO) (measured as nitrites and nitrates μmol/l, R&D systems Europe, Ltd., United Kingdom), serum malondialdehyde (MDA, as an indicator of lipid peroxidation), serum reduced glutathione (GSH) were measured using spectrophotometer (Shimadzu/Double beam spectrophotometer U.V 150, Germany). Serum homocysteine (HCY) was measured by an enzymatic immunoassay (R&D, UK).

Aortic tissue (0.1 gm) was perfused with phosphate buffered saline solution, pH 7.4 containing 0.16 mg/ml heparin to remove any red blood cells and clots. Then, the tissue was homogenized in 10 ml/mg tissue ice-cold 50 mM potassium phosphate buffer, pH 7.4 containing 1 mM EDTA and 1 ml/L tritonX-100 using polytron homogenizer (PT 3100). The homogenate was centrifuged at 4000 rpm at 4 °C for 15 min. The aortic tissue catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity were assessed by spectrophotometer as above.

*2.7. Histopathological analysis*

Kidney and aortic samples were washed with saline and then fixed in buffered formalin (10%) for 24 h, then embedded in paraffin wax and serially sectioned 3–5 μm for staining with hematoxylin and eosin (H&E). Aortic sections were immunostained for $CD34^+$ (mouse monoclonal antibody specific for $CD34^+$) assay based on the immunoperoxidase technique using Lab Vision Kit.

*2.8. Statistical analysis*

The data collected were tabulated and analyzed by SPSS statistical package version 11 on IBM compatible computer. Quantitative data were expressed as mean and standard error (mean ± SE) and analyzed by applying student *t*-test for comparison of two groups of normally distributed variables and Mann Whitney *U* test for not normally distributed ones. All these tests were used as tests of significance at $P < 0.05$.

## 3. Results

*3.1. Effect of FA on body weight in ACTH-induced hypertension rats*

Change in body weight after treatment was increased in saline control group (241 ± 5 g T0–298 ± 2 g T12), FA control group (245 ± 3–289 ± 6 g), FA + ACTH-prevention study group (243 ± 4–248 ± 2 g) and ACTH + FA reversal study group (238 ± 5–241 ± 6 g), but not in ACTH-induced hypertension group (242 ± 2–237 ± 2 g). Body weight increase was lower in ACTH-treated rats compared with saline- or FA-treated rats ($P < 0.05$). FA had no significant effect on body weight in ACTH-treated rats (FA prevention or reversal study) ($P > 0.05$).

*3.2. Effect of FA on SBP in ACTH-induced hypertension rats*

There was no difference in SBP between FA and saline-treated rats (120.16 ± 1.47 *vs* 108 ± 2.36 mmHg; $P > 0.05$). SBP was significantly higher in ACTH-treated rats compared with saline-treated rats (161.1 ± 5.01 *vs* 108 ± 2.36 mmHg, $P < 0.05$). SBP was significantly lower in FA prevention study (FA + ACTH group, 117.50 ± 1.87 mmHg) and also, in FA

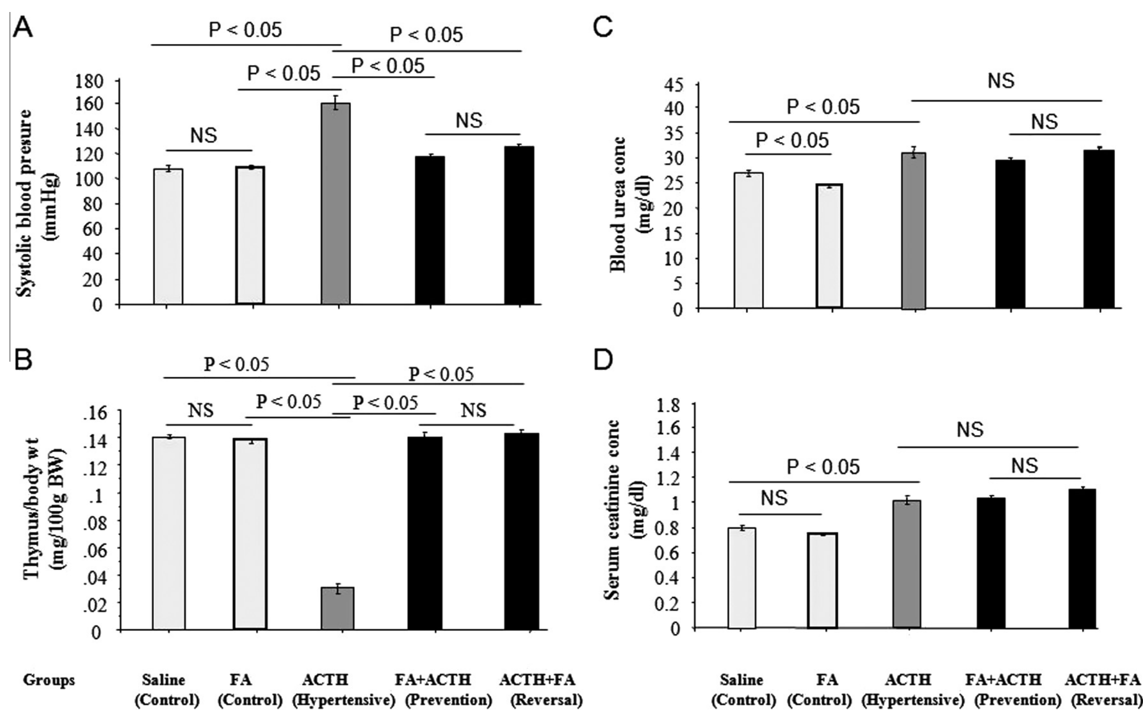


**Figure 1** Effect of folic acid (FA) on systolic blood pressure (SBP) (A), thymus weight/body weight (glucocorticoid activity) (B), blood urea (C) and serum creatinine in various groups. Rats were treated with saline (1.0 ml/kg/day, S.C.) (Saline, control groups), FA (0.04 g/l, P.O.) (FA, control groups), ACTH (0.2 mg/kg/day, S.C) (ACTH-induced hypertension groups), FA 5 days before ACTH (FA + ACTH) (FA prevention study groups), and ACTH followed by FA for the last 5 days of study duration (ACTH + FA) (FA reversal study groups). Data are shown as mean ± SE ($n = 10$), NS = $P$ value > 0.05 was not significant, $P < 0.05$ was significant. P.O. = per orally, S.C. = subcutaneously injection.

reversal study (ACTH + FA group, 120.83 ± 1.83 mmHg) compared with ACTH-treated rats ($P < 0.05$) (Fig. 1A).

### *3.3. Effect of FA on thymus weight/body weight ratio in ACTH-induced hypertension rats*

There was no difference in thymus weight/body weight ratio between FA and saline treated rats (0.138 ± 0.00 *vs* 0.140 ± 0.004 mg/100 g BW; $P > 0.05$). Thymus weight/body weight ratio was significantly lower in ACTH-treated rats compared with saline-treated rats (0.031 ± 0.005 *vs* 0.140 ± 0.004 mg/100 g BW, $P < 0.05$). Thymus weight/body weight ratio was significantly higher in FA prevention study (FA + ACTH group, 0.140 ± 0.003 mg/100 g BW) and also, in FA reversal study (ACTH + FA group, 0.143 ± 0.005 mg/100 g BW) compared with ACTH-treated rats ($P < 0.05$) (Fig. 1B).

### *3.4. Effect of FA on kidney function test in ACTH-induced hypertension rats*

Treatment of rats with FA resulted in a significant decrease in serum urea concentration ($p = 0.043$) as compared to saline-treated rats. Treatment of rats with ACTH resulted in a significant increase in serum urea concentration ($P = 0.033$) as compared to saline-treated rats. Urea concentrations were non-significantly changed in FA prevention study (FA + ACTH group) and FA reversal study (ACTH + FA group) compared with ACTH-treated rats ($P > 0.05$) (Fig. 1C).

Treatment of rats with FA resulted in a non-significant increase in serum creatinine concentration ($P = 0.043$) as compared to saline-treated rats. Treatment of rats with ACTH resulted in a non-significant decrease in serum creatinine concentration ($P > 0.05$) as compared to saline-treated rats. Serum creatinine concentrations were non-significantly higher in FA prevention study (FA + ACTH group) and in FA reversal study (ACTH + FA group) compared with ACTH-treated rats ($P > 0.05$) (Fig. 1D).

### *3.5. Effect of FA on serum NO in ACTH-induced hypertension rats*

There was no difference in NO concentrations between FA and saline-treated rats (12.51 ± 0.77 *vs* 13.11 ± 0.76 µmol/L; $P > 0.05$). NO concentrations were significantly lower in ACTH-treated rats compared with saline-treated rats (7.28 ± 0.89 *vs* 13.11 ± 0.76 µmol/L; $P < 0.05$). NO concentrations were significantly higher in FA prevention study (FA + ACTH group, 12.66 ± 0.41 µmol/L) and also, in FA reversal study (ACTH + FA group, 12.56 ± 0.40 µmol/L) compared with ACTH-treated rats ($P < 0.05$) (Fig. 2A).

### *3.6. Effect of FA on serum HCY in ACTH-induced hypertension rats*

There was no difference in HCY concentrations between FA and saline-treated rats (13.73 ± 0.25 *vs* 13.80 ± 0.08 µmol/L; $P > 0.05$). HCY concentrations were significantly higher in

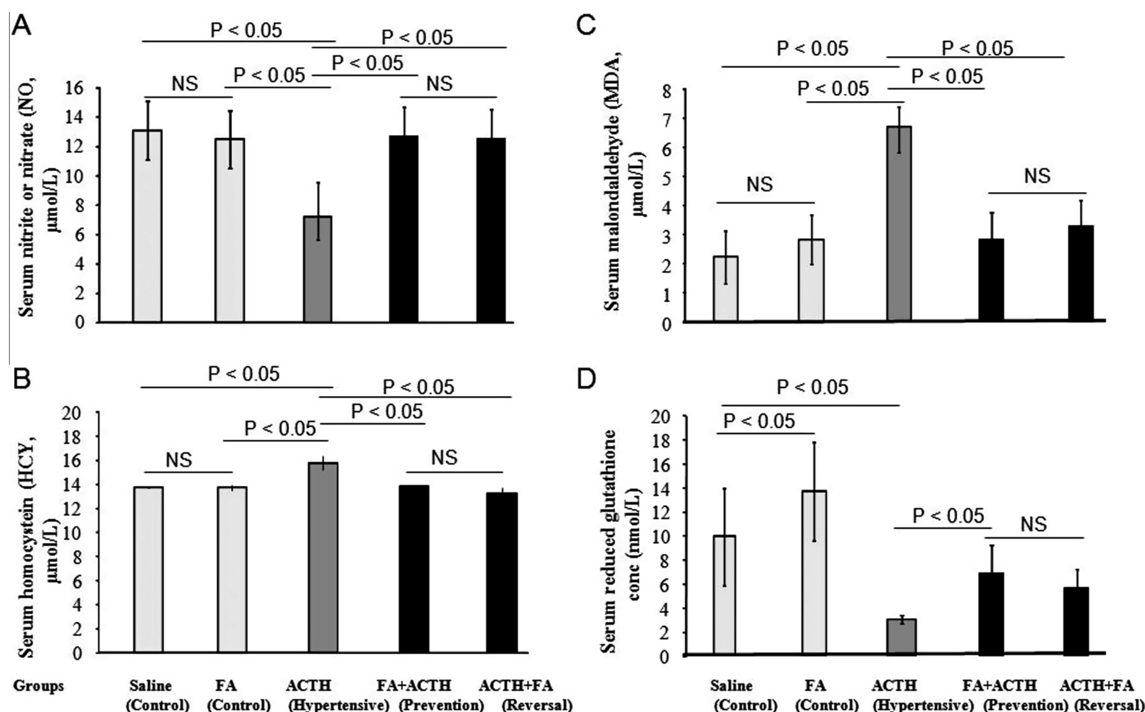


**Figure 2** Effect of folic acid (FA) on serum nitric oxide (NO) (A), homocysteine (HCY) (B), malondialdehyde (MDA) (C) and reduced glutathione (GSH) (D) level in various groups. Rats were treated with saline (1.0 ml/kg/day, S.C.) (Saline, control groups), FA (0.04 g/l, P.O.) (FA, control groups), ACTH (0.2 mg/kg/day, S.C) (ACTH-induced hypertension groups), FA 5 days before ACTH (FA + ACTH) (FA prevention study groups), and ACTH followed by FA for the last 5 days of study duration (ACTH + FA) (FA reversal study groups). Data are shown as mean ± SE) ($n = 10$), NS = $P$ value > 0.05 was not significant, $P < 0.05$ was significant. P.O. = per orally, S.C. = Subcutaneously injection.

ACTH-treated rats compared with saline-treated rats (15.73 ± 0.69 *vs* 13.80 ± 0.08 µmol/L; $P < 0.05$). HCY concentrations were significantly lower in FA prevention study (FA + ACTH group, 13.85 ± 0.10 µmol/L) and also, in FA reversal study (ACTH + FA group, 13.23 ± 0.53 µmol/L) compared with ACTH-treated rats ($P < 0.05$) (Fig. 2B).

### 3.7. Effect of FA on serum MDA in ACTH-induced hypertension rats

There was no difference in MDA concentrations between FA acid and saline-treated rats (2.83 ± 0.85 *vs* 2.21 ± 0.92 µmol/L; $P > 0.05$). MDA concentrations were significantly higher in ACTH-treated rats compared with saline-treated rats (6.48 ± 0.99 *vs* 2.21 ± 0.92 µmol/L; $P < 0.05$). MDA concentrations were significantly lower in FA prevention study (FA + ACTH group, 2.81 ± 0.96 µmol/L) and also, in FA reversal study (ACTH + FA group, 3.28 ± 0.90 µmol/L) compared with ACTH-treated rats ($P < 0.05$) (Fig. 2C).

### 3.8. Effect of FA on serum GSH in ACTH-induced hypertension rats

Treatment of rats with FA resulted in a significant increase in serum GSH concentration as compared to saline-treated rats ($P = 0.003$). GSH concentrations were significantly reduced in ACTH-treated rats compared with saline-treated rats ($P = 0.001$). GSH concentrations were significantly higher in FA prevention study (FA + ACTH group, $p = 0.0001$) but non-significantly higher in FA reversal study (ACTH + FA group) compared with ACTH-treated rats ($P > 0.05$) (Fig. 2D).

### 3.9. Effect of FA on EDD% in ACTH-induced hypertension rats

There was no difference in endothelium-dependent vasorelaxation (EDD) in aortic rings produced by acetylcholine (ACh, $1 \times 10^{-4}$ M) between FA and saline-treated rats (89 ± 11 *vs* 90 ± 1%; $P > 0.05$). Aortic ring relaxation produced by ACh was significantly lower in ACTH-treated rats compared with saline-treated rats (43 ± 5 *vs* 90 ± 1%; $P < 0.05$). Aortic ring relaxation produced by ACh was significantly higher in FA prevention study (FA + ACTH group, 70 ± 5%) and also, in FA reversal study (ACTH + FA group, 75 ± 5%) compared with ACTH-treated rats ($P < 0.05$) (Fig. 3A). However, there was no significant difference in endothelium-independent vasorelaxation in aortic rings produced by SNP ($1 \times 10^{-4}$ M) among all groups were 92%, 93%, 87%, 89%, 84% for saline group, FA group, ACTH group, FA + ACTH group (prevention study), ACTH + FA group (reversal study), respectively (Fig. 3B).

### 3.10. Effect of FA on antioxidant activity of aortic tissue in ACTH-induced hypertension rats

Treatment of rats with FA resulted in a non-significant increase in aortic tissue CAT activity as compared to saline-

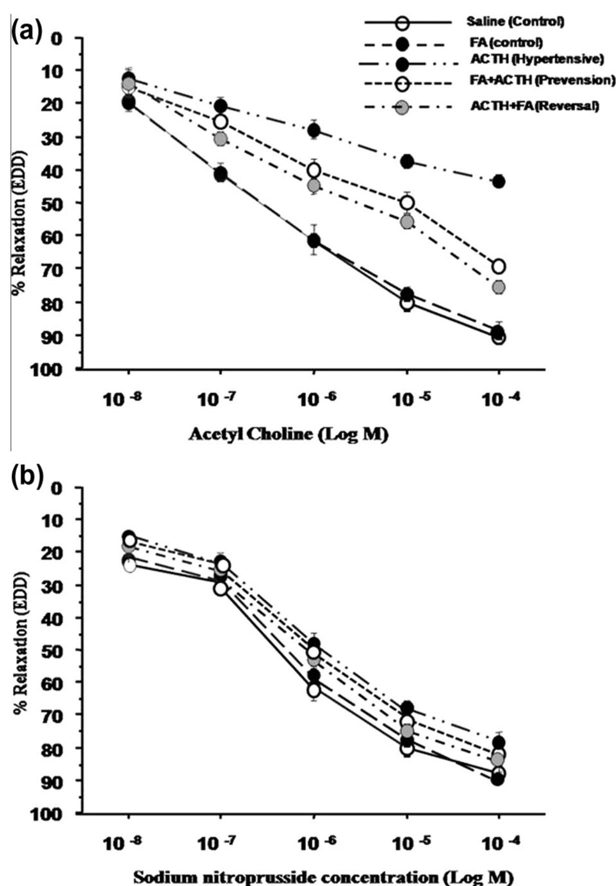


**Figure 3** Effect of folic acid (FA) on aortic rings vasorelaxation in various groups. Vasorelaxation to acetylcholine (ACh, endothelium-dependent vasorelaxation) (A) and sodium nitroprusside (SNP, endothelium-dependent vasorelaxation) (B) in phenylephrine-precontracted aortic segments isolated from rats that treated with saline (1.0 ml/kg/day, S.C.) (Saline, control groups), FA (0.04 g/l, P.O.) (FA, control groups), ACTH (0.2 mg/kg/day, S.C) (ACTH-induced hypertension groups), FA 5 days before ACTH (FA + ACTH) (FA prevention study groups), and ACTH followed by FA for the last 5 days of study duration (ACTH + FA) (FA reversal study groups). Data are shown as mean ± SE ($n$ = 10), NS = $P$ value > 0.05 was not significant, $P$ < 0.05 was significant. P.O. = per orally, S.C. = subcutaneously injection.

treated rats ($P > 0.05$). Aortic tissue CAT activity was significantly reduced in ACTH-treated rats compared with saline-treated rats ($P = 0.003$). Aortic tissue CAT activity was significantly higher in FA prevention study (FA + ACTH group, $p = 0.005$) but non-significantly lower in FA reversal study (ACTH + FA group) compared with ACTH-treated rats ($P > 0.05$) (Fig. 4A).

There was no difference in aortic tissue SOD activity between FA- and saline-treated rats ($P > 0.05$). Aortic tissue SOD activity was significantly reduced in ACTH-treated rats compared with saline-treated rats ($P = 0.004$). Aortic tissue SOD activity was significantly higher in FA prevention study (FA + ACTH group, $p = 0.0002$) but non-significantly lower in FA reversal study (ACTH + FA group) compared with ACTH-treated rats ($P > 0.05$) (Fig. 4B).

Treatment of rats with FA resulted in a non-significant increase in aortic tissue GPx activity as compared to saline-treated rats ($P > 0.05$). Aortic tissue GPx activity was significantly reduced in ACTH-treated rats compared with saline-treated rats ($P = 0.003$). Aortic tissue GPx activity was significantly higher in FA prevention study (FA + ACTH group, $p = 0.005$) but non-significantly lower in FA reversal study (ACTH + FA group) compared with ACTH-treated rats ($P > 0.05$) (Fig. 4C).

### *3.11. Effect of FA on histopathology of kidney in ACTH-induced hypertension rats*

Kidney sections of saline-treated rats revealed normal kidney architecture, normal glomeruli with mild widening in Bowman's space, normal tubules, and normal blood vessels in all studied animals (Fig. 5A). In FA-treated rats, kidney sections revealed apparently normal kidney with mild widening in Bowman's space, and mild degenerative changes in proximal tubules (cloudy swelling) in all studied animals (Fig. 5B and B1). In ACTH-treated rats, two animals out of the eight studied animals showed focal interstitial hemorrhage with mild tubular degeneration (cloudy swelling). Five animals revealed moderate widening in Bowman's space, slightly hypercellular glomerular tuft of capillaries with hyaline casts associated with tubular degeneration, and increased mononuclear cellular infiltration (Fig. 5C). Three animals showed thickened prominent arteriolar walls associated with severe congestion (Fig. 5 C1). In FA prevention study (FA + ACTH), kidney sections of rats, revealed mild tubular degeneration (cloudy swelling) (Fig. 5D), but on the other hand in FA reversal study (ACTH + FA), revealed widening of Bowman's space and tubular degeneration with focal cellular tubular casting in all studied animals (Fig. 5E).

### *3.12. Effect of FA on histopathology of aortic endothelium in ACTH-induced hypertension rats*

Aortic sections in saline-treated rats revealed normal arterial architecture regarding endothelial lining as flat continuous layer with regular nuclear pattern and distribution, normal intima and media of the vessel wall in all studied animals (Fig. 5F). Rats treated with FA revealed normal arterial architecture in eight animals out of the ten studied animals. Two animals showed mild focal endothelial changes in the form of focal endothelial swelling (Fig. 5G). On the other hand, in ACTH-treated rats, five animals out of ten studied revealed edematous endothelial cells, irregular lumen due to extensive protrusion and bridging of endothelium into lumen, endothelial sloughing, and the integrity of endothelial lining was locally destroyed. Two animals showed endothelial cells assuming bizarre shapes and bulging toward the lumen with thickening and discontinuity. Some endothelial cells appeared to be sloughed into lumen. Three animals showed diffuse endothelial cell loss leading to denudation of arterial intimal surface with prominent basement membrane like material (Fig. 5H). In FA prevention study (FA + ACTH), aortic sections of rats revealed nearly normal endothelial cell layer with mild diffuse endothelial swelling and mild irregular luminal layer in all studied animals (Fig. 5I). On the other hand, in FA reversal study (ACTH + FA), aortic sections of rats revealed a marked focal endothelial swelling with irregular cell contours and undulation in eight animals out of ten studied

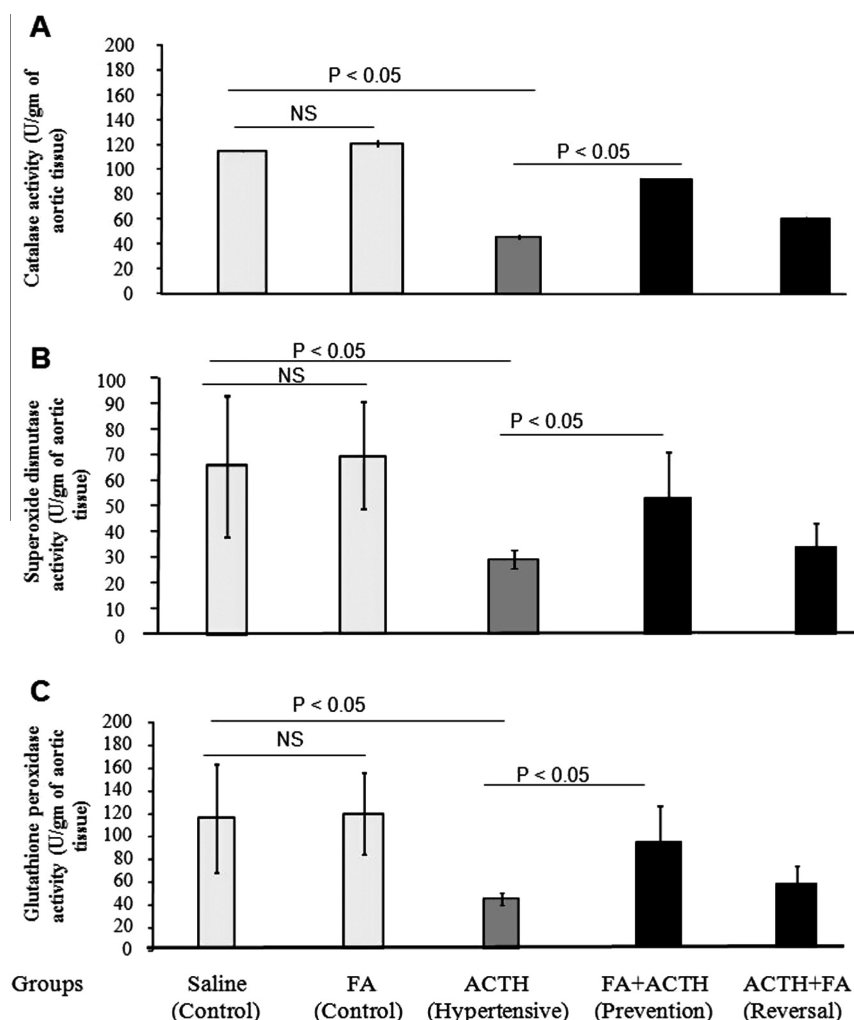


**Figure 4** Effect of folic acid (FA) on aortic tissue antioxidant such as catalase (CAT) (B), superoxide dismutase (SOD) (C) and glutathione peroxidase (GPx) (D) activities in various groups. Rats were treated with saline (1.0 ml/kg/day, S.C.) (Saline, control groups), FA (0.04 g/l, P.O.) (FA, control groups), ACTH (0.2 mg/kg/day, S.C) (ACTH-induced hypertension groups), FA 5 days before ACTH (FA + ACTH) (FA prevention study groups), and ACTH followed by FA for the last 5 days of study duration (ACTH + FA) (FA reversal study groups). Data are shown as mean ± SE ($n$ = 10), NS = $P$ value > 0.05 was not significant, $P$ < 0.05 was significant. P.O. = per orally, S.C. = subcutaneously injection.

animals. Two animals showed focal endothelial swelling (Fig. 5J).

### 3.13. Effect of FA on CD34$^{+}$ expression of aortic endothelium in ACTH-induced hypertension rats

Finally, aortic sections showed strongly positive (+ + +) cytoplasmic staining for CD34$^{+}$ in saline studied animals (Fig. 5K). Rats treated with FA revealed normal arterial wall and continuous regular endothelial lining with focal isolated swollen endothelial cells. Aortic sections showed strongly positive (+ + +) cytoplasmic staining for CD34$^{+}$ in all studied animals (Fig. 5L). On the other hand, aortic sections of rats treated with ACTH revealed endothelial swelling and denudation of the intimal surface. Aortic sections showed weakly positive (±) cytoplasmic staining for CD34$^{+}$ in eight animals out of the ten studied animals. Two animals showed mildly positive (+) cytoplasmic staining for CD34$^{+}$ (Fig. 5M). In FA prevention study (FA + ACTH), aortic sections of rats, revealed continuous layer of endothelial lining with mildly swollen endothelial cells. Aortic sections showed moderately positive (+ +) cytoplasmic staining for CD34$^{+}$ in all studied animals (Fig. 5N). On the other hand, in FA reversal study (ACTH + FA), aortic sections showed swollen endothelial cells with focally sloughed endothelial lining with mildly positive (+) cytoplasmic staining for CD34$^{+}$ in all studied animals (Fig. 5O).

## 4. Discussion

FA is a B-vitamin (known as vitamin $B_9$, vitamin M or folacine) which acts as a co-factor with cobalamin dependant enzyme methionine synthase and vitamin $B_{12}$ in the HCY remethylation pathway.[18] The present study showed that FA has a role in cardiovascular protective in ACTH-induced hypertension: (i) induced NO production and antioxidant

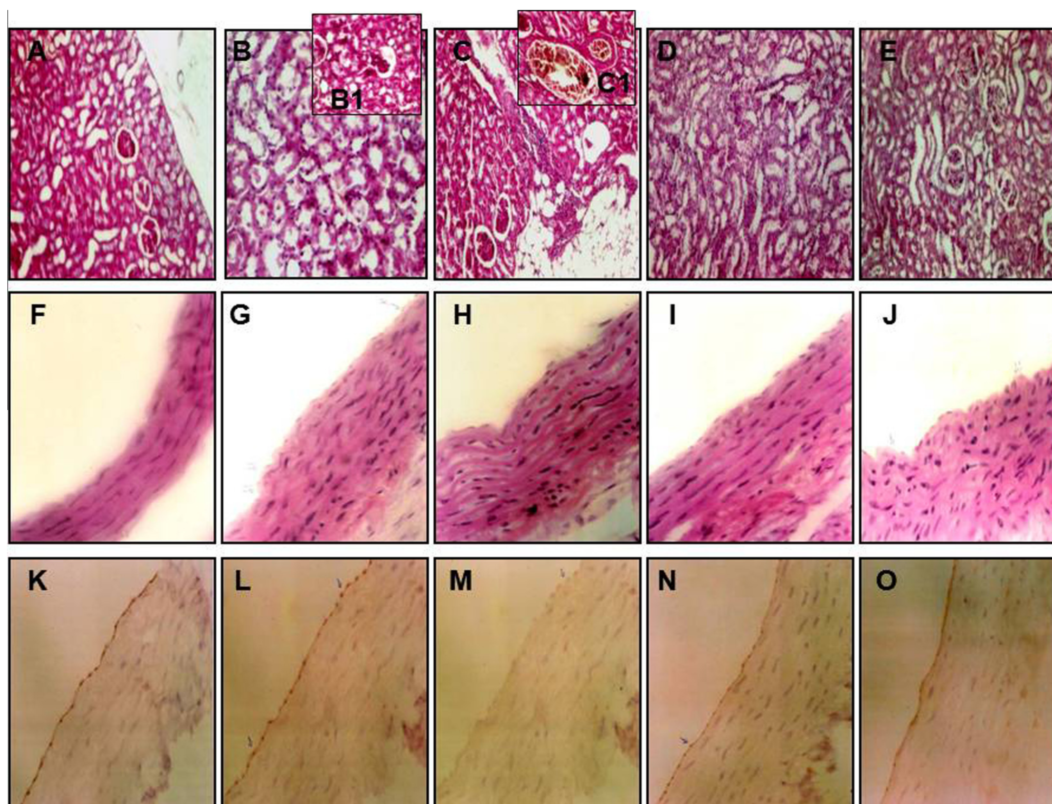


**Figure 5** Effect of folic acid (FA) on histopathology of kidney tissue (H&E X125, A–E) (H&E X250, B1 and C1) and aortic tissue section (H&E X250, F–J) and $CD34^{+}$ expression of aortic tissue sections (PAP stains X250, K–O) in various groups. Rats were treated with saline (1.0 ml/kg/day, S.C.) (Saline, control groups, A, F and K), FA (0.04 g/l, P.O.) (FA, control groups, B, G and L), ACTH (0.2 mg/kg/day, S.C) (ACTH-induced hypertension groups, C, H and M), FA 5 days before ACTH (FA + ACTH) (FA prevention study groups, D, I and N), and ACTH followed by FA for the last 5 days of study duration (ACTH + FA) (FA reversal study groups, E, J and O).

activity such as GSH, CAT, GPx and SOD; (ii) reduced HCY and MDA (oxidative stress); and (iii) improved endothelial function by improving EDD% and increasing endothelial progenitor cell (EPC) numbers that enhance vascular repair and tissue regeneration of endothelial dysfunction.

There was a significant weight loss in ACTH-treated groups and explained by decrease food consumption or severe protein catabolism induced by ACTH.[3] FA supplementation prevented and partially reversed systolic hypertension in ACTH-treated groups. This was in agreement with other studies that oral FA as a nutritional factor can be used untraditionally as an antihypertensive therapy in animals and humans.[3,19,20] Kidney function tests of ACTH-treated groups showed a significant increase in serum urea but non-significant change in serum creatinine concentration versus saline-treated groups. This result was in agreement with the other study that showed the increase of serum urea due to an increase in protein catabolism induced by ACTH.[21]

FA administration improved significantly NO production that led to smooth muscle cell relaxation (EDD, vasorelaxation) in hypertensive preventive study and reversal groups. This was consistent with other studies.[4,22] Also, other workers suggested that reduced NO activity led to impaired vasodilatation which had been significantly improved in hypertensive FA-treated groups.[23]

Elevated HCY in hypertensive groups indicated the role of HHCY in creating a state of oxidative stress. On the other hand, HCY level decreased in experimental glucocorticoid-induced hypertension.[3] While, there was no effect of Dex on HCY level.[24] Moreover, elevated HCY has been suggested to evoke hypertension through many actions such as an alteration of the elastic properties of the vascular wall, endothelial dysfunction and proliferation of vascular smooth muscle cells.[25] Our results showed FA supplementation significantly decreased HCY levels; this was in agreement with other studies.[23,26]

FA supplementation significantly reduced serum MDA, indicating its role in the reduction of lipid peroxidation in prevention and reversal groups. These results were also consistent with other previous study.[3] In addition, marked elevated HCY has been confirmed in folate deficient rats that also showed significantly elevated plasma MDA, GPx and SOD.[27] Dietary FA in some studies in human had no effect on MDA.[26]

GSH level was significantly decreased in ACTH versus saline-treated groups, indicating the GSH may be implicated in hypertension induced by ACTH. This was in agreement with previous studies.[28] FA-treated groups showed significantly increased GSH concentration in FA prevention study (but, not with FA reversal study) versus saline- or ACTH-treated groups. These results were in agreement with other

studies that showed FA supplementation reduced HCY and increased glutathione level in type-2 diabetes mellitus, and may both mediate improvement in vascular function and outcome.[29]

There was a significant decrease in maximal relaxation EDD% produced by ACh in FA-treated hypertensive group versus ACTH-treated groups. Similarly, in various experimental models of hypertension, reduction in the responsiveness of large arteries to ACh and other endothelium-dependent relaxing agents has been reported.[17,30] In the aorta, inhibitors of NOS abolished the relaxation to ACh, indicating that it was mediated by NO.[30] Thus, in hypertension, the ability of endothelium to release NO was blunted.[30] Therefore, there was a defect in NO production or bioavailability rather than an impaired vascular response to NO, and that NO formation was increased during FA supplementation.

FA administration in treated groups resulted in a significant decrease in HCY level, while GSH, GPx, and SOD were significantly increased. These results were in agreement with other studies that showed the reduction of antioxidant activity (CAT, GPx, and SOD) and ROS scavengers (vitamin E, C and glutathione), may contribute to oxidative stress involved in the pathogenesis of human hypertension.[8] Moreover, MDA was decreased concomitantly in patients with high coronary risk or manifest atherosclerotic disease.[31] Also, folate depletion decreased Cu–Zn-SOD and GPx activities, but had no effect on CAT activity in rat liver homogenates.[32]

Histopathologically, kidney sections of FA prevention study revealed mild tubular degeneration (cloudy swelling). These changes were milder than those observed in ACTH-treated rats. On the other hand, in FA reversal study, widening of Bowman's space and tubular degeneration with focal cellular tubular casting were observed.

Our results were in agreement with other studies.[33] FA supplementation may have a beneficial effect on kidney function in subjects with low content of FA (0.4 mg/day, orally for 3 months) in the diet.[34] FA therapy can reduce cardiovascular disease (CVD) risk in patients with ESRD/or advanced chronic kidney disease (ACKD) by 15%.[35] Also, in rats, dietary FA restriction or FA deficiency was found to induce kidney oxidative stress (tissue MDA)[3,33]; and that these changes were ameliorated by FA supplementation. Unfortunately, parenterally large doses of FA (240–250 mg/kg, I.P.; or 300 mg/kg, S.C, in vehicle 0.2 ml of 0.3 M $NaHCO_3$), impaired kidney function tests and induced renal tubulointerstitial injury with marked inflammatory cell infiltrations in experimental model (*folic acid nephropathy*).[36–38] However, the precise mechanism of injury remains unclear.

From previous results concerning kidney function tests and histopathological examination of the kidney, we notice conflicting results thus; the effect of FA on the kidney needs further investigation. Our results with oral intake 5 mg/day/rat of FA are more than 0.4 mg/day of FA (recommended daily allowance,[19] this is much more less than the dose which induces alterations in rat kidney). FA based HCY lowering does not reduce cardiovascular events in people with kidney disease. Therefore, FA based regimens should not be used for the prevention of cardiovascular events in people with kidney disease.[39]

Also, aortic sections revealed nearly normal endothelial cell layer with mild diffuse endothelial swelling and mild irregular luminal layer in FA prevention study but, revealed marked focal endothelial swelling with irregular cell contours and undulation in FA reversal study. These results were consistent with other studies.[40] Also, 3 month supplementation with FA and vitamin $B_6$ decreased endothelial injury in HHCY patients.[41]

The intensity of staining $CD34^+$ expression reflected the degree of EPC affection by ACTH-treatment in the presence of FA, which revealed moderately positive cytoplasmic staining for $CD34^+$ in FA prevention study. While in FA reversal study, examination revealed mildly positive cytoplasmic staining for $CD34^+$. These findings seem to be consistent with results that reported the addition of 10 μM of HCY to smooth muscle cells cultured medium caused a significant increase in cell proliferation and death through apoptosis and necrosis, respectively. The addition of FA to the culture medium significantly reduced both HCY concentration and the effects of HCY on the proliferation, apoptosis and necrosis of cells in the culture. The percentages for apoptotic cells and for cells with a necrotic morphology continued to increase as HCY concentration increased, although the absolute values were lower in the culture treated than not treated with FA.[42] Also, in the patients with type-I diabetes have reduced levels of EPCs and FA normalized EPCs gene expression profiles of those patients. Also, few oxidative stress-related genes were affected by FA.[43] Therefore, FA may be potential therapeutic targets to improve EPC function.

Therefore, in hypertension, there was an elevated HCY level which led to endothelial dysfunction possibly due to reduction of NO or vascular hypertrophy. HCY was toxic to vascular endothelium and impaired endothelial function by: inhibiting NO production,[27] increasing its degradation *via* the generation of ROS such as superoxide ($O_2^-$), peroxynitrite ($OONO^-$) and $H_2O_2$; or by modification of proteins and peroxidation of lipid resulting in formation of oxidized low density lipoprotein (LDL) which impaired expression of NOS and directly degrades NOS.[44] ROS may interact with NO, thus reducing its bioavailability, leading to vascular constriction and elevated resistance.[23] MDA was a secondary lipid peroxidation generated by oxidative stress.[27] MDA was reactive toward protein causing polymerization of membrane components altering intrinsic membrane properties such as deformability, ion transport and enzyme activities, MDA also reacted with nitrogenous bases of DNA. It appeared in blood and urine, used as an indicator of lipid peroxidation. Because FA was soluble in water, it is suggested to inhibit microsomal lipid peroxidation.[23]

Finally, the FA has an antioxidant action by itself or through its effect on the reduction of levels of HCY levels.[45] FA supplementation may increase recycling of HCY to methionine,[18] so reversing the effects of HHCY leading to the improvement of endothelial function (directly or through increased bioavailability of NO), large artery stiffness[10] and renovascular remodeling.[30]

Clinically, oral FA supplementation (5 mg/day for 4–6 weeks) reduced SBP and plasma HCY and enhanced endothelium-dependent vasodilatation in smokers[4] and coronary heart disease (CAD) patients,[46] but did not provide any additional cardiovascular system benefits in another study.[47] Other study showed oral FA supplementation (10 mg/day for 2 weeks) improved levels of oxidative stress markers in patients with essential hypertension.[48] Finally, low-dose FA supplementation (0.4 mg/day for 3 months), has a beneficial effect

on blood lipid profiles (decreasing concentrations of total cholesterol and LDL-C and increasing concentrations of apoAI) and increases NO-mediated endothelium-dependent vasomotor responses in CAD patients[49]; and also on serum uric acid in hypertensive patients.[22,50]

## 5. Conclusion

FA supplementation is a safe and inexpensive tool for clinical use in hypertensive patients, and avoids many adverse effects of other antihypertensive drugs. Therefore, FA could be used as an adjuvant therapy for prevention and treatment of glucocorticoid-induced hypertension in humans and in other free radical related diseases.

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## Conflict of interests

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## References

1. Tsai WC. Treatment options for hypertension in high-risk patients. *Vasc Health Risk Manag* 2011;**7**:137–41.
2. Hu Z, Guan W, Wang W, Zhu Z, Wang Y. Folacin $C_{60}$ derivative exerts a protective activity against oxidative stress-induced apoptosis in rat pheochromocytoma cells. *Bioorg Med Chem Lett* 2010;**20**:4159–62.
3. Miao Y, Zhang Y, Lim PS, Kanjanapan Y, Mori TA, Croft KD, et al. Folic acid prevents and partially reverses glucocorticoid-induced hypertension in the rat. *Am J Hypertens* 2007;**20**:304–10.
4. Mangoni AA, Sherwood RA, Swift CG, Jackson SH. Folic acid enhances endothelial function and reduces blood pressure in smokers: a randomized controlled trial. *J Intern Med* 2002;**252**:497–503.
5. Wen C, Li M, Fraser T, Wang J, Turner SW, Whitworth JA. L-arginine partially reverses established adrenocorticotrophin-induced hypertension and nitric oxide deficiency in the rat. *Blood Press* 2000;**9**:298–304.
6. Moens AL, Kass DA. Therapeutic potential of tetrahydrobiopterin for treating vascular and cardiac disease. *J Cardiovasc Pharmacol* 2007;**50**:238–46.
7. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 2003;**111**:1201–9.
8. Touyz RM, Schiffrin EL. Reactive oxygen species in vascular biology: implications in hypertension. *Histochem Cell Biol* 2004;**122**:339–52.
9. Moat SJ, Doshi SN, Lang D, McDowell IF, Lewis MJ, Goodfellow J. Treatment of coronary heart disease with folic acid: is there a future? *Am J Physiol Heart Circ Physiol* 2004;**287**:H1–7.
10. Bazzano LA, Reynolds K, Holder KN, He J. Effect of folic acid supplementation on risk of cardiovascular diseases: a meta-analysis of randomized controlled trials. *JAMA* 2006;**296**:2720–6.
11. Terzolo M, Allasino B, Bosio S, Brusa E, Daffara F, Ventura M, et al. Hyperhomocysteinemia in patients with Cushing's syndrome. *J Clin Endocrinol Metab* 2004;**89**:3745–51.
12. Ng X, Boyd L, Dufficy L, Naumovski N, Blades B, Travers C, et al. Folate nutritional genetics and risk for hypertension in an elderly population sample. *J Nutrigenet Nutrigenomics* 2009;**2**:1–8.
13. Symons JD, Zaid UB, Athanassious CN, Mullick AE, Lentz SR, Rutledge JC. Influence of folate on arterial permeability and stiffness in the absence or presence of hyperhomocysteinemia. *Arterioscler Thromb Vasc Biol* 2006;**26**:814–8.
14. Thambyrajah J, Landray MJ, McGlynn FJ, Jones HJ, Wheeler JN, Townend JN. Does folic acid decrease plasma homocysteine and improve endothelial function in patients with predialysis renal failure? *Circulation* 2000;**102**:871–5.
15. Malinow MR, Duell PB, Hess DL, Anderson PH, Kruger WD, Phillipson BE, et al. Reduction of plasma homocyst(e)ine levels by breakfast cereal fortified with folic acid in patients with coronary heart disease. *N Engl J Med* 1998;**338**:1009–15.
16. Mangoni AA, Sherwood RA, Asonganyi B, Swift CG, Thomas S, Jackson SH. Short-term oral folic acid supplementation enhances endothelial function in patients with type 2 diabetes. *Am J Hypertens* 2005;**18**:220–6.
17. Noguchi K, Hamadate N, Matsuzaki T, Sakanashi M, Nakasone T, Uchida T, et al. Increasing dihydrobiopterin causes dysfunction of endothelial nitric oxide synthase in rats in vivo. *Am J Physiol Heart Circ Physiol* 2011;**301**:H721–9.
18. Hagar HH. Folic acid and vitamin B(12) supplementation attenuates isoprenaline-induced myocardial infarction in experimental hyperhomocysteinemic rats. *Pharmacol Res* 2002;**46**:213–9.
19. Xun P, Liu K, Loria CM, Bujnowski D, Shikany JM, Schreiner PJ, et al. Folate intake and incidence of hypertension among American young adults: a 20-y follow-up study. *Am J Clin Nutr* 2012;**95**:1023–30.
20. Moens AL, Claeys MJ, Wuyts FL, Goovaerts I, Van Hertbruggen LC, Wendelen LC, et al. Effect of folic acid on endothelial function following acute myocardial infarction. *Am J Cardiol* 2007;**99**:476–81.
21. Brown MA, Whitworth JA. Hypertension in human renal disease. *J Hypertens* 1992;**10**:701–12.
22. Mao G, Hong X, Xing H, Liu P, Liu H, Yu Y, et al. Efficacy of folic acid and enalapril combined therapy on reduction of blood pressure and plasma glucose: a multicenter, randomized, double-blind, parallel-controlled, clinical trial. *Nutrition* 2008;**24**:1088–96.
23. Joshi R, Adhikari S, Patro BS, Chattopadhyay S, Mukherjee T. Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. *Free Radic Biol Med* 2001;**30**:1390–9.
24. Brotman DJ, Girod JP, Garcia MJ, Patel JV, Gupta M, Posch A, et al. Effects of short-term glucocorticoids on cardiovascular biomarkers. *J Clin Endocrinol Metab* 2005;**90**:3202–8.
25. Dobarro D, Gomez-Rubin MC, Sanchez-Recalde A, Moreno R, Galeote G, Jimenez-Valero S, et al. Current pharmacological approach to restore endothelial dysfunction. *Cardiovasc Hematol Agents Med Chem* 2009;**7**:212–22.
26. Moat SJ, Lang D, McDowell IF, Clarke ZL, Madhavan AK, Lewis MJ, et al. Folate, homocysteine, endothelial function and cardiovascular disease. *J Nutr Biochem* 2004;**15**:64–79.
27. Diez N, Perez R, Hurtado V, Santidrian S. Hyperhomocysteinaemia induced by dietary folate restriction causes kidney oxidative stress in rats. *Br J Nutr* 2005;**94**:204–10.
28. Vasdev S, Gill V. The antihypertensive effect of arginine. *Int J Angiol* 2008;**17**:7–22.
29. Child DF, Hudson PR, Jones H, Davies GK, De P, Mukherjee S, et al. The effect of oral folic acid on glutathione, glycaemia and lipids in Type 2 diabetes. *Diabetes Nutr Metab* 2004;**17**:95–102.

30. Pushpakumar SB, Kundu S, Metreveli N, Sen U. Folic acid mitigates angiotensin-II-induced blood pressure and renal remodeling. *PLoS One* 2013;**8**:e83813.
31. Mayer Jr O, Simon J, Rosolova H, Hromadka M, Subrt I, Vobrubova I. The effects of folate supplementation on some coagulation parameters and oxidative status surrogates. *Eur J Clin Pharmacol* 2002;**58**:1–5.
32. Huang RF, Hsu YC, Lin HL, Yang FL. Folate depletion and elevated plasma homocysteine promote oxidative stress in rat livers. *J Nutr* 2001;**131**:33–8.
33. Kumagai H, Katoh S, Hirosawa K, Kimura M, Hishida A, Ikegaya N. Renal tubulointerstitial injury in weanling rats with hyperhomocysteinemia. *Kidney Int* 2002;**62**:1219–28.
34. Mierzecki A, Makarewicz-Wujec M, Kloda K, Kozlowska-Wojciechowska M, Pienkowski P, Naruszewicz M. Influence of folic acid supplementation on coagulation, inflammatory, lipid, and kidney function parameters in subjects with low and moderate content of folic acid in the diet. *Kardiol Pol* 2015;**73**:280–6.
35. Qin X, Huo Y, Langman CB, Hou F, Chen Y, Matossian D, et al. Folic acid therapy and cardiovascular disease in ESRD or advanced chronic kidney disease: a meta-analysis. *Clin J Am Soc Nephrol* 2011;**6**:482–8.
36. Yang HC, Zuo Y, Fogo AB. Models of chronic kidney disease. *Drug Discov Today Dis Models* 2010;**7**:13–9.
37. Tsutsumi T, Ichihara T, Kawabe M, Yoshino H, Asamoto M, Suzuki S, et al. Renal toxicity induced by folic acid is associated with the enhancement of male reproductive toxicity of di(n-butyl) phthalate in rats. *Reprod Toxicol* 2004;**18**:35–42.
38. Doi K, Okamoto K, Negishi K, Suzuki Y, Nakao A, Fujita T, et al. Attenuation of folic acid-induced renal inflammatory injury in platelet-activating factor receptor-deficient mice. *Am J Pathol* 2006;**168**:1413–24.
39. Dashti HM, Bo-Abbas YY, Asfar SK, Mathew TC, Hussein T, Behbahani A, et al. Ketogenic diet modifies the risk factors of heart disease in obese patients. *Nutrition* 2003;**19**:901–2.
40. Doshi SN, McDowell IF, Moat SJ, Payne N, Durrant HJ, Lewis MJ, et al. Folic acid improves endothelial function in coronary artery disease via mechanisms largely independent of homocysteine lowering. *Circulation* 2002;**105**:22–6.
41. Constans J, Blann AD, Resplandy F, Parrot F, Renard M, Seigneur M, et al. Three months supplementation of hyperhomocysteinaemic patients with folic acid and vitamin $B_6$ improves biological markers of endothelial dysfunction. *Br J Haematol* 1999;**107**:776–8.
42. Buemi M, Marino D, Di Pasquale G, Floccari F, Ruello A, Aloisi C, et al. Effects of homocysteine on proliferation, necrosis, and apoptosis of vascular smooth muscle cells in culture and influence of folic acid. *Thromb Res* 2001;**104**:207–13.
43. van Oostrom O, de Kleijn DP, Fledderus JO, Pescatori M, Stubbs A, Tuinenburg A, et al. Folic acid supplementation normalizes the endothelial progenitor cell transcriptome of patients with type 1 diabetes: a case-control pilot study. *Cardiovasc Diabetol* 2009;**8**:47.
44. van der Molen EF, van den Heuvel LP, te Poele Pothoff MT, Monnens IA, Eskes TK, Blom HJ. The effect of folic acid on the homocysteine metabolism in human umbilical vein endothelial cells (HUVECs). *Eur J Clin Invest* 1996;**26**:304–9.
45. Cano MJ, Ayala A, Murillo ML, Carreras O. Protective effect of folic acid against oxidative stress produced in 21-day postpartum rats by maternal-ethanol chronic consumption during pregnancy and lactation period. *Free Radic Res* 2001;**34**:1–8.
46. McRae MP. High-dose folic acid supplementation effects on endothelial function and blood pressure in hypertensive patients: a meta-analysis of randomized controlled clinical trials. *J Chiropr Med* 2009;**8**:15–24.
47. Naruszewicz M, Klinke M, Dziewanowski K, Staniewicz A, Bukowska H. Homocysteine, fibrinogen, and lipoprotein(a) levels are simultaneously reduced in patients with chronic renal failure treated with folic acid, pyridoxine, and cyanocobalamin. *Metabolism* 2001;**50**:131–4.
48. Stiefel P, Arguelles S, Garcia S, Jimenez L, Aparicio R, Carneado J, et al. Effects of short-term supplementation with folic acid on different oxidative stress parameters in patients with hypertension. *Biochim Biophys Acta* 2005;**1726**:152–9.
49. Mierzecki A, Kloda K, Bukowska H, Chelstowski K, Makarewicz-Wujec M, Kozlowska-Wojciechowska M. Association between low-dose folic acid supplementation and blood lipids concentrations in male and female subjects with atherosclerosis risk factors. *Med Sci Monit* 2013;**19**:733–9.
50. Li H, Qin X, Xie D, Tang G, Zhang Y, Li J, et al. Effects of combined enalapril and folic acid therapy on the serum uric acid levels in hypertensive patients: a multicenter, randomized, double-blind, parallel-controlled clinical trial. *Intern Med* 2015;**54**:17–24.