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Original Article

Effect of losartan with folic acid on plasma homocysteine and vascular ultrastructural changes in spontaneously hypertensive rats

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Abstract: Elevated homocysteine (Hcy) is a high risk factor of hypertension due to its function in endothelial dysfunction. Its level in the blood is strongly influenced by folic acid. In order to investigate the effects of losartan with folic acid on plasma level of Hcy and vascular ultrastructural changes, thirty spontaneously hypertensive rats (SHR) involved and randomly divided into three groups (n=10): SHR-C group (control), SHR-L group (losartan 25 $mg \cdot kg^{-1} \cdot d^{-1}$), SHR-L+Y group (losartan 25 $mg \cdot kg^{-1} \cdot d^{-1}$ + folic acid 0.4 $mg \cdot kg^{-1} \cdot d^{-1}$). Another 10 Wistar Rats involved as WKY-C group for normal control. The level of plasma Hcy was measured dynamically by LS-MS, the vascular ultrastructural changes were analyzed by light and electron microscopy. Moreover, the thickness and area of aorta was measured. The results showed the Hcy levels in four groups were WKY-C 7.49 ± 1.95 μmol/L; SHR-C 8.45 ± 1.90 μmol/L; SHR-L 8.28 ± 2.11 μmol/L; SHR-L+Y 7.53 ± 2.02 μmol/L at 80 days. There was no significant change for plasma Hcy ($P>0.05$). The morphological change showed the subendothelial space didn't increased significantly, the endothelial cells have a more smooth and intact cellular membrane in SHR-L+Y group. In conclusion, Losartan combined with folic acid could improve arterial endothelial structure in SHR which has no significant correlation with plasma Hcy.

Keywords: Losartan, folic acid, endothelium, SHR, homocysteine

Introduction

Hypertension is a common disease which causes a large number of serious complications such as cardiovascular disease, converging stroke, ischemic and peripheral vascular disease [1]. Hypertension results from a complex interaction of genes and environmental factors, about 95 percent of hypertensive patients is idiopathic and multifactorial [2]. Therefore, preventing potential risk factors is very important for the treatment of hypertension.

Elevated homocysteine (Hcy) is one of such risk factors, which has been suggested to cause hypertension due to its function in endothelial dysfunction [3]. The "Hordal and Homocysteine Study" about 16,000 people with no history of hypertension showed the plasma homocysteine levels were positively related to blood pressure [2]. It has been well proved that the higher plasma Hcy, compared the blood stress in hypertensive patients to healthy volunteers, resulting in more advanced systemic arterial stiffness and greater blood pressure [4, 5]. There is growing evidence that Hcy involves increased oxidative injury to the endothelium, proliferation of vascular smooth muscle cells, and inhibition and degrading of arterial structural components such as collagen, elastin, and proteoglycans [3, 6, 7].

Folic acid and its active metabolite 5-methyltetrahydrofolate (5-MTHF) are essential nutrients in humans and play a significance role in nucleotide synthesis and methylation reactions. Hcy-induced endothelial dysfunction in humans was improved with oral administration of folic acid [8-10]. Folic acid can normalize high Hcy concentrations [11] and may reduce cardiovascular events [12]. Clinical studies have shown that

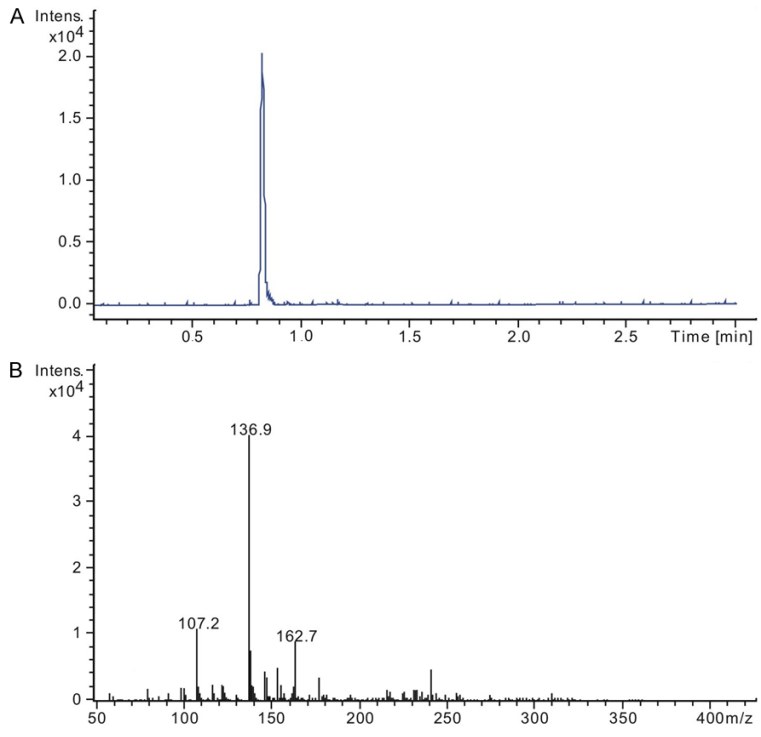

Figure 1. Representative LC-MS chromatograms of Hcy in rat plasma.

long-term oral folic acid can control the level of plasma Hcy, effectively reduce the risk of pregnancy hypertension, mend arterial endothelial structure and have potential implications for avoiding the happening of atherosclerosis in hyperhomocysteine patients [13, 14]. Therefore, folic acid is routinely used in hypertensive patients with high Hcy and pregnant women.

However, high Hcy is not appeared in every hypertensive patients, it's still a controversial theory that folic acid improve endothelial structure by reducing Hcy concentration [15-17]. Therefore, whether the hypertensive patients should add a small dose of folic acid to control the level of plasma Hcy and improve arterial endothelial structure is still unsure. Accordingly, we investigated the effects of losartan combined with low dose folic acid on vascular endothelial structure and plasma Hcy in spontaneously hypertensive rats.

Materials and methods

Animals and chemicals

Male SHR and Wistar-Kyoto (WKY) rats aged 12 weeks were purchased from Shanghai Institute of Hypertension which both weighing approximately 250-350 g. The rats were housed individually in standard plastic cages and maintained on normal rat purina chow and fresh water in a room controlled for temperature (23-25°C) and lighting (8:00-20:00 light, 20:00-8:00 dark). After the 1-wk acclimatization period, the rats were used for experiments and all efforts were made to minimize any animal suffering. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College, and were in accordance with the Guide for the Care and Use of Laboratory Animals. Antihypertensive drugs used in this study are as follows: losartan (Moshadong Pharmaceutical Co, Hangzhou, China) and folic acid (Feiying Pharmaceutical Co, Tianjin, China).

Drug administration and sample collection

A total of 30 male SHR were randomly divided into 3 groups (n=10 for each group): SHR-C control group, SHR-L group (losartan 25 mg·kg^{-1}·d^{-1}), and SHR-L+Y group (losartan 25 mg·kg^{-1}·d^{-1} + folic 0.4 mg·kg^{-1}·d^{-1}). WKY non-hypertensive was served as WKY-C normal control group (n=10). Each treatment group of rats received one dosage, and SHR-C group and WKY-C group were given normal saline. Drugs were administered by gastric irrigation once a day for 12 weeks.

Blood samples were drawn from the vena caudalis of each rat every 10 days until death, and then put into tube containing 80 IU heparin. The samples were immediately centrifuged at 5000 r/min within 10 min. The plasma separated and stored at -80°C until further analysis.

Determination of plasma Hcy

A 1200 Series Agilent liquid chromatograph and a Bruker Esquire HCT mass spectrometer

Table 1. Dynamic monitoring plasma homocysteine levels from 1 to 80 days in four groups (mean ± SD, μmol/L)

Days	WKY-C	SHR-C	SHR-L	SHR+L+Y
1	6.98 ± 1.82	8.04 ± 1.64	8.42 ± 1.20	8.30 ± 1.07
10	7.50 ± 1.00	8.10 ± 1.00	8.00 ± 1.00	7.90 ± 1.00
20	7.32 ± 1.52	8.95 ± 1.80	8.43 ± 1.44	8.74 ± 0.96
30	7.04 ± 1.42	8.36 ± 1.80	8.46 ± 2.25	8.04 ± 2.24
40	7.60 ± 2.51	7.69 ± 1.38	8.59 ±2.37	7.57 ± 1.74
50	7.45 ± 1.83	7.95 ± 2.34	8.37 ± 1.59	8.42 ± 1.77
60	7.54 ± 1.05	8.07 ± 1.35	8.88 ± 1.24	7.63 ± 1.31
70	7.90 ± 1.00	7.60 ± 1.50	7.60 ± 1.60	7.90 ± 1.00
80	7.49 ± 1.95	8.45 ± 1.90	8.28 ± 2.11	7.53 ± 2.02

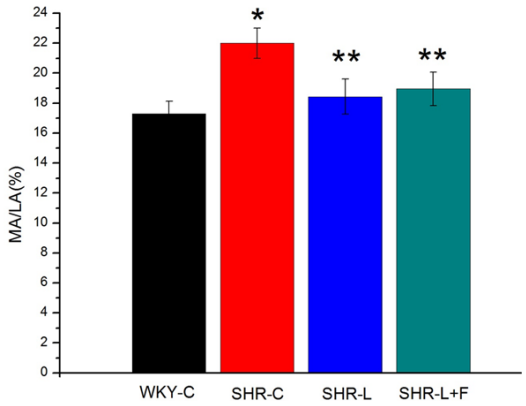

Figure 2. Media cross-sectional area/lumen area (MA/LA) of WKY-C, SHR-C, SHR-L and SHR-L+Y. *P<0.01 vs. WKY-C; **P<0.01 vs. SHR-C.

equipped with an electrospray ion source (Bruker Technologies, Germany) were used in this study. The chromatographic separation was achieved on an Agilent Zorbax SB-C_{18} column (150 mm×2.1 mm, 3.5 μm particle) at 30°C. The mobile phrase was methanol vs. water (40:60, V/V) at 300 L/min. The positive electrospray ionization technique operated in the selected reaction mode was used to measure the samples. Drying gas flow and nebulizer pressure was set to 7 L/min and 30 psi. The protonated [M+H] + molecule in a positive ion mode were obtained: m/z 136 (Hcy). The analysis was performed with dry gas temperature adjusted to 350°C and capillary voltage of the system was 3,000 V.

Sample preparation and calibration

Before sample extraction, 100 μL Plasma was added to 1.5 mL EP tubes, and 50 μL of trichloroacetic acid and methyl alcohol (1:9) was added for protein precipitation. The mixture was vortex-mixed for 0.5 min and centrifugated at 12000 rpm/10 min. The supernatant was separated and 50 μL was transferred quantitatively into a vase. A 5 μL aliquot was injected into LC-MS for analysis.

The plasma calibration curve of Hcy was constructed in the range of 1-64 mol/L. Eight calibration standards samples (0.5, 1, 2, 4, 8, 16, 32, 64, mol/L) were prepared by spiking blank rat plasma with appropriate amounts of the working solutions of Hcy.

Light and electron microscopy

At week 12, rats were deeply anesthetized with 10% chloral hydrate (i.p., 20 mg/kg). The thoracic aortic rings near the first intercostal artery were rapidly isolated and freed from adherent adipose and connective tissues. Thereafter, they were immersed in freshly prepared 4% w/v formaldehyde (0.1 phosphate buffer, pH 7.2) for 48 h and then embedded in paraffin. Then 5-μm-thick histological sections were prepared and stained with hematoxylin and eosin by routine HE method. The morphological changes of aorta and mesenteric blood vessels were observed under light microscope. In addition, the media wall thickness and lumen diameter, and the ratio of the media cross-sectional area to lumen area were calculated.

Other specimens were immediately fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and 0.25% tannic acid post-fixed in 1% osmium tetroxide, and then embedded in Epon. Ultrathin sections were obtained with an ultramicrotome Leica Ultracut-UCT, counterstained with uranyl acetate and lead citrate, and then examined with a Hitachi H-7500 transmission electron microscope.

Statistical analysis

SPSS 16.0 statistical package was used for statistical analysis. Data were presented as mean ± standard deviation (mean ± SD). Statistical comparisons were made using one-way ANOVA. The differences among four groups were compared pairwise by LSD-t. $P<0.05$ was considered as statistically significant. Results of repeatedly measured analysis of variance

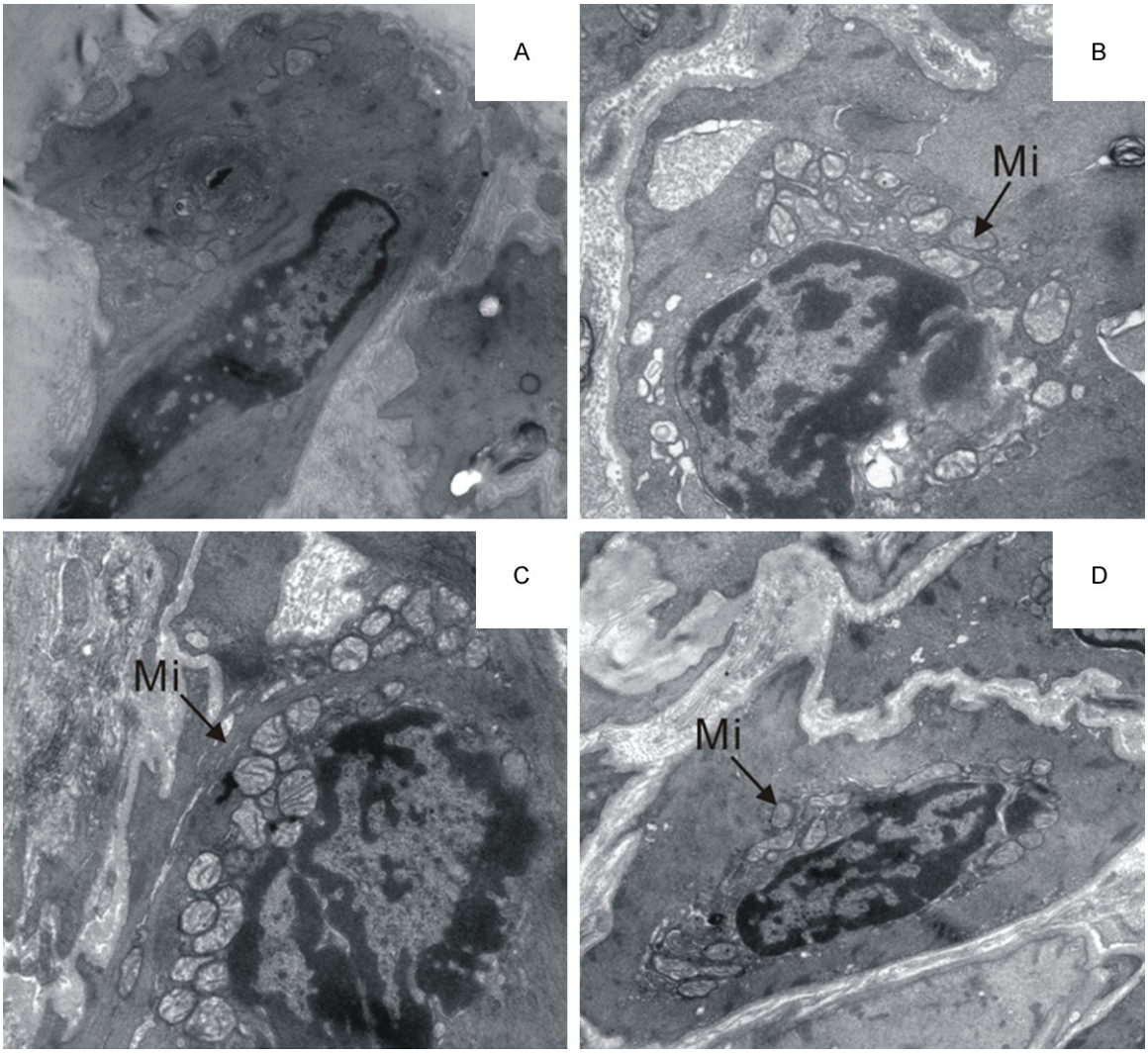

Figure 3. The ultrastructural changes of smooth muscle cell (×10000) A: WKY-C. B: SHR-C. C: SHR-L. D: SHR-L+Y; mitochondria (Mi) had obvious hyperplasia in SHR-C and SHR-L.

were used for Hcy detection, judging Hcy trends in each group.

Results

Dynamic monitoring of plasma Hcy levels

There was a good linear regressions of the peak area ratios versus concentrations over the range of 1-64 ng/mL for Hcy. Typical equation of the calibration curve was: y=297.51 x + 145. 29, r=0.9964, in which x represented the Hcy peak area and y represented the plasma concentration. The typical chromatograms of a blank plasma sample spiked with Hcy were shown in **Figure 1**.

Plasma Hcy in four groups were dynamically tested at day 1, 10, 20, 30, 40, 50, 60, 70, 80 by LC-MS method. The values of the measured levels of Hcy at first and last days were WKY-C 6.98 ± 1.82 and 7.49 ± 1.95 μmol/L; SHR-C 8.04 ± 1.64 and 8.45 ± 1.90 μmol/L; SHR-L 8.42 ± 1.20 and 8.28 ± 2.11 μmol/L; SHR-L+Y 8.30 ± 1.07 and 7.53 ± 2.02 μmol/L. There was no significant difference in the four groups, include SHR-L+Y and SHR-C group. The results of dynamically tested Hcy levels were shown in **Table 1**.

Value of media cross-sectional area/lumen area

The changes of vascular structure in hypertensive patients were mainly characterized for the ratio of the vessel wall and lumen increased. The ratio of the media cross-sectional area

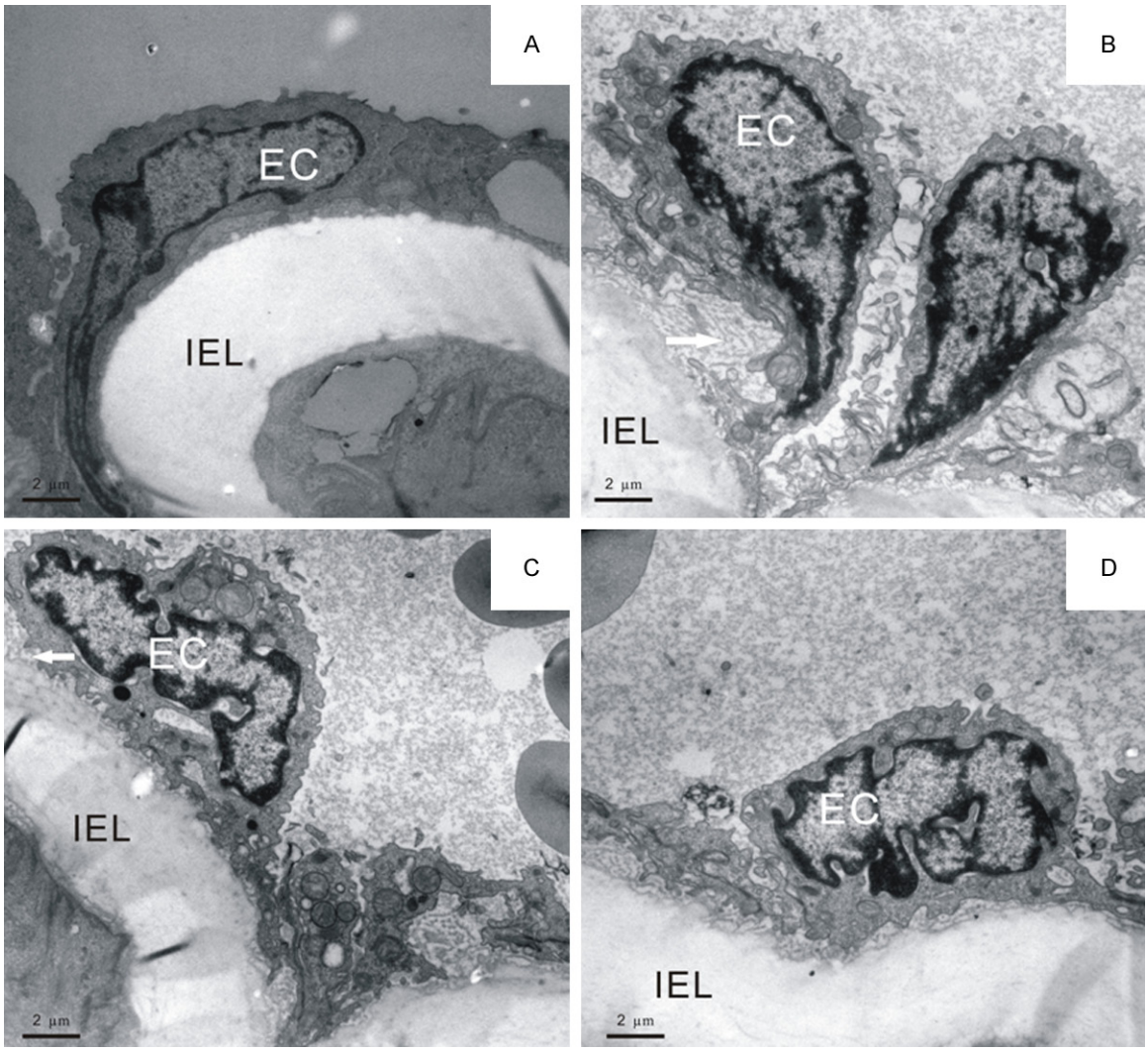

Figure 4. The ultrastructural changes of endothelial cell (×10000): A: WKY-C. B: SHR-C. C: SHR-L. D: SHR-L+Y; EC, endothelial cell; IEL, internal elastic lamina.

(MA) to lumen area (LA) was calculated through the data of aortic media wall thickness and vascular standardized inner diameter and represented in **Figure 2**. LSD tests showed that the value of media cross-sectional area vs. lumen area in the SHR-L+Y and SHR-L groups were significantly decreased when compared to the SHR-C group rats ($P<0.01$), While no difference was found between the SHR-L+Y and SHR-L groups ($P>0.05$).

Ultrastructural examination

In the SHR-C group, aortal wall was thickened, tunica intima was not smooth, internal elastic lamina was segregated and broken, vascular smooth muscle cells of tunica media were irregularly arranged, and nuclei number per unit area was increased. In the SHR-L+Y group, intima was smooth and had an integrated structure on the whole, the hyperplasia of smooth muscle cells was not obviously increased, the internal elastic fibrous membrane of tunica media was continuous, and aortal wall became thinner compared with the SHR-C group. As for the SHR-L group, the vascular smooth muscle cells of tunica media showed thinner than the SHR-C group, but tunica intima was similar not smooth. The ultrastructural changes of smooth muscle cells were showed in **Figure 3**.

The endothelial cell layer and internal elastic membrane structure of artery were showed in **Figure 4** by the electron microscopic examina-

tions. In SHR-C group there was a serious alterations in the structure of mesenteric wall. The endothelial cells were activated, the volume of nucleus widened, the subendothelial spaces between IEL increased and endothelial cells almost separated from it (**Figure 4B**). Due to the treatment with the losartan-folic (SHR-L), a significantly better morphology of vascular wall could be observed. Although the structure of endothelial cells in SHR-L+Y didn't same to the WKY-C (**Figure 4A**), their subendothelial space didn't increased significantly and the endothelial cells have a more smooth and intact cellular membrane than SHR-L (**Figure 4C**).

Discussion

Hypertension can result in large and medium arteries damaged and atherosclerosis. Hcy is an independent risk element for cardiovascular disease [18] and induces endothelial dysfunction and arterial stiffening [19, 20]. Plasma Hcy levels could be lowered by folic acid safely and effectively [21]. During our dynamic monitor of the concentration of Hcy, we found folic acid seems to exert beneficial effects on endothelial structure that are not completely dependent on the Hcy lowering effects. As the concentration of Hcy have not markedly changed when folic acid was co-administered, there was no difference between four groups ($P>0.05$).

An explanation for improved arterial endothelial structure is an independent vascular effect of folic acid, as suggested by Verhaar et al. demonstrating that administration of 5-MTHF, an active form of folic acid, reduced superoxide generation and provided antioxidant potential, thus restoring impaired endothelial structure [22]. Doshi et al. also observed that folic acid improves endothelial structure in coronary artery disease by reduction of intracellular superoxide [23]. Another explanation for the beneficial effects of folic acid on endothelial structure refers to its effects on the enzyme endothelial nitric oxide synthase. Stroes et al. revealed that 5-MTHF decreases superoxide generation through NO synthase [24].

This study demonstrated that losartan combined with low doses of folic acid can significantly improve the whole blood viscosity when compared with the SHR-C group rats ($P<0.05$). On the other hand, a modest decrease of the whole blood viscosity was found in comparison with SHR-L group, indicating more pronounced effects. In addition, aorta and mesenteric arteries for histological examination showed that arterial intima was more complete, endothelial cells and internal elastic membrane was more clearly visible in the SHR-L+Y group. Moreover, the ratio of media cross-sectional area vs. lumen area were similar in the SHR-L+Y and SHR-L group, but was distinctively decreased when compared with the SHR-C group.

Conclusion

Losartan with low folic acid can alleviate the vascular endothelial dysfunction and provide cardiovascular protection from vascular remodeling which was largely independent from the Hcy lowering effects. Thus, a simple, nontoxic, and relatively inexpensive vitamin intervention might be useful in hypertensive prevention.

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Disclosure of conflict of interest

None.

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References

[1] Lip GY, Barnett AH, Bradbury A, Cappuccio FP, Gill PS, Hughes E, Imray C, Jolly K and Patel K. Ethnicity and cardiovascular disease prevention in the United Kingdom: a practical approach to management. J Hum Hypertens 2007; 21: 183-211.

[2] Sen U, Mishra PK, Tyagi N and Tyagi SC. Homocysteine to hydrogen sulfide or hypertension. Cell Biochem Biophys 2010; 57: 49-58.

[3] Rodrigo R, Passalacqua W, Araya J, Orellana M and Rivera G. Homocysteine and essential hypertension. J Clin Pharmacol 2003; 43: 1299-1306.
[4] Tayama J, Munakata M, Yoshinaga K and Toyota T. Higher plasma homocysteine concentration is associated with more advanced systemic arterial stiffness and greater blood pressure response to stress in hypertensive patients. Hypertens Res 2006; 29: 403-409.
[5] Brett SE, Jiang BY, Turner C, Ritter JM and Chowienczyk PJ. Elevation of plasma homocysteine by methionine loading increases the diastolic blood pressure response to exercise. J Hypertens 2006; 24: 1985-1989.
[6] Upchurch GR Jr, Welch GN, Fabian AJ, Freedman JE, Johnson JL, Keaney JF Jr and Loscalzo J. Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 1997; 272: 17012-17017.
[7] Zhang X, Li H, Jin H, Ebin Z, Brodsky S and Goligorsky MS. Effects of homocysteine on endothelial nitric oxide production. Am J Physiol Renal Physiol 2000; 279: F671-678.
[8] Chao CL, Chien KL and Lee YT. Effect of short-term vitamin (folic acid, vitamins B6 and B12) administration on endothelial dysfunction induced by post-methionine load hyperhomocysteinemia. Am J Cardiol 1999; 84: 1359-1361, A1358.
[9] Constans J, Blann AD, Resplandy F, Parrot F, Renard M, Seigneur M, Guerin V, Boisseau M and Conri C. Three months supplementation of hyperhomocysteinaemic patients with folic acid and vitamin B6 improves biological markers of endothelial dysfunction. Br J Haematol 1999; 107: 776-778.
[10] Title LM, Cummings PM, Giddens K, Genest JJ Jr and Nassar BA. Effect of folic acid and antioxidant vitamins on endothelial dysfunction in patients with coronary artery disease. J Am Coll Cardiol 2000; 36: 758-765.
[11] Brattstrom LE, Israelsson B, Jeppsson JO and Hultberg BL. Folic acid--an innocuous means to reduce plasma homocysteine. Scand J Clin Lab Invest 1988; 48: 215-221.
[12] Malinow MR, Duell PB, Hess DL, Anderson PH, Kruger WD, Phillipson BE, Gluckman RA, Block PC and Upson BM. 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: 1009-1015.
[13] Woo KS, Chook P, Chan LL, Cheung AS, Fung WH, Qiao M, Lolin YI, Thomas GN, Sanderson JE, Metreweli C and Celermajer DS. Long-term improvement in homocysteine levels and arterial endothelial function after 1-year folic acid supplementation. Am J Med 2002; 112: 535-539.
[14] Hernandez-Diaz S, Werler MM, Louik C and Mitchell AA. Risk of gestational hypertension in relation to folic acid supplementation during pregnancy. Am J Epidemiol 2002; 156: 806-812.
[15] Mangoni AA, Sherwood RA, Swift CG and Jackson SH. Folic acid enhances endothelial function and reduces blood pressure in smokers: a randomized controlled trial. J Intern Med 2002; 252: 497-503.
[16] van Oort FV, Melse-Boonstra A, Brouwer IA, Clarke R, West CE, Katan MB and Verhoef P. Folic acid and reduction of plasma homocysteine concentrations in older adults: a dose-response study. Am J Clin Nutr 2003; 77: 1318-1323.
[17] Woo KS, Chook P, Lolin YI, Sanderson JE, Metreweli C and Celermajer DS. Folic acid improves arterial endothelial function in adults with hyperhomocystinemia. J Am Coll Cardiol 1999; 34: 2002-2006.
[18] Mangoni AA and Jackson SH. Homocysteine and cardiovascular disease: current evidence and future prospects. Am J Med 2002; 112: 556-565.
[19] Woo KS, Sanderson JE, Sun YY, Chook P, Cheung AS, Chan LT, Metreweli C, Lolin YI and Celermajer DS. Hyperhomocyst(e)inemia is a risk factor for arterial endothelial dysfunction in humans. Circulation 2000; 101: E116.
[20] Bortolotto LA, Safar ME, Billaud E, Lacroix C, Asmar R, London GM and Blacher J. Plasma homocysteine, aortic stiffness, and renal function in hypertensive patients. Hypertension 1999; 34: 837-842.
[21] Clarke R. Lowering blood homocysteine with folic acid-based supplements: meta-analysis of randomised trials. Indian Heart J 2000; 52: S59-64.
[22] Verhaar MC, Wever RM, Kastelein JJ, van Dam T, Koomans HA and Rabelink TJ. 5-methyltetrahydrofolate, the active form of folic acid, restores endothelial function in familial hypercholesterolemia. Circulation 1998; 97: 237-241.
[23] Doshi SN, McDowell IF, Moat SJ, Lang D, Newcombe RG, Kredan MB, Lewis MJ and Goodfellow J. Folate improves endothelial function in coronary artery disease: an effect mediated by reduction of intracellular superoxide? Arterioscler Thromb Vasc Biol 2001; 21: 1196-1202.
[24] Stroes ES, van Faassen EE, Yo M, Martasek P, Boer P, Govers R and Rabelink TJ. Folic acid reverts dysfunction of endothelial nitric oxide synthase. Circ Res 2000; 86: 1129-1134.