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# The intervention of enalapril maleate and folic acid tablet on the expressions of the GRP78 and CHOP and vascular remodeling in the vascular smooth muscle cells of H-hypertensive rats with homocysteine

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**Abstract. – OBJECTIVE: GRP78 and CHOP play essential roles in endoplasmic reticulum stress (ERS) of the vascular smooth muscle cells. We aim to investigate the effect of enalapril maleate and folic acid tablet on the expressions of GRP78 and CHOP and vascular remodeling in a homocysteine (HCY)-treated hypertensive rat model.**

**MATERIALS AND METHODS: The hypertensive rat model was established with the technique of coarctation in the abdominal aorta, and the blood pressure of the rat was measured with the non-destructive tail-cuff method two weeks after operation. Thirty-six rats with hypertension were randomly divided into 3 groups (n=12 in each group). The control group received common diet and double distilled water, methionine group received 30 g/L methionine diet and double distilled water, while enalapril maleate and folic acid tablet group received 30 g/L methionine diet and 0.2 mg.kg-1.d-1 solution of enalapril maleate and folic acid tablet. Samples were collected at week 4 and week 8 for analysis. The plasma homocysteine was measured by homocysteine detector; MAP was detected through carotid artery incubation and aortic media thickness was determined by image analyses software. The expression of GRP78 and CHOP in the vascular smooth muscle cells were identified by immunohistochemistry and Western blot.**

**RESULTS: Compared with the control group, the concentration of HCY in the serum of rats in methionine group was increased significantly after 4 weeks ($p < 0.01$), and even more significant after 8 weeks ($p < 0.01$). Compared with that of methionine group, the level of HCY in enalapril maleate and folic acid tablet group rats was significantly decreased ($p < 0.01$). The level of MAP in methionine group was increased significantly after 8 weeks compared with that of control group ($p < 0.05$). However, the MAP in enalapril maleate and folic acid tablet group was decreased significantly compared with that of methionine group. Compared with control group, the media thickness of vascular smooth muscle of rats in the methionine group was increased significantly ($p < 0.05$) while was statistically reduced in the enalapril maleate and folic acid tablet group ($p < 0.05$). The expressions of GRP78 and CHOP in methionine group were significantly elevated compared to that of control in a time dependent manner ($p < 0.05$), which were remarkably down regulated in enalapril maleate and folic acid tablet group compared with that in methionine group.**

**CONCLUSIONS: The administration of enalapril maleate and folic acid tablet can maintain the normal state of cells via the alleviation of ERS and vascular damages, reduction of HCY and the thickness of arterial media as well as the improvement of vascular remodeling.**

*Key Words:*
Homocysteine, H-hypertensive, Endoplasmic reticulum stress, Vascular remodeling, GRP78, CHOP, Rats.

## Introduction

"H-type" hypertension refers to the hypertensive patient with increasing level of HCY. There are about 150 million patients suffering "H-type" hypertension in China, and the incidence is much higher than that in foreign countries. Previous studies[1-5] showed that synergetic damages of hypertension were aggravated by hyperhomocysteine (HHcy). Clinical studies showed that there was close relationship between the serum level of Hcy and the occurrence of the vascular remodeling of the hypertension. However, complicated causes included multiple molecular mechanisms,



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such as endothelial cell dysfunction[6,7], proliferation of vascular smooth muscle cells[8], platelet aggregation[9], insulin resistance and imbalance of the pro-coagulant and anti-coagulant reactions[10,11].

The endoplasmic reticulum stress (ERS), at cellular level, represents a self-protection mechanism with the imbalance of homeostasis of endoplasmic reticulum. The stress apoptosis factors including CHOP, caspase12 and the endurable and strong stress, lead to the apoptosis of vascular smooth muscle cells of aorta, and result in the vascular remodeling. Moreover, the metabolism factors such as HCY, cholesterol, glucose and fatty acid also motivate ERS[12,13]. However, whether the ERS can be one of the molecular mechanisms for the vascular remodeling of the hypertension induced by the HHcy remains poorly understood.

The enalapril maleate and folic acid tablet are a second-generation angiotensin converting enzyme inhibitor formulated with folic acid for the treatment of essential hypertensive patients with HHcy. Enalapril mainly produces the antihypertensive effect by inhibiting RAS system, while folic acid can promote Hcy methylation process, and reduce serum Hcy. A research[14] has shown that the enalapril maleate and folic acid tablet not only lower blood pressure and serum Hcy level, but also reduce the occurrence of adverse events concerning cardiovascular disease. We aimed to examine the intervention as well as the mechanism of enalapril and folic acid tablet on vascular remodeling of hypertension.

## Materials and Methods

### Materials

There were 60 male adult SD rats approved by the Ethics Committee of Tangdu Hospital of the Fourth Military University, with a body weight of 200-240 g and age of 8-10 weeks (provided by the Laboratory Animal Center of the Fourth Military University Xi'an, Shan'xi, China). Reagents were: GRP78 antibody of the rabbit anti-rat and the CHOP antibody of the rabbit anti-rat (Cambridge, CA, USA), rabbit anti-goat labeled with AP (Santa Cruz Company, Santa Cruz, CA, USA), methionine (Sigma-Aldrich, St. Louis, MO, USA), enalapril folic acid tablets, HCY detection kit and AUSA 340 II HCY tester (Shenzhen Aokang Pharmaceuticals Co., Ltd., Shenzhen, Guangdong, China). Diaminobenzidine (DAB) detection kit was obtained from Shanghai Yanjing Biological Technology (Shanghai, China). BX41 optical microscope and DP71 microscope camera systems were purchased by Japanese Olympus Optical Co., Ltd., (Shinjuku, Japan) and Image ProPlus 6.0 image analysis software was from Meyer Instruments (Houston, TX, USA). BP-100 rat non-destruction tail artery blood pressure testing and analysis software were from Chengdu Taimeng Technology Co., Ltd. (Chengdu, Sichuan, China).

## Methods

### Experimental Subgroup Established for H-type Hypertensive Rat Model

60 adult male SD rats were processed with technique of coarctation in the abdominal aorta to establish the hypertensive rat model. Briefly, rats were anesthetized through injecting 1% pelltobarbitalum natricum in the abdomens. Operation area was disinfected and covered with shop-hole towel. The abdomen of each rat was opened with an incision right in the middle, and the arterial section of the abdomen between aortas in the left and right kidneys by pulling out the stomach and intestines was isolated by the cotton ball soaked with the physiological saline solution. The abdomen aorta for the operation was removed by curved forceps and the organs in the abdomen were avoided to be pulled out of the bodies. No.7 non-tip injection needle was placed in the abdomen aorta in parallel manner. 5/0 operation thread was bound, and the needle was extracted out, in order to cause the abdomen aorta coarctation. The rats' abdomens were stitched layer by layer after being injected with 1 ml gentamycin. The incision was sterilized. The blood pressure of the quiet rats was detected by the technique of the non-destruction tail cuff at 2 weeks after the operation. 36 rats (MAP > 150 mmHg) were divided into the control group, methionine group and the enalapril maleate and folic acid tablet randomly, with each group of 12 (n = 12). The control group received diet and double distilled water, the methionine group received 30 g/L methionine diet and double distilled water, and the enalapril maleate and folic acid tablet group received 30 g/L methionine diet and 0.2 mg/kg/d solution of the enalapril maleate and folic acid tablet. The methionine diet, the enalapril maleate, and folic acid tablet were used for treatment after 4 week and 8 week, which were defined as 4W subgroup and 8W subgroup, respectively.

### Detection of HCY Concentration in Serum

2 ml blood were extracted from the vein of the lower abdomen, which was left still for 30 min, and its supernatant liquid was taken after being centrifuged for 10 min at 3000 r/min. The supernatant was stored in Eppendorf (EP) tube and placed in the refrigerator at -80°C for testing.

### Measurement of MAP

The sober rats were placed in the cylindrical fixture, put into preheating tank at 37°C for 30 min, with local vascular expansion in slightly red of its tail. The cuff of the sphygmomanometer was placed around the tail near the heart of the rat. The highly sensitive pulse transducer was placed around 1/3 above its tail, whose surface was fixed with the cuff at the side of the tail near its abdomen. The blood pressure and heart rates were measured. SBP and DBP, MBP = DBP+1/3 (SBP-DBP).

### Measurement of the Media Thickness of the Aorta

After blood pressure was recorded, the chest was opened quickly. The aorta was incised in about 1.0 cm, flushed with phosphate-buffered saline (PBS), fixed with PFA (40 g/L), wrapped and buried with the paraffin wax. Among pieces of slices in the thickness of 55 μm, 5 were chosen from the paraffin slices of the chest aorta in every subgroup, with 3 fields of vision chosen from every slice randomly. Image-proplus 6.0 medical analysis system was used for measuring the thickness of the media in the aorta of the rat, with the mean taken.

### Expressions of GRP78 and CHOP in VSMCS of the Aorta Detected with the Immunohistochmistry

After conventional dewaxing, the paraffin wax slice was added with 30 ml/L $H_2O_2$ for 10 min, and the citrate buffer solution (0.01 mol/L, PH6.0) was used for restoring the tissue antigen in the microwave oven for 15 min, flushed with phosphate-buffered saline (PBS) after cooling. 50 ml/L BSA sealing liquid was dripped and sealed at room temperature for 20 min; 1:100 mouse-anti-rat GRP78 antibody (through the night 4°C, flushed with PBS), anti-rabbit IgG-Biotin antibody (incubated at 37°C for 30 min, flushed with PBS) and SABC (incubated at 37°C for 20 min, flushed with PBS) were dripped in turn. After being stained with DAB, the hematoxylin was re-stained slightly, and the results were observed in the microscope after being dehydrated, transparent, and sealed.

### Analysis of the Protein Content of GRP78 and CHOP by Western Blot

The protein content was detected with Bradford method, after RIPA splitting solution in 10 times of tissue volume was used for extracting total protein of the vascular smooth muscles. After the upper sample volume was calculated, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transmembrane were conducted. 1:1000 rabbit-anti rat GRP78 antibody and 1:1000 rabbit-anti rat CHOP antibody were added respectively, followed by incubation at 4°C overnight. It was flushed with PBS buffer for three times, with each of time for 5 min. Goat-anti rabbit IgG with 1:500 AP label was added and placed at room temperature for incubation for 30 min, shaken and detained by the shaker for 3 times, 5 min each time. The strip was put into double distilled water and the reaction was terminated after being stained by dripping AP alkali phosphatase chromogenic agent. Gray scale analysis was conducted after scanning. The analysis was conducted with the ratio between the gray scale value of the protein from GRP78 and CHOP and corresponding gray scale value of β-actin.

### Statistical Analysis

The experimental data were denoted in mean ± standard deviation (SD), and SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the significance between groups. ANOVA of single factor was used for comparing the mean of many samples, and LSD-*t* test was used for comparing the means in two groups. $p < 0.05$ indicated a statistical significance.

## Results

### The Change of the Blood Pressure of the Rats in Various Groups

After 4 weeks, compared with control group, MAP was decreased significantly after being intervened by the enalapril maleate and folic acid tablet, compared with methionine group ($p < 0.05$). After 8 weeks, compared with control group, MAP in methionine group was increased

significantly ($p < 0.05$), which was decreased in the enalapril maleate and folic acid tablet group compared with that in methionine group ($p < 0.05$) (Table I).

### The Change of Concentration of HCY for the Rates in Various Groups

The concentration of HCY in methionine group was significantly higher than that in control group ($p < 0.01$). After being intervened by enalapril maleate and folic acid tablet, HCY level was decreased significantly compared with methionine group ($p < 0.01$) (Table II).

### The Change of GRP78 Expressions in the Vascular Smooth Muscles of the Rats in Various Groups

The arterial vascellum in the chest of rats was taken for immunohistochemistry analysis at 4 weeks and 8 weeks post intervention. It was discovered that GRP78 expressions in the arterial vascular smooth muscle tissues of rats were basically located in the cytoplasm with pale brown particles shown in the immunohistochemistry. The result also showed that the OD value of GRP78 expression in control group, methionine group and enalapril maleate and folic acid tablet group after 4 weeks and 8 weeks were 0.74 ± 0.030, 0.51 ± 0.032; 0.82 ± 0.031, 0.63 ± 0.029; 0.41 ± 0.030,0.33 ± 0.033, respectively. Compared with control group, GRP78 protein expression in VSMCs of methionine group was increased significantly ($p < 0.05$). However, the treatment of the enalapril maleate and folic acid tablet significantly decreased GRP78 protein expression compared with methionine group ($p < 0.05$) (Figure 1 and 2).

### The Change of CHOP Expression in the Vascular Smooth Muscles of Rats in Various Groups

Similar to the GRP78, it was discovered that the CHOP expressions in the arterial vascular smooth muscle tissues of the rats were basically located in the cytoplasm. The OD values of CHOP expression in VSMCs in methionine group after 4 weeks and 8 weeks were 0.68 ± 0.030 and 0.87 ± 0.034, respectively, which were significantly increased compared with that in the control group (0.56 ± 0.029 and 0.76 ± 0.031) ($p < 0.05$). Nevertheless, the OD values of CHOP expressions of VSMCs in the enalapril maleate and folic acid tablet group were 0.31 ± 0.032 and 0.43 ± 0.033, respectively, which were significantly decreased, compared with the corresponding subgroups of methionine group after 4 weeks and 8 weeks ($p < 0.05$) (Figure 3 and 4).

### The Change of GRP78 and CHOP Protein Volume in the Vascular Smooth Muscles in the Rats of Various Groups

The levels of GRP78 and CHOP were also assessed by Western blot analysis. The result showed that GRP78 expressions in the VMSCs from various groups were decreased gradually. The levels of GRP78 and CHOP in methionine group were remarkably elevated compared to control group while in enalapril maleate and folic acid tablet group, the expressions were significantly reduced (Figure 5-7).

**Table I.** The change of the MAP of the rats in various groups (n = 6, ± s, mmHg)

| Group /time | Control group | Methionine group | Enalapril maleate and folic acid tablets group |
|---|---|---|---|
| 4W | 162.25 ± 6.71 | 166.35 ± 8.17 | 130.78 ± 6.97[b] |
| 8W | 170.98 ± 9.29 | 178.53 ± 9.66[a] | 140.56 ± 5.36[b] |

[a]Compared to control group, $p < 0.05$; [b]Compared to methionine group, $p < 0.05$.

**Table II.** The comparison of HCY values for the rats in various group (n = 12, ± s, μmol/L).

| Group /time | Control group | Methionine group | Enalapril maleate and folic acid tablets group |
|---|---|---|---|
| 4W | 6.45 ± 1.56 | 41.65 ± 8.27[a] | 10.89 ± 6.58[b] |
| 8W | 7.49 ± 1.36 | 71.93 ± 10.36[a] | 16.75 ± 8.63[b] |

[a]Compared to control group, $p < 0.05$; [b]Compared to methionine group, $p < 0.05$.

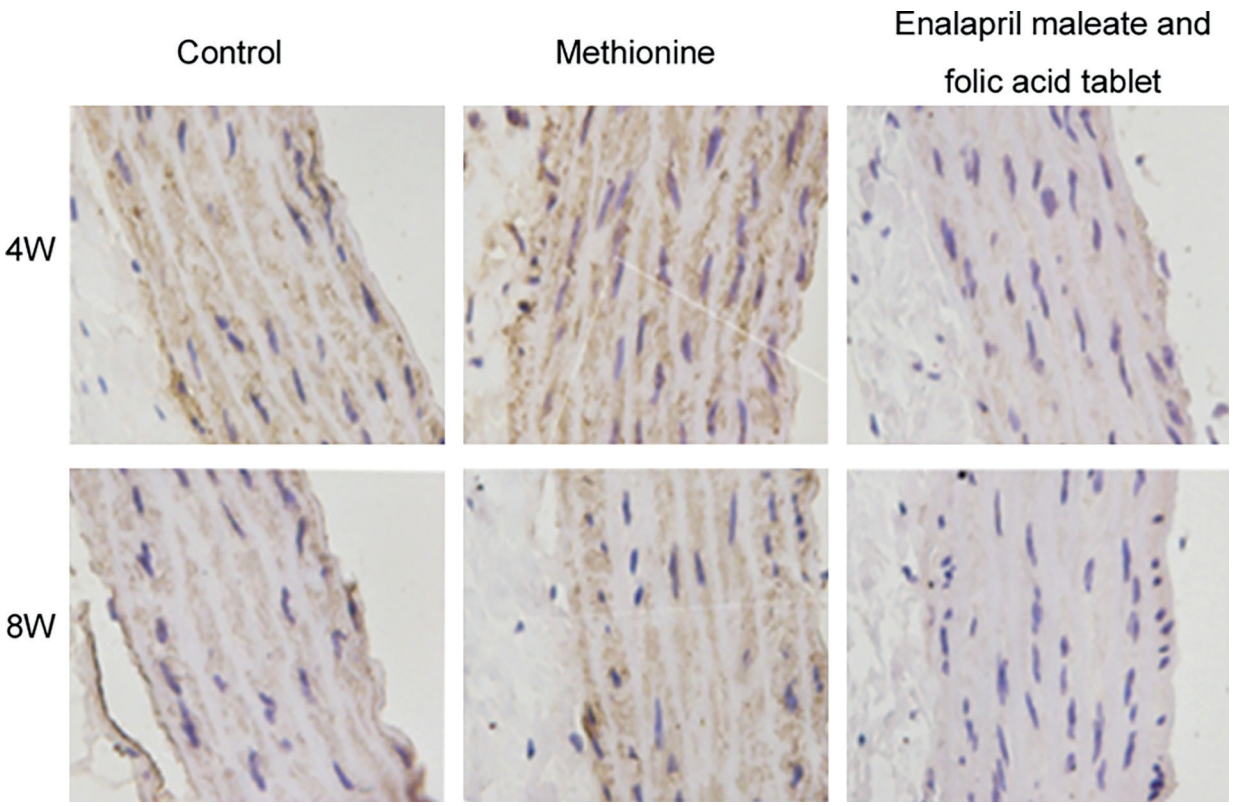


**Figure 1.** The GRP78 expressions of the vascular smooth muscles of the rates in various groups (×400).

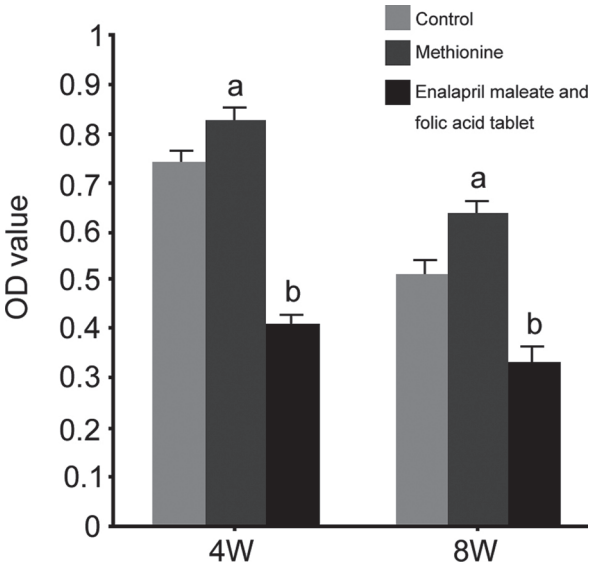


**Figure 2.** The expressions of GRP78 in the vascular tissues of the rats in various groups. The comparison between the methionine group and the control group ($^{a}p < 0.05$); The comparison between the enalapril maleate and folic acid tablet group and the methionine group ($^{b}p < 0.05$).

### *The Change of the Arterial Media Thickness of the Rats in Various Groups*

Compared with control group, the arterial media thickness of the chest in methionine group was increased significantly ($p < 0.05$); compared with methionine group, the arterial media thickness after being intervened by the enalapril maleate and folic acid tablet was decreased significantly ($p < 0.05$) (Table III).

## Discussion

There are 260 million hypertensive patients in China currently, among whom the patients with increasing HCY account for 75.5%, and more than 80% adult hypertensive patients suffer from the vascular remodeling characterized as thickening vascular wall. A multitude of factors are involved in the development of hypertension while hypertension presents a critical cause to diverse diseases[15]. Graham et al[16] demonstrated that there was significant synergy between hypertension and HHcy in the occurrence of cardio-cerebrovascular disease[17-19]. This study firstly

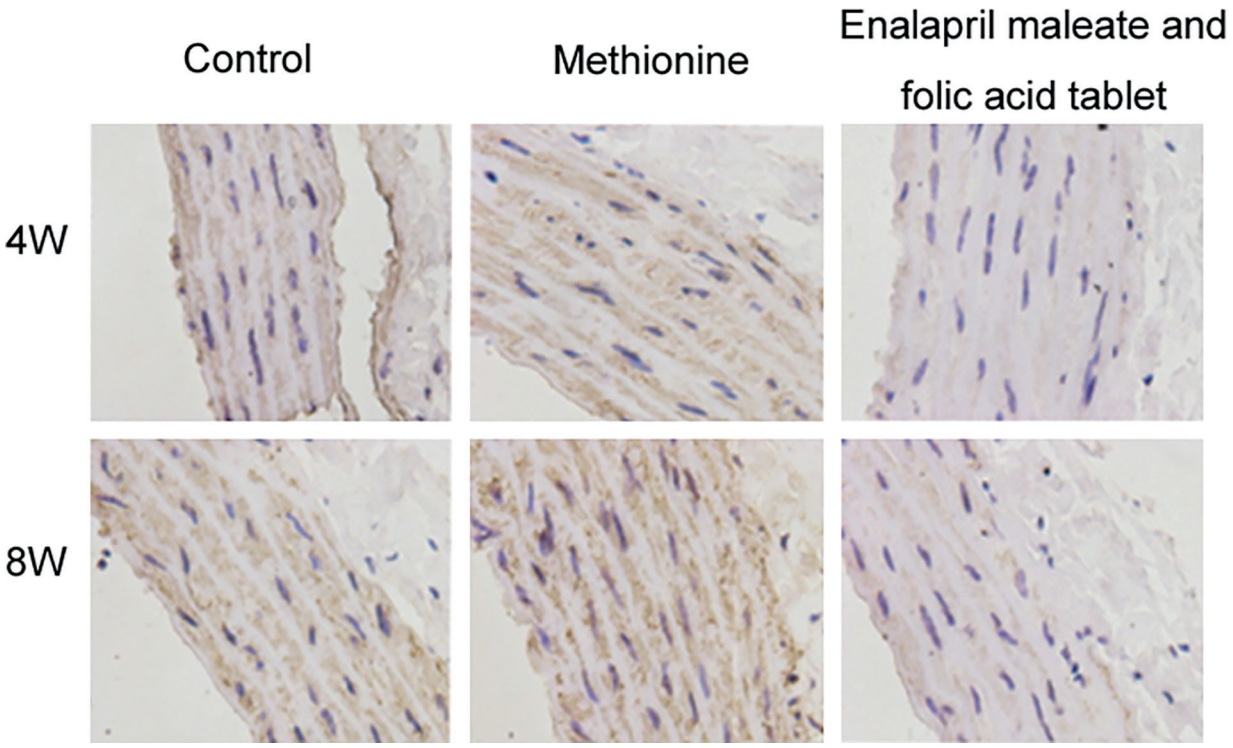


**Figure 3.** The expressions of the CHOP of the vascular smooth muscles of the rats in various groups (×400).

established the hypertension rat model with the diet of methionine in high concentration (according to relevant report[20]), in order to prepare hypertensive rat with HHcy after 4 weeks. High-dose of methionine increased the intermediate metabolism product from the cycle of HCY in the blood, leading to growing level of HCY in blood. The establishment of this model simulated the effects of the increasing level of HCY in the serum on the vascular remodeling of the hypertension and showed the MAP, blood pressure, vascular wall of rats were remarkably changed in methionine group, compared with control group. HE staining showed that the muscle cells of the vascular smooth muscles were hypertrophic, the

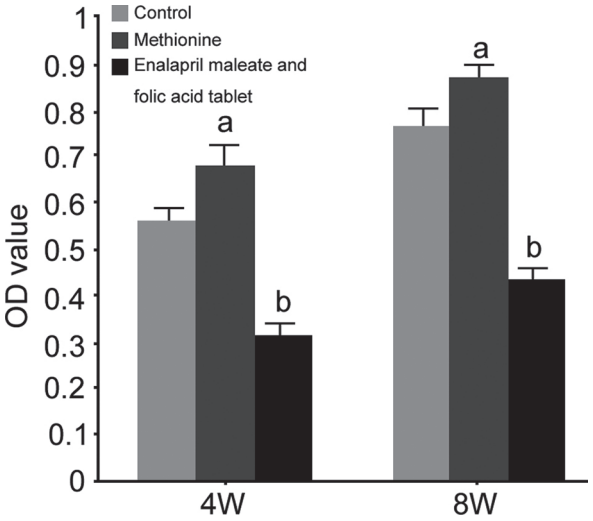


**Figure 4.** The expressions of the CHOP in the vascular tissues of the rats in various groups. The comparison between the methionine group and the control group ($^{a}p < 0.05$); The comparison between the enalapril maleate and folic acid tablet group and the methionine group ($^{b}p < 0.05$).

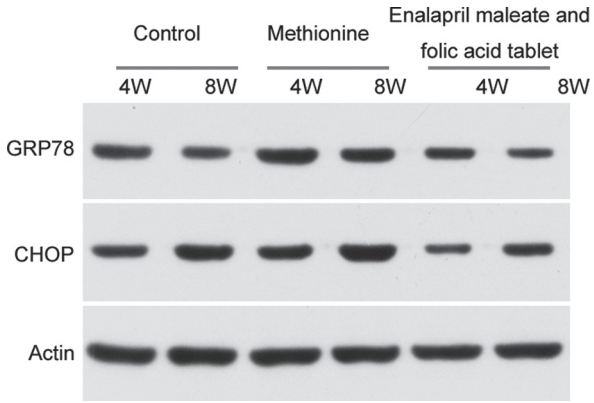


**Figure 5.** The picture of Western blot for GRP78 and CHOP protein expressions of the vascular smooth muscles in the rats of various groups. The quantitative analysis of GRP78 expressions.

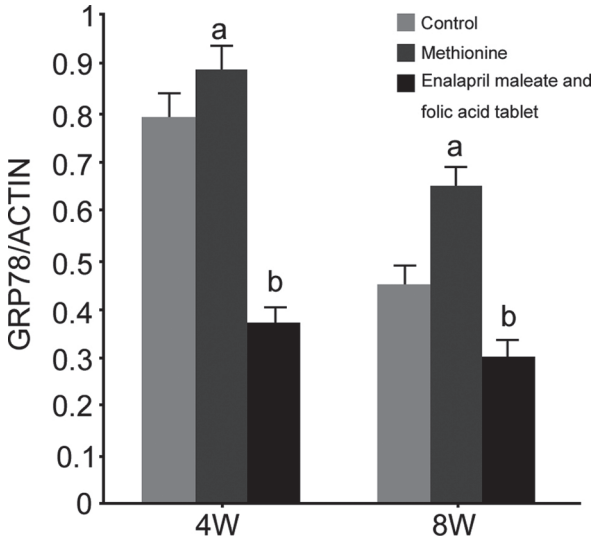


**Figure 6.** The comparison between the methionine group and the control group ($^{a}p < 0.05$). The comparison between the enalapril maleate and folic acid tablet group and the methionine group ($^{b}p < 0.05$). The quantitative analysis of CHOP expressions.

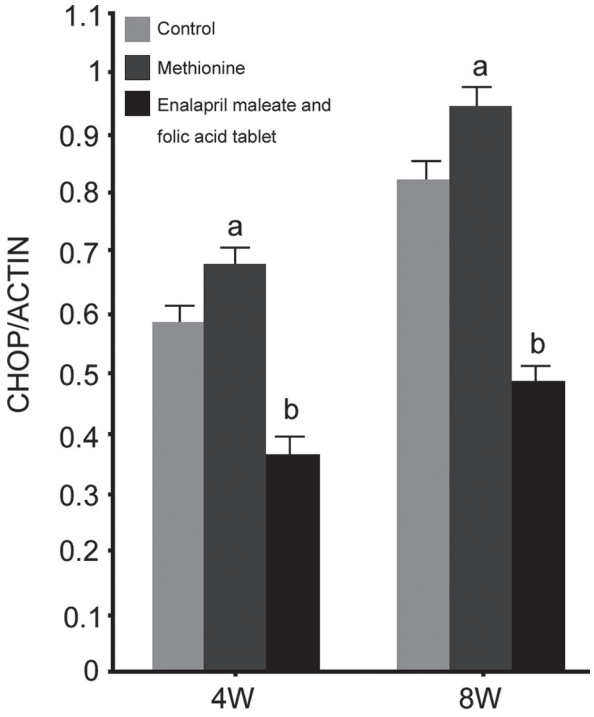


**Figure 7.** The comparison between the methionine group and the control group ($^{a}p < 0.05$); The comparison between the enalapril maleate and folic acid tablet group and the methionine group ($^{b}p < 0.05$).

nucleus was abnormal and the arrangement was chaotic, indicating that the high concentration of HCY in serum can damage the vascular smooth muscle cells, and cause the vascular smooth muscle cells hypertrophic.

Our previous research demonstrated that the long-term and endurable vascular pressure load can induce the dissociation of the chaperonin GRP78, GRP94 and transmembrane protein IRE1, PERK, ATF on the surface of the endoplasmic reticulum in the vascular smooth muscle cells. The transmission of the stress signal for the occurrence of stress reaction was activated, and the expressions of GRP78 was increased at the beginning of hypertension. The accumulation of unfold protein was prevented by protease, and homeostasis of the endoplasmic reticulum in the cells was maintained[21,22]. However, the endurable and overly strong ERS can activate the pro-apoptosis factor caspase-12 and CHOP, mediate vascular smooth muscle cells hypertrophic, and cause the disorder of the cell metabolism[23,24], leading to the apoptosis of the cells and the vascular remodeling[20]. This study showed that the GRP78 and CHOP expressions of the vascular smooth muscle cells were increased significantly in the vascular smooth muscle cells. It indicated that the early hypertension activated the ERS of the vascular smooth muscle cells, and the increase of GRP78 expression recovered the homeostasis of the endoplasmic reticulum and maintained the survival of the cells, with continuous increase of the blood pressure. ERS initiated the channel of the apoptosis signal, and the increase of the CHOP expressions of the pro-apoptosis factor mediated the apoptosis of the cells, which was consistent as the previous study. The increase of the HCY level in the serum allowed overly early and strong

**Table III.** The change of the arterial media thickness of the rats in various groups (n = 6, ±, μm).

| Group /time | Control group | Methionine group | Enalapril maleate and folic acid tablets group |
|---|---|---|---|
| 4W | 102.96 ± 2.91 | 109.76 ± 3.09[a] | 94.78 ± 2.79[b] |
| 8W | 109.01 ± 3.53 | 118.42 ± 2.81[a] | 97.03 ± 2.96[b] |

[a]Compared to control group, $p < 0.05$; [b]Compared to methionine group, $p < 0.05$.

ERS production in the vascular smooth muscle cells of hypertensive rats, and the apoptosis of the vascular smooth muscle cells mediated by the ERS was affected, with the increase of the HCY level in the serum, resulting in the vascular remodeling of the hypertension. Therefore, the ERS may be one of the molecular mechanisms on the synergy between the HHcy and hypertension.

The enalapril maleate and folic acid tablet is an innovative compound formulation combining the folic acid and the enalapril and contributes to general prevention and treatment of the multiple dangerous factors. It not only reduces the level of HCY and side effects of long-time oral administration of folic acid, but also down regulates the level of blood pressure effectively. Of note, the unique effects of the enalapril maleate and folic acid tablet on the treatment of hypertension with HHcy were identified, consistently with above report. The study also showed that the thickness of the vascular wall of the rat suffering hypertension and HHcy was decreased significantly after being intervened by the enalapril maleate and folic acid tablet for 4 weeks, compared with the rat without intervention, suggesting that the enalapril maleate and folic acid tablet can protect the vascular smooth muscles effectively and reduce the synergetic damages of hypertension with HHcy on the vascellum while treating the hypertension with HHcy. The enalapril maleate and folic acid tablet significantly down regulated the expressions of GRP78 and CHOP proteins of the vascular muscle tissues of the rats, compared with the methionine group, indicating that the enalapril maleate and folic acid tablet can inhibit the expressions of the CHOP protein in the pro-apoptotic factors effectively, protect the vascular smooth muscle cells and reduce the synergetic damages to the vascular smooth muscle cells caused by the HHcy and hypertension.

## Conclusions

Our data demonstrated that enalapril maleate and folic acid tablet can reduce the blood pressure and HCY level in the serum effectively, protect the vascular smooth muscles by inhibiting the ERS, and relieve the vascular remodeling. Our investigation provides new fundamental theory basis for standardized treatment of the cardio-cerebrovascular diseases caused by "H"-type hypertension.

### Acknowledgements

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### Conflict of Interest

The Authors declare that they have no conflict of interests.

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