Vascular protective effects of *Astragalus membranaceus* and its main constituents in rats with chronic hyperhomocysteinemia

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**Abstract.** Hyperhomocysteinemia (HHcy) is an important factor in cardiovascular disease. However, is currently no cure available in western medicine for HHcy-evoked cardiovascular disease. The present study explored the vascular protective effects of *Astragalus membranaceus* (AM), a traditional Chinese medicine. Rats with HHcy were induced by feeding high-methionine diets and treated with total extract of AM (TEA) and its constituents, including *Astragalus* saponins (ASP), *Astragalus* total flavonoids (ATF) and *Astragalus* polysaccharides (APS). Examination of the rats indicated that TEA and ASP controlled blood pressure and ameliorated HHcy-induced impairment of endothelium-dependent vasorelaxation by increasing the nitric oxide content and nitric oxide synthase activity of the abdominal aorta. Furthermore, they decreased the accumulation of hydrogen peroxide and superoxide anion, and attenuated the inhibition of superoxide dismutase and catalase activities in rats with HHcy. Additionally, TEA and ASP attenuated the HHcy-induced increases of matrix metalloproteinase (MMP)-2 and -9 concentrations. However, similar effects were not observed for ATF and APS. In conclusion, TEA and ASP are beneficial to vascular disease, and their effects may be attributed to protective actions against oxidation, activity of the MMPs and endothelial dysfunction.

**Introduction**

Cardiovascular disease, which is characterized by dysfunctional conditions of the heart, arteries and veins, remains the most common cause of morbidity and mortality and is a serious threat to human health (1). Age, gender, cigarette smoking, physical inactivity, high blood pressure and serum cholesterol, elevated serum glucose, obesity and family history have been identified as classic risk factors for the development of cardiovascular disease (2). However, even when all these risk factors are considered, they only explain approximately one-half to two-thirds of the occurrence of cardiovascular disease (1). It appears that additional crucial risk factors remain to be identified. Elevated plasma levels of homocysteine (Hcy) have been identified as classic risk factors for the development of cardiovascular disease (3).

Hcy is the main metabolite of methionine, and its levels can be inordinately increased due to genetic enzymatic deficiencies and nutritional defects, both of which affect the proper metabolism of methionine (4). Clinical and animal studies have identified that hyperhomocysteinemia (HHcy) may be important in the damage of the vascular structure and function (5-7). Although a unifying hypothesis has not been established thus far, stress, endothelial dysfunction and vascular remodeling are considered as the primary pathological mechanisms underlying vascular diseases following chronic exposure to Hcy (1). However, in a review of several randomized controlled trials, it was shown that Hcy-lowering interventions were not able to prevent cardiovascular events (8). It appears that there is not yet a significant cure for HHcy-evoked vascular disease in western medicine.

According to the original recording in Shen Nong’s *Materia Medica* >2,000 years ago (9), *Astragalus membranaceus* (AM) has been widely used in traditional Chinese medicine to treat patients with cardiovascular disease. *Astragalus* saponins (ASP), *Astragalus* total flavonoids (ATF) and *Astragalus* polysaccharides (APS) have been identified as the main bioactive chemicals in AM (9). Observations in previous studies revealed that this herb was able to prevent lipid peroxidation by improving antioxidant enzyme activity and scavenging free radicals, improve vasorelaxation by increasing levels of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP), and hinder the process of pathological vascular remodeling (10-12). Furthermore, previous in vitro studies from the present author's laboratory demonstrated that AM and its main components may be advantageous in the treatment of Hcy-mediated endothelial dysfunction by stabilizing the NO/NO synthase (NOS) pathway (13,14).
The aim of the present study was to investigate the vascular protective effect of AM and its main constituents in rats with chronic HHcy and to explore the underlying mechanism.

Materials and methods

Materials. AM roots were collected at Longxi (Gansu, China). A total extract of AM (TEA; yield, 9.8%) and bioactive constituents (yield: APS, 6.4%; ATF, 3.6%; ASP, 1.1%) from the AM roots was prepared by the Department of Pharmacy at the First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China) as previously described (14).

Animals and experimental protocol. A total of 72 male 8-week-old Sprague-Dawley rats (weighing 240-260 g) were obtained from Laboratory Animal Center in the Chinese Academy of Science (Shanghai, China). Rats were housed in a room at a constant temperature (23-26°C) and humidity (40-60%) with a 12-h light/dark cycle. They were randomly divided into 6 groups (each n=12): i) Control, ii) HHcy, iii) TEA, iv) ASP, v) APS and vi) ATF groups. HHcy was induced in the rats by feeding a high-methionine (2% by weight) diet (15). Rats in the control group received a regular diet. In the TEA, ASP, APS and ATF groups, rats fed a high-methionine diet were treated with TEA (196 mg/kg), APS (128 mg/kg), ATF (72 mg/kg) and ASP (22 mg/kg), respectively, by intraperitoneal injection once per day. The rats in the control and HHcy groups were intraperitoneally administered the same amount of double-distilled water. The above experimental protocol was maintained for 6 weeks prior to examination.

The body weight and systolic blood pressure/diastolic blood pressure (SBP/DBP) of conscious rats were monitored weekly (16). At the end of the experiment in week 6, blood samples were collected from each rat by cardiac puncture using an EDTA-treated syringe following anesthesia. Serum was prepared by centrifugation at 3,000 x g for 20 min at 4°C and then stored at -80°C until assayed. The aorta was extracted from the animal immediately following sacrifice. A section of the abdominal aorta was kept at -71°C and chopped into small segments, homogenized in PBS on ice and then centrifuged at 1,000 x g for 10 min prior to Performing a biochemical analysis. The remainder was frozen and prepared with a 10-µm thick section using a cryostat microtome for reactive oxygen species (ROS) measurement. In order to analyze the isometric force, the surrounding fat and connective tissues of the thoracic aorta were carefully dissociated. The study protocol was approved by the Animal Care and Use Committee of The First Affiliated Hospital, College of Medicine, Zhejiang University.

Measurement of biochemical parameters in aortic homogenates. The abdominal aorta was used to measure ROS, catalase, superoxide dismutase (SOD), endothelial NOS (eNOS), NO and matrix metalloproteinases (MMPs). The protein content was accessed in an aliquot of the homogenate by the bicinchoninic acid assay method (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China).

Determination of total serum Hcy, glucose and cholesterol levels. Total serum glucose and cholesterol levels were determined by standard enzymatic techniques (17). Total serum Hcy concentrations were measured using a commercially available ELISA kit (CSB-E13376r; Cusabio Biotech Co., Ltd., College Park, MD, USA).

Measurement of ROS levels. Aortic tissue was prepared as previously described (18,19). The fluorescent probes 2,7-dichlorofluorescin-diacetate (DCFH-DA) and dihydroethidium (DHE) (both from Molecular Probes, Inc., Eugene, OR, USA) were used to evaluate the amount of hydrogen peroxide and superoxide anion, respectively. In the cytoplasm, the ester groups of DCFH-DA are hydrolyzed by cellular esterase to form DCFH, which is subsequently oxidized to intracellular ROS to highly fluorescent DCF, whereas DHE reacts with ROS and forms ethidium bromide, which binds to DNA and produces red fluorescence. The fluorescence (excitation 488 nm and emission 525 nm; excitation 518 nm and emission 605 nm) was monitored using a fluorescence microscope (Nikon TE2000; Nikon Corporation, Tokyo, Japan). The fluorescence intensities were recorded and analyzed using a charge-coupled device camera (CoolSNAP HQ) with an image analysis system (MetaMorph) (both from Nippon Roper K.K., Chiba, Japan).

Measurement of catalese and SOD activities. The activities of catalese (catalase assay kit; cat no. A007-1) and SOD (superoxide dismutase assay kit; cat no. A001-3) were determined using commercially available kits following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Research Institute). The catalese activity assay was based on the reaction of methanol with the enzyme in the presence of an optimal concentration of hydrogen peroxide, while the SOD activity assay utilized xanthine oxidase and hypoxanthine to generate superoxide radicals.

Measurement of the levels of NO and the activity of eNOS. NO is rapidly consumed to generate peroxynitrite in the presence of superoxide (20). Therefore, aortic nitrite levels were measured as a level of NO inactivation due to superoxide. Nitrite was estimated colorimetrically using Griess reagent (21). The activity of eNOS was determined from the rate of conversion of L-arginine to L-citrulline. All assays were performed according to the manufacturer’s instructions (NO assay kit; cat no. A012; NOS typed assay kit; cat no. A014-1; Nanjing Jiancheng Bioengineering Research Institute).

Measurement of the concentrations of MMP-2 and -9. MMP-2 and -9 were assessed using commercial ELISA kits (Rat MMP-2 ELISA kit, cat no. RA20502; Rat MMP-9 ELISA kit, cat no. RA20128; BioSwamp, Wuhan, China) according to the manufacturer’s instructions (22).

Measurement of vasodilation using isometric tension recordings. Prepared thoracic aortic rings were suspended isometrically between two stirrups in an organ bath filled with 10 ml modified Krebs solution at 37°C, which was ventilated continuously with 95% O₂ and 5% CO₂. The isometric tension...
was recorded using a force transducer (JZ101; metrical range, 0-5.0 g) and MedLab 5.0v recording system (Nanjing Medease Science and Technology Co., Ltd., Nanjing, China). A resting tension of 2.0 g was maintained for each ring. All rings were allowed to equilibrate for 60 min prior to the start of the experiment and then challenged twice by KCl (60 mmol/l) to ensure the repeatability of contractions. Thereafter, phenylephrine (PE; 1 µmol/l) was used to induce a steady contraction. Once a stable tension was established, vessel relaxation was determined as the response to an accumulative addition of an endothelium-dependent dilator agent, acetylcholine (Ach; 0.1 nmol/l to 1 µmol/l), and an endothelium-independent dilator agent and sodium nitroprusside (SNP; 0.1 nmol/l to 1 µmol/l). A number of vessels were incubated for 30 min with 0.1 mmol/l N(omega)-nitro-L-arginine-methyl ester (L-NAME), an inhibitor of NOS, and the responses to Ach (0.1 nmol/l to 1 µmol/l) were then assessed.

**Statistical analysis.** All values are presented as the mean ± standard deviation of n experiments from different rats. Vasorelaxation is expressed as the percentage of the tension evoked by PE (1 µmol/l). The data was analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analysis was performed by Student-Newman-Keuls test or one-way analysis of variance. P<0.05 was considered to be statistically significant.

**Results**

**Body weight, blood pressure and metabolic parameters.** At the end of the experiment, there was no significant difference observed in the body weight among any of the groups (data not shown).

As shown in Table I, the high-methionine diet significantly increased the SBP/DBP (115.91±3.70/81.83±2.86 mmHg) in rats of the HHcy group compared with the control group (103.08±3.60/65.33±2.71 mmHg). TEA (108.50±3.75/74.83±2.92 mmHg) and ASP (109.42±4.29/75.00±3.02 mmHg) but not APS (114.92±4.34/79.83±2.69 mmHg) and ATF (115.18±2.86/80.58±2.94 mmHg) significantly attenuated the HHcy-induced increase in SBP/DBP.

Serum Hcy levels in rats fed with high-methionine diets [group (µmol/l): HHcy (34.78±1.39), TEA (33.91±1.69), ASP (33.78±1.74), APS (33.93±2.00) and ATF (34.41±2.32)] were significantly higher compared with those in rats fed with regular diets (control group, 6.92±0.61 µmol/l). Treatment with TEA, ASP, APS or ATF did not influence the levels of serum Hcy. Serum cholesterol levels (1.03±0.17 µmol/l) in rats with high-methionine diets were higher than those in the control group (0.85±0.11 µmol/l). However, treatment with TEA (1.00±0.17 µmol/l), ASP (1.04±0.16 µmol/l) or ATF (1.03±0.17 µmol/l) did not exhibit any significant difference compared with the HHcy group. Additionally, there were no significant differences in serum glucose levels among any of the groups (Table I).

**Redox system biomarker levels.** The aortic DCFH-DA fluorescence intensity, which reflects the hydrogen peroxide level, was elevated in the rat aorta of the HHcy group when compared with the control group. Treatment with TEA or ASP, but not APS nor ATF, significantly reduced the hydrogen peroxide level in HHcy rats. A similar response was observed in the determination of the arterial DHE fluorescence intensity, which reflects the superoxide anion level (Fig. 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>Hcy (µmol/l)</th>
<th>Cholesterol (mmol/l)</th>
<th>Glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>450.8±15.0</td>
<td>103.08±3.60</td>
<td>65.33±2.71</td>
<td>6.92±0.61</td>
<td>0.85±0.11</td>
<td>4.96±0.32</td>
</tr>
<tr>
<td>HHcy</td>
<td>448.0±13.8</td>
<td>115.91±3.70</td>
<td>81.83±2.86</td>
<td>34.78±1.39</td>
<td>1.03±0.17</td>
<td>4.86±0.32</td>
</tr>
<tr>
<td>TEA</td>
<td>449.7±13.5</td>
<td>108.50±3.75</td>
<td>74.83±2.92</td>
<td>33.91±1.69</td>
<td>1.00±0.17</td>
<td>4.93±0.34</td>
</tr>
<tr>
<td>ASP</td>
<td>452.2±13.9</td>
<td>109.42±4.29</td>
<td>75.00±3.02</td>
<td>33.78±1.74</td>
<td>1.03±0.17</td>
<td>4.87±0.35</td>
</tr>
<tr>
<td>APS</td>
<td>449.3±14.7</td>
<td>114.92±4.34</td>
<td>79.83±2.69</td>
<td>33.93±2.00</td>
<td>1.04±0.16</td>
<td>4.86±0.30</td>
</tr>
<tr>
<td>ATF</td>
<td>444.9±13.8</td>
<td>115.18±2.86</td>
<td>80.58±2.94</td>
<td>34.41±2.32</td>
<td>1.03±0.17</td>
<td>4.97±0.32</td>
</tr>
</tbody>
</table>

Table I. Body weight, blood pressure and metabolic parameters.

SBP, systolic blood pressure; DBP, diastolic blood pressure; Hcy, homocysteine; HHcy, hyperhomocysteinemia; TEA, total extract of Astragalus membranaceus; ASP, Astragalus saponins; APS, Astragalus polysaccharides; ATF, Astragalus total flavonoids. *P<0.01 and **P<0.05 vs. the control group; ***P<0.01 vs. the HHcy group.
By contrast, the aortic SOD (6.57±1.75 U/mg protein) and catalase (43.17±5.62 U/mg protein) activities were evidently reduced in HHcy rats as compared with the control group (SOD, 16.30±1.65 U/mg protein; catalase, 68.24±7.76 U/mg protein, P<0.01). Additionally, TEA and ASP significantly increased the activities of SOD (TEA, 14.40±1.62 U/mg protein; ASP, 12.29±1.58 U/mg protein) and catalase (TEA, 56.84±8.48 U/mg protein; ASP, 53.85±8.40 U/mg protein) compared with those in the HHcy group. The other components, APS and ATF, had no ameliorating effects on the activities of SOD and catalase (Fig. 2).

Levels of NO and the activity of eNOS. The aortic NO levels of various groups are shown in Fig. 3. The NO levels were significantly lower in the HHcy group (5.29±0.56 µmol/l/g protein) compared with the control group (8.37±0.90 µmol/l/g protein). Furthermore, TEA (7.45±0.92 µmol/l/g protein) and ASP (7.41±0.87 µmol/l/g protein) treatment significantly increased aortic NO contents as compared with those in the HHcy group. Finally, there was no significant change in the aortic NO levels of the APS (5.35±1.09 µmol/l/g protein) and ATF (5.50±0.81 µmol/l/g protein) groups compared with the HHcy group.

As shown in Fig. 4, chronic exposure to Hcy decreased the eNOS activity by 63.9%, which was markedly reversed by treatment with TEA and ASP, respectively. The eNOS activities in aortic homogenates from the APS and ATF groups demonstrated no difference in comparison with the HHcy group.

Tissue contents of MMP-2 and -9. When HHcy was successfully induced by a high-methionine diet, significantly increased MMP-2 (HHcy vs. control: 17.86±1.01 vs. 5.28±0.19 ng/mg protein) and MMP-9 (HHcy vs. control: 10.62±1.33 vs. 2.93±0.43 ng/mg protein) concentrations were observed. The increase in MMP-2 content was clearly restrained by TEA (10.32±1.21 ng/mg protein) or ASP (11.03±0.97 ng/mg protein) compared with the HHcy group (17.86±1.01 ng/mg protein). MMP-9 was also reduced to a similar extent by TEA or ASP. However, APS and ATF did not appear to influence the concentration of MMP-2 or -9 (Fig. 5).

Relaxation response of the thoracic aorta to Ach or SNP. In a dose-dependent manner, Ach (0.1 nmol/l to 1 µmol/l) elicited greater relaxations of the aortic rings from the control group compared with those in the HHcy group. Furthermore, TEA or ASP, but not APS or ATF, significantly increased the dilatation of aortic rings compared with those in the HHcy group. However, the relaxation responses of aortic rings from TEA or ASP groups did not differ markedly from those of the HHcy group (Fig. 6).

Following incubation for 30 min with L-NAME, Ach-induced relaxation of aortic rings from the control and
TEA group, but not from the HHcy group, decreased significantly (Fig. 7). Furthermore, in a dose-dependent manner, SNP elicited similar dilations of aortic rings from the control, HHcy and TEA groups (Fig. 8).

Discussion

Epidemiological studies have revealed that moderate HHcy is highly prevalent in the general population and HHcy has been recognized as an increased risk factor for the development of cardiovascular disease (23).

The present study observed a sustained increase of the resting SBP/DBP and significantly elevated cholesterol levels when moderate HHcy was induced by chronic methionine feeding. These results were consistent with previous clinical and animal studies (24,25). Hcy levels have been reported to be higher in patients with essential hypertension compared with normotensive subjects, with higher levels of Hcy being associated with an increased risk of hypertension (26). Furthermore, increased SBP/DBP returned to control values when Hcy levels were normalized following the discontinuation of Hcy-elevating treatment (27). Additionally, the accumulation of lipids in the arterial wall, particularly cholesterol, is a major event preceding the formation of atherosclerotic lesions (28-30). Thus, it may be hypothesized that there is a causal correlation between HHcy and cardiovascular changes.

According to previous studies, one of the mechanisms by which HHcy impairs the vessel is Hcy-elicited endothelial dysfunction (5-7). Our previous in vitro studies (13,14) and the present in vivo study indicated that Hcy impaired Ach-induced endothelium-dependent vasodilation, but did not alter SNP-induced endothelium-independent vasorelaxation. Additionally, pretreatment with L-NAME, an inhibitor of
NOS, did not induce further relaxation of aortic rings from HHcy rats. Furthermore, it was demonstrated that chronic exposure to Hcy decreased the aortic NO content and inhibited eNOS activity. These results suggest that HHcy induced endothelial dysfunction by the endogenous NO/NOS pathway.

One of the mechanisms by which every component of the NO metabolism, including synthesis, release or action, may be impaired is an increased formation of ROS and the diminished expression and activity of antioxidant enzymes. The results of the present study indicated that hydrogen peroxide and superoxide anion were significantly accumulated in the aorta of the HHcy group compared with the control group, while the activities of SOD and catalase were evidently reduced.

An increase in oxidative stress accompanied by decreased NO bioavailability may lead to the increased activity of MMPs. MMPs, a family of specialized proteases, initiate the process of breakdown and synthesis of the extracellular matrix that is responsible for vascular remodeling (31). In the present study, an increased concentration of MMP-2 and -9 in the aorta of HHcy rats was also observed.

Thus, it has been unequivocally established that oxidative toxicity, the contribution of the endogenous NO/NO system and MMP-mediated vascular remodeling are primary pathophysiological mechanisms underlying Hcy-mediated vascular dysfunction. However, western medicines for the cure of HHcy-evoked vascular disease are lacking.

AM has long been prescribed as a ‘qi supplement’ in traditional Chinese medicine (9). Experimental and clinical data have shown that AM and its constituents have cardiovascular protective effects, including the amelioration of atherosclerosis, induction of vasodilation and lowering of blood pressure (32,33).

ASP is the most predominant of the bioactive compounds in the roots of AM. Meng et al (34) reported that ASP is able to increase free radical removal and decrease lipid peroxidation in isoproterenol-treated cardiomyocytes. Previous studies have indicated that astragaloside IV-induced improvements in vasorelaxation are associated with increased levels of aortic NO and cGMP (11,13,14). In a study by Huang et al (35), the results revealed that ASP protects against ischemia-reperfusion injury by modulation of MMP-9/tissue inhibitor of metalloproteinases 1.

The present results demonstrate that TEA and its constituents have no influence on the serum levels of Hcy, cholesterol and glucose. TEA and ASP clearly controlled the HHcy-induced increase in SBP/DBP in rats. Furthermore, they decreased the levels of hydrogen peroxide and superoxide anion, increased the activities of SOD and catalase, restrained the concentrations of MMP-2 and -9, and increased the aortic NO contents and eNOS activities in aortic homogenates from HHcy rats. Finally, they significantly enhanced the endothelium-dependent vasorelaxation of aortic rings of HHcy rats.

It is worthy of note that although APS and ATF are considered the most important components of this plant and have been attributed with multiple biological activities (9,32,33), ATF and APS were not observed to be as efficacious as TEA and ASP in the present study. Wang et al (36) reported that ATF benefits cardiovascular disease via its potent antioxidant activity and Zhang et al (37) indicated that APS reversed palmitate-induced cardiomyocyte toxicity partially through the suppression of ROS accumulation. However, the antioxidant effects of ATF and APS have been demonstrated to be significantly lower than those of ASP (38). Additionally, our previous in vitro study demonstrated that ASP, but not APS, protected endothelium-dependent relaxation against the acute injury from Hcy through NO regulatory pathways (14).

In conclusion, the present study demonstrated that TEA and its ASP constituents are able to improve vascular dysfunction and remodeling in HHcy, which may be attributed to its potent effects against oxidation, activity of the MMPs and endothelial dysfunction.

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References


