Cardiac Protective Effect of Astragalus on Viral Myocarditis Mice: Comparison with Perindopril

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Abstract: In clinical practice, Astragali Radix (Astragalus), the root of *Astragalus membranaceus* Bunge, has been widely applied to treat patients with viral diseases, including viral myocarditis in China. The present study was designed to evaluate the protective effects of Astragalus on the function of sarcoplasmic reticulum calcium ATPase (SERCA2) activity and endothelin system at acute and chronic periods of myocarditis mice induced by CVB3 infection. Astragalus feeding (2.2 mg/kg/day) could significantly increase the survival rate, alleviate pathological alterations and serum cardiac troponin I (cTnI), as well as restore impaired SERCA activity at the acute stage. Low affinity and capacity of ETR were reversed with Astragalus after the first CVB3 inoculation up to 7 days and after the second virus inoculation up to 150 days. In the meantime, the contents of cardiac ET-1 and ANP were reduced. Comparison the myocarditis mice treated with Perindopril (0.44 mg/kg/day), an ACE inhibitor, shows that Astragalus achieved a similar effect on survival rate, SERCA2 and ET system. These results indicated that the beneficial effects of Astragalus and Perindopril for treating viral myocarditis might be partly mediated by preserving the functions of SERCA 2 activity and ET system.

Keywords: Astragalus; Perindopril; Myocarditis; Endothelin; Sarcoplasmic Reticulum.

Introduction

Astragali Radix (Astragalus), one of the thousands of Chinese herbs named HuangQi, is derived from the root of *Astragalus membranaceus* Bunge and contains medicinally active
compounds, including polysaccharides, saponins, flavones and essential trace minerals, such as selenium (Zhang et al., 1998; Ma et al., 2002; Yin et al., 2004). As an exciting and potentially promising herb, Astragalus has been prepared as fresh, capsules, concentrated drops, injections and extracts and has been used for clinical application in China for over hundreds of years. Based on previous knowledge that it could strengthen the body’s overall vitality, improve digestion and immunity, and boost multiple organ functions, Astragalus was given a single or in combination with other herbs by practitioners of Traditional Chinese Medicine to patients with a variety of conditions and illnesses, especially those with viral diseases (Wu et al., 2001; Zou and Liu, 2003; Yin et al., 2004). Though its precise pharmacological effects on the cardiovascular system are unknown, it was also proven that patients with cardiovascular disorders could benefit from Astragalus, for example, those with viral myocarditis (Yang, 2001). Further research may provide convincing and exact evidence that Astragalus is invaluable for medical treatment.

Viral myocarditis (VMC) is an inflammatory disease of the cardiac muscle caused by viral infection. It can be in the phases of acute, subacute, or chronic. From the pathological view, there may be either focal or diffuse involvement of the myocardium. The most frequently implicated viruses are enteroviruses, including coxsackievirus B (CVB) (Kearney et al., 2001). Experimental and clinical studies indicated that many factors, such as virulent, selenium deficiency (Beck, 1997), cytokines (Kishimoto et al., 2003), autoantibodies (Takata et al., 2004), Ca\(^{2+}\) balance (Hu et al., 2001), free radicals (Chen and Zhou, 2001), and endothelin (ET) (Ono et al., 1999; Baba et al., 2000; Seta et al., 2000) etc. are related to the pathogenesis and prognosis of VMC, and each with their corresponding therapeutic option, including immunosuppressants, immunomodulators, angiotensin-converting enzyme inhibitor (ACEI), calcium channel blockers and nitric oxide inhibition (Anandasabapathy and Frishman, 1998). The mechanisms both for and against the virus are not completely clear and need to be further elucidated.

We attempted to compare the protective effects of Astragalus on the alterations of sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA 2) activity and on the behavior of the endothelin system in CVB\(_3\) induced viral myocarditis mice with Perindopril.

Materials and Methods

Chemicals and Drugs

Astragalus injections were obtained from the Fuda Pharmaceutical Co., Ltd., Shanghai, China, and contained 2 g crude material per milliliter with astragalosides, isoflavonoids, pterocarpan and isoflavan, in which Astragaoside IV was no less than 0.08 mg/ml. Perindopril was endowed by SERVIER, France, and dissolved in distilled water. Endothelin-1 (ET-1) was purchased from MERCK, Germany, and labeled with \(^{125}\)I by National Science Nuclear Institute, Beijing, China. All other chemicals used were of analytical grade.

The concentrations of cardiac ET-1 and atrial natriuretic peptide (ANP) were determined by using radio-immunoassay kits which were provided by North Biology Ltd., Beijing, China.
Animals

Three-week-old male BALB/c mice (12–15 g) were obtained from SIPPR/BK Co., Ltd., Shanghai, China (BALB/c ICR C57, No 153). They were housed in standard environmental conditions. Investigations using experimental animals were conducted in accordance with internationally accepted principles for laboratory animal use and care. The experimental protocol was approved by the local ethical committee.

Experimental Protocol

Acute viral myocarditis mice were induced by intraperitonial (i.p.) injection with 0.1 ml \(2 \times 10^4\) TCID\(_{50}\) coxsackievirus B\(_3\) (TCID\(_{50}\) was \(10^{-5.83}\)) in all groups except for group N. Group A (Astragalus treated group) contained 31 mice treated orally with Astragalus 2.2 mg/kg/day. Thirty-three mice in Group P (Perindopril treated group) were treated orally with Perindopril 0.44 mg/kg/day. Group M (model) and group N (normal control) which contained 36 and 31 mice respectively, were treated with normal distilled water. After 7 days of treatment, all animals were sacrificed under the deep anesthesia.

Chronic viral myocarditis mice were induced and classed by the same method as the acute ones but housed for 150 days. Some mice were reinoculated in the same manner with CVB\(_3\) at the 14th day. Thus, group M1 (n = 43), A1 (n = 35) and P1 (n = 34) represented single infected VMC mice which received corresponding treatments, and group M2 (n = 29), A2 (n = 24) and P2 (n = 25) represented the reinfected ones. Astragalus (2.2 mg/kg/day) and Perindopril (0.44 mg/kg/day) were given daily for one month, then twice a week for two months.

Mice were weighed before being sacrificed. Blood was collected without an anticoagulant. Serum was separated for the assay of cardiac troponin I (cTnI) by the method of “sandwich” ELISA described previously (Zhang et al., 1999). The hearts were excised from the mice immediately. The cardiac apical section was fixed in 10% formalin, embedded in paraffin, sectioned into slices of 4-µm thickness and stained with hematoxylin and eosin. The extents of myocardial inflammation and necrosis were graded as described by Baba et al. (2000). The remaining heart tissue was immediately immersed into liquid nitrogen. Radioligand binding studies for the estimation of endothelin receptor (ETR) maximum binding capacity (Bmax) and equilibrium dissociation constant (Kd) were performed by the method of Modesti et al. (1999) with little modification. In brief, cardiac myocyte membrane was isolated through homogenizing and centrifuging repeatedly. Cardiac membranes (300 µg/ml) were incubated with \(^{125}\text{I}-\text{ET-1}\) ranged from 10 to 370 pM for total binding at 22ºC for 60 minutes. Nonspecific binding was obtained by adding unlabeled ET-1 (0.5 µM). The binding was terminated by rapid vacuum filtration through GF/C filters presoaked with 3% bovine serum albumin (BSA). Filters were punched out and radioactivity was counted by a gamma counter. SERCA activity in the homogenate was assessed by pNPPase method of Larsen and Kjeldsen (1995). The concentrations of cardiac ET-1 and atrial natriuretic peptide (ANP) were determined by using radio-immunoassay kits.
Statistical Analysis

The results in Table 1 are represented as mean ± SD and analyzed by one-way analysis of variance (ANOVA) except the survival rate which was analyzed by chi-square, cTnI was analyzed by rank-sum test for non-normal distribution. The results in Table 2 and Fig. 1 are expressed as mean ± SEM and analyzed by one-way ANOVA among matched groups. Further analysis between the groups was statistically evaluated by Newman-Keuls test. p < 0.05 was regarded as significant.

Results

As shown in Table 1, Astragalus and Perindopril significantly increased the survival rates of acute virus myocarditis mice within 7 days (p < 0.05). Meanwhile, severe pathological changes were noted in the mice of group M. Focal myocardial lesions, which contained an extensively inflammatory cell infiltration, especially mononuclear cells, with cardiac myocytes denatured, necrosis and erythrocyte effusion, were observed. In contrast, myocardial inflammation and/or necrosis were attenuated significantly by Astragalus and Perindopril (p < 0.05). The levels of serum cardiac troponin I, as a high-sensitivity and high-specificity serum marker for myocardial lesions (Zhang et al., 1999), were consistent with pathological findings.

None or only one to two new deaths of VMC mice in each group were recorded after the acute stage, even if some of them repeatedly inoculated thereafter. Thus, the survival rate of each group at the 150th day only slightly varied from that of the 7th day. Myocardial necrosis or cellular infiltration was found in a few VMC mice at the chronic stage with occasional calcification and collage proliferation. The pathological scores and cTnI values in VMC mice, no matter what treatment, approximately recovered to normal levels at the 150th day and there was no obvious difference between single infected and re-infected mice (data not shown).

Activity of myocardium SERCA in VMC mice decreased to half of that in normal mice at the 7th day (Table 1, p < 0.05), and was significantly recovered by Astragalus and

| Table 1. Effects of Astragalus and Perindopril on Survival Rates, Pathological Scores, Serum cTnI, and SERCA Activity of VMC Mice at the 7th Day |
|---|---|---|---|---|
| Group | Survival Rate (n) | Pathological Scores (n) | CtnI (mg/l) (n) | SERCA Activity (µmol/g pro.min) (n) |
| | | Inflammation | Necrosis | |
| | | | | |
| N | 100% (31)a | 0 ± 0 (17)a | 0 ± 0 (17)a | 1.99 ± 0.29 (17)a |
| M | 66.7% (36) | 3.36 ± 0.74 (14) | 3.1 ± 40.86 (14) | 6.79 ± 5.53 (14) |
| A | 97% (31)a | 2.00 ± 0.94 (12)a | 2.18 ± 1.07 (12) | 1.47 ± 2.36 (12)a |
| P | 87.9% (33)a | 2.00 ± 0.93 (18)a | 1.88 ± 1.25 (18)a | 2.66 ± 3.83 (18) |

Values are represented as mean ± SD and analyzed by one-way analysis of variance (ANOVA) except the survival rate which is analyzed by chi-square, cTnI by rank-sum test for non-normal distribution. A p < 0.05 was regarded as significant. (N) Normal control; (M) VMC model; (A) Astragalus (2.2 mg/kg) treated per day; (P) Perindopril (0.44 mg/kg) treated per day. *p < 0.05 vs group M.
Table 2. Effects of Astragalus and Perindopril on ETR Maximum Binding Capacity (Bmax), Equilibrium Dissociation Constant (Kd), ET-1 and ANP Concentrations of VMC Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>ETR Bmax (fmol/mg.pro) (n)</th>
<th>ETR Kd (nmol/l) (n)</th>
<th>ET-1 (pg/ml.pro) (n)</th>
<th>ANP (ng/ml.pro) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 Days</td>
<td>150 Days</td>
<td>7 Days</td>
<td>150 Days</td>
</tr>
<tr>
<td>N</td>
<td>54.52 ± 7.21 (6)</td>
<td>17.82 ± 2.50 (7)</td>
<td>0.073 ± 0.013 (6)</td>
<td>0.062 ± 0.016 (7)</td>
</tr>
<tr>
<td>M1</td>
<td>67.22 ± 11.42 (12)</td>
<td>21.32 ± 4.30 (6)</td>
<td>0.191 ± 0.029 (12)</td>
<td>0.046 ± 0.009 (6)</td>
</tr>
<tr>
<td>M2</td>
<td>–</td>
<td>37.96 ± 14.90 (6)</td>
<td>–</td>
<td>0.125 ± 0.041 (6)</td>
</tr>
<tr>
<td>A1</td>
<td>47.07 ± 6.79 (5)</td>
<td>23.62 ± 3.29 (7)</td>
<td>0.092 ± 0.010 (5)</td>
<td>0.081 ± 0.029 (7)</td>
</tr>
<tr>
<td>A2</td>
<td>–</td>
<td>32.95 ± 7.82 (6)</td>
<td>–</td>
<td>0.049 ± 0.017 (6)</td>
</tr>
<tr>
<td>P1</td>
<td>N.D.</td>
<td>26.53 ± 11.79 (5)</td>
<td>N.D.</td>
<td>0.048 ± 0.017 (6)</td>
</tr>
<tr>
<td>P2</td>
<td>–</td>
<td>21.65 ± 6.86 (5)</td>
<td>–</td>
<td>0.054 ± 0.016 (5)</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM and analyzed by one-way ANOVA among groups N, M1, M2, and N, M1, A1, P1 and N, M2, A2, P2, respectively. Further analysis between the groups was statistically evaluated by Newman-Keuls test. N.D.: Not determined. (N) Normal control; (M1) VMC single-infected model; (M2) VMC reinfected model; (A1) single-infected VMC treated with Astragalus (2.2 mg/kg/day); (A2) reinfected VMC treated with Astragalus (2.2 mg/kg/day); (P1) single-infected VMC treated with Perindopril (0.44 mg/kg/day); (P2) reinfected VMC treated with Perindopril (0.44 mg/kg/day). Astragalus and Perindopril were given daily for one month, then twice a week for two more months to chronic VMC mice. \(^p < 0.05\) vs group M1. \(^\ddag p < 0.05\) vs group M2.
Perindopril (p < 0.05). Meanwhile, the increasing ETR Kd value in group M1 (Table 2, p < 0.05 vs N and A1) suggested the decreasing affinity of ETR after virus inoculation. Furthermore, at the 150th day, the tendency of decreasing affinity of ETR was retained in reinfected VMC mice other than single infected VMC mice, and recuperated by Astragalus and Perindopril treatments. No significant difference in changes of cardiac ETR densities (Bmax) among age-matched or condition-matched groups was found, though there was a slight increasing trend in virus infected groups. In contrast to single infected VMC mice, reinfected VMC mice showed obviously elevated ET-1 and ANP concentrations in heart homogenates (Table 2, p < 0.05 vs N), which were decreased in Astragalus and Perindopril treated groups, especially the latter one.

Figure 1. Representative of saturation curve (A) and scatchard plot (B) of [125I]-endothelin-1 specific binding to heart homogenate of group N (normal control), M (VMC model) and A (Astragalus treated VMC) mice at the 7th day. Each point represents the mean ± SEM.
Discussion

Previous studies in vivo and in vitro have shown that Astragalus, or its extraction, exerted beneficial effects for viral diseases via virus replication inhibition, interferon system activation and free radical scavenging (Peng et al., 1995; Lu and Zhang, 1998; Yang, 2001; Meng et al., 2005). In the present study, the observed lower mortality, pathological changes and serum cTnI values in mice treated with Astragalus and Perindopril confirmed the cardiac protective effects of Astragalus and Perindopril for treating viral myocarditis. Besides the above mentioned antiviral effects, we hypothesized that Astragalus might play a role in attenuating some pathogenetic process of viral myocarditis, such as regulation of calcium and ET system defect, which protect virus infected myocardium.

In cardiac myocytes, intracellular Ca\(^{2+}\) tidal change is involved in cardiac diastolic and systolic functions and is finely tuned via complex interactions between channels, pumps, transporters, and binding proteins located in sarcolemma and sarcoplasmic reticulum (SR). Defects in Ca\(^{2+}\) cycling, dominantly Ca\(^{2+}\) overload due to increased Ca\(^{2+}\) entry and/or decreased Ca\(^{2+}\) reuptake, is recognized as a common pathobiochemical finding in numerous experimental models and human heart diseases, including viral myocarditis and cardiomyopathies. SERCA is localized in SR and responsible for the uptake of Ca\(^{2+}\) from the cytoplasm into the SR lumen. During each cardiac contraction, the Ca\(^{2+}\) released through the ryanodine receptor (RyR) from SR is roughly proportional to the amount of SR Ca\(^{2+}\) content. In this manner, SERCA activity is a critical determinant of relaxation and contractility of cardiomyocytes. Moreover, SERCA was once used as an autoimmune antigen to induce experimental myocarditis and cardiomyopathy (Sharaf et al., 1994), in which, 75% cardiac SERCA enzymatic activity was inhibited by its monoclonal antibody. In this study, SERCA activity was depressed by CVB\(_3\) inoculation and preserved by Astragalus and Perindopril treatments to near normalcy. Thus, it is reasonable to deduce that myocardial damage mediated by Ca\(^{2+}\) overload could be partly alleviated by Astragalus and Perindopril in this model.

As one of the most important biological systems in the cardiovascular, the endothelin system became a new target of therapeutic intervention for the treatment of heart failure (Duchman et al., 2000) at the beginning of this century. Cardiac ET system activation, with increased tissue ET-1 concentration and changes of ETR expression and distribution, was observed and has been demonstrated to play an inducible role in cardiac hypertrophy and to accelerate the severity of heart failure in experimental and clinical investigations (Zolk et al., 1999; Duchman et al., 2000; Rothermund et al., 2000). Therefore, during the process of viral myocarditis, a specific blockade of ET-1 was the dominant treatment for cardiac hypertrophy and chronic heart failure (CHF) at the terminal stage (Seta et al., 2000). With the finding that the ET system is involved in the process of the early stage of inflammation (Ono et al., 1999; Petkova et al., 2000), pretreatment with an endothelin receptor antagonist, such as bosentan, resulted in a reduction in the extent of myocardial necrosis without modifying viral replication in a murine model of myocarditis. In the present study, the down-regulation of ETR affinity was observed in the hearts up to 7 days after the first CVB\(_3\) inoculation and up to 150 days after the second inoculation,
and was accompanied by a slight up-regulation of ERT density. This suggested that the disorder of the ET system occurred in the acute and chronic stage of viral myocarditis. The administration of Astragalus could reverse the alterations of ETR, which might be one of the reasons for its alleviation of cardiac injury induced by viruses.

In the clinic, most people with myocarditis have been previously exposed to more than one enterovirus before myocarditis onset. It is believed that the murine model of virus-induced myocarditis after a secondary virus exposure may have greater clinical relevance than models using a single inoculation. It was confirmed that successive infection additively caused myocardial damage that resembled chronic myocarditis and dilated cardiomyopathy (DCM), which is one of the most complicated and serious sequelae of myocarditis (Beck et al., 1990; Okada et al., 1992; Nakamura et al., 1999). We designed repetitively infected VMC mice for this study, which showed exacerbated endothelin system disorder and ANP without exhibiting increased mortality and pathological changes compared with single infected ones. We postulated that the neutralizing antibodies against CVB₃ induced by the first infection might produce a protective effect on the heart at the time of the second infection (14th day). It was estimated that the second CVB₃ inoculation, though producing a lack of additional morphological changes, still induced the functional alterations, which might ascribe to endothelin system disorder and might favor the transition to DCM later on.

The relationship between the activity of the ET system and myocyte Ca²⁺ handling was supported by the fact that SR Ca²⁺ reuptake, was negatively correlated with all components of the ET system (preproET-1, endothelin-converting enzyme-1, ETA receptor) in Ren2 rats (Rothermund et al., 2000). The causal interaction between the ET system and cardiomyocyte Ca²⁺ handling was confirmed in the same study by the observation that the ETA receptor blockade completely normalized SR Ca²⁺ handling in the Ren2-30/Lu135252 rats. It provided another method by which to understand that Astragalus simultaneously preserved SERCA activity and the ET system in VMC mice.

In a comparative study, an angiotensin II subtype-1 receptor antagonist (ATA) and captopril had similar effects on preventing the thickening of left ventricular wall and cavity dimension in a murine model of dilated cardiomyopathy induced by encephalomyocarditis virus (Kanda et al., 1995). Baba et al. (2000) reported that the effect of ATA on the reduction of endothelin had a different pathway from ACEI, including captopril and enalapril, though all of them repressed cardiac hypertrophy in VMC mice. Furthermore, they suggested that the different properties of ACEI, such as the sulfhydryl structure, determined the different effects on treating VMC. In our study, it seemed that Perindopril, without sulfhydryl structure, maintained the levels of the evaluated parameters to near normalcy and showed a beneficial effect on treating VMC through its influence on regulating SERCA activity and the endothelin system.

Our study strongly suggests the protective effects of Astragalus on SERCA activity and ETR defects in CVB₃-infected and re-infected VMC mice. The beneficial effect of Astragalus on VMC mice was similar to that of Perindopril. Further studies are being carried out for a better understanding of the beneficial mechanism of Ca²⁺ cycling and to explore the most effective component of Astragalus on viral myocarditis.
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REFERENCES


