Chinese medicinal herb Radix Astragali suppresses cardiac contractile dysfunction and inflammation in a rat model of autoimmune myocarditis

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\textbf{A B S T R A C T}

Radix Astragali, a Chinese medicinal herb, consists of polysaccharides and flavonoids as its main active ingredients. It has been widely used for treatment of cardiovascular diseases such as heart failure, angina pectoris, myocardial infarction and stroke in Asian countries. This study was designed to evaluate the effect of Radix Astragali on myocardial dysfunction, cardiac remodeling and morphological alteration in an experimental model of autoimmune myocarditis, a clinical condition often resulting in dilated cardiomyopathy. Experimental autoimmune myocarditis was established with a subcutaneous injection of porcine cardiac myosin into rear footpad in Lewis rats. Radix Astragali treatment was delivered via an intravenous injection (0.2 ml/100 g body weight, daily) for 3 weeks. Results from transthoracic echocardiography indicated that experimental autoimmune myocarditis led to impaired myocardial contractile function which was reconciled by Radix Astragali. The experimental autoimmune myocarditis triggered profound inflammation and fibrosis in myocardium as assessed by hematoxylin and eosin (H and E) and Masson’s trichrome staining. Interestingly, Radix Astragali significantly attenuated autoimmune myocarditis-induced myocardial inflammation and fibrosis. Similarly, Radix Astragali treatment alleviated autoimmune myocarditis-triggered overt lymphocyte proliferation. Furthermore, Radix Astragali significantly attenuated elevated levels of the Th1 cytokines (IFN-\(\gamma\) and IL-2), and increased the Th2 cytokines (IL-4 and IL-10) in autoimmune myocarditis. Collectively, our data revealed that Radix Astragali effectively protected against cardiac functional and morphological aberrations in experimental autoimmune myocarditis.

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\textbf{1. Introduction}

Myocarditis, a heterogeneous myocardial inflammatory disease, leads to both acute and chronic heart failure manifested by dilated cardiomyopathy. The etiology of myocarditis appears to be rather complicated involving idiopathic, infectious or autoimmune factors (Caforio and Illiceto, 2008). Autoimmunity and immune mediators are known to play a critical role in the pathogenesis of an array of cardiovascular diseases leading to compromised ventricular function (Afanasyeva et al., 2004; Maisch et al., 2005). A wide variety of immune cells including CD4+ T cell, CD8+ T cells, granulocytes and mast cells may result in focal myocardial damage en route to cardiomyocyte death, replacement fibrosis and ultimately cardiac contractile dysfunction (Afanasyeva et al., 2004). In addition to apoptosis and fibrosis, other factors such as humoral, cellular immune dysregulation, environmental factors and genetic predisposition have also been consolidated to play a role in the autoimmune-induced myocardial dysfunction (Maisch et al., 2005). Nonetheless, it is still unclear as to what extent these factors contribute to the immunologically-mediated myocardial damage. Given the poorly defined etiology of autoimmune myocarditis, the current clinical management against this devastating myocardial problem is somewhat dismay with symptom management being the main focus.

Radix Astragali is a traditional Chinese medicinal herb derived from the root of Astragalus membranaceus with polysaccharides and flavonoids being the active constituents (Qi, 1987; Li, 2000). The polysaccharides of Radix Astragali include two glucans, AG-1 (astragalus glucan-1) and AG-2 (astragalus glucan-2), as well as two heteroglycans, AH-1 (astragalus heteroglycan-1 and AH-2 (astragalus heteroglycan-2). The flavonoids comprise predominantly...
7,3-dimercapto-4,1-methoxyisoflavone, 3-dimercapto-7,4,1-methoxyisoflavone, catycosin, kumatakenin and fomononetin. Certain amino acids (e.g., folic acid, nicotinamide, and linoleic acid), β-sterol, lupeol, hexanol, palmitic acid, 6-0-β-0-pyranoglucoside, 3-0-β-xylopyranose and carotenol can also be extracted from A. membranaceus (Qi, 1987; Li, 2000). It has been used clinically for several thousands of years in China and other southeast Asian countries for the treatment of heart, liver and kidney diseases, as well as diabetes mellitus, viral infection and immune disorders (Gui et al., 2006; Chan et al., 2007, 2008; Ai et al., 2008; Ryu et al., 2008; Xu et al., 2008). Pharmacological studies have demonstrated cardiovascular benefit of this product including its cardioprotective effect against heart failure, angina pectoris and myocardial infarction (Chan et al., 2007, 2008; Ryu et al., 2008; Xu et al., 2008).

Up-to-date, a number of mechanisms have been speculated for the cardioprotective role of Radix Astragali including improved energy metabolism and antioxidant capacity (Xuejiang et al., 2001). Nonetheless, the precise mechanism(s) of action behind the beneficial effects of Radix Astragali on myocardial injury especially under the condition of autoimmun myocarditis remains elusive.

In the present study, we took advantage of an established experimental model of autoimmune myocarditis model induced by cardiac myosin reminiscent of human myocarditis (Pummerer et al., 1996; Li et al., 2004). Autoimmune myocarditis often develops following acute viral myocarditis in both humans and rodents (Afanasyeva et al., 2004). Experimental autoimmune myocarditis (EAM) induced by cardiac myosin offers a virus-free model to examine the immunopathogenic mechanisms of autoimmune myocarditis with severity peaking around 21 days after immunization (Pummerer et al., 1996; Matsui et al., 2004; Li et al., 2008). The therapeutic potential of Radix Astragali on cardiac myosin-induced experimental autoimmune myocarditis was examined both functionally and morphologically. Considering the close association between proinflammatory cytokines and prevalence of myocardial dysfunction especially in autoimmunity, release of proinflammatory cytokines was monitored in serum from Lewis rats with experimental autoimmune myocarditis in the presence or absence of Radix Astragali treatment.

2. Materials and methods

2.1. Experimental animals and research reagents

Twenty-four adult male Lewis rats (6-week-old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were maintained in the animal facility of the Shandong University School of Medicine. All animals were housed with controlled temperature (22–26 °C), humidity (50–60%) and lighting (12/12 h circadian cycle) with free access to standard rat chow and sterile water throughout the duration of our study. All animal experimental procedures were performed in accordance with the guideline for animal experiments of the Medical College of Shandong University and were approved by the Institutional Committee for Laboratory Animal Care. Purified porcine cardiac myosin and complete Freund’s adjuvant used for induction of EAM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mycobacterium tuberculosis H37RA was obtained from Difco (Detroit, MI, USA). Commercial Radix Astragali injections, which are manufactured in compliance with GMP, were purchased from Chiatai Qingchunbao Chemical Co. (St. Louis, MO, USA). Mycobacterium tuberculosis H37RA was obtained from the State Pharmacopoeia Commission. Given that the clinical RA administration ranges between 10 and 20 ml/day/person, a healthy individual weighing 60 kg should receive 0.167–0.333 mg/kg of RA daily. Based on the dose translation equation between rodents and human (rat dose = human dose × human Km factor/rat Km factor) (Reagan-Shaw et al., 2008), the dosage of RA was calculated to be 0.103–0.206 ml/100 g in rats. A volume of 0.2 ml/100 g body weight was used in our present study. Echocardiographic examination was performed 3 weeks after immunization immediately before sacrifice when serum samples were collected and myocardial samples were fixed in formalin for histopathological examination.

2.2. Induction of EAM and experimental protocols

Lewis rats (n = 24) were randomly divided into three groups, namely control, myocarditis and myocarditis with RA-treatment. Porcine cardiac myosin was dissolved in 10 mM potassium phosphate buffer (pH 7.4), containing 0.6 M potassium chloride with a final concentration of 10.0 g/L. The protein was then mixed with an equal volume of Freund’s complete adjuvant containing 10.0 g/L of heat-killed mycobacterium tuberculosis. A total volume of 0.2 ml of the emulsion was injected subcutaneously into the rear footpad of rats in myocarditis and RA-treated myocarditis groups on days 0 and 7, respectively. Rats in control group were given an equal volume of potassium phosphate buffer in Freund’s complete adjuvant (as vehicle control). The vehicle control produced little effect on cardiac function and morphology based on previous findings (Wang et al., 2006). Rats in RA-treated group received intravenous Radix Astragali injection (0.2 ml/100 g body weight, daily) for 3 weeks prior to sacrifice. Each millilitre of RA injection emulsion contains 2 g of crude A. membranaceus compound based on the Chinese Pharmacopoeia from the State Pharmacopoeia Commission. Given that the clinical RA administration ranges between 10 and 20 ml/day/person, a healthy individual weighing 60 kg should receive 0.167–0.333 mg/kg of RA daily. Based on the dose translation equation between rodents and human (rat dose = human dose × human Km factor/rat Km factor) (Reagan-Shaw et al., 2008), the dosage of RA was calculated to be 0.103–0.206 ml/100 g in rats. A volume of 0.2 ml/100 g body weight was used in our present study. Echocardiographic examination was performed 3 weeks after immunization immediately before sacrifice when serum samples were collected and myocardial samples were fixed in formalin for histopathological examination.

2.3. Echocardiography examination

Twenty-one days after the first immunization, transthrachoric echocardiography was performed in rats using an Agilent Sonos 5500 echocardiograph with an 11–13L transducer. All animals were anesthetized with pentobarbital sodium (20–35 mg/kg, i.p.). The M-mode echocardiogram was obtained along the short axis view of the left ventricles at the chordae tendineae level. Left ventricular end-diastolic dimension (LVIDd), left ventricular end-systolic dimension (LVIDs), left ventricular diastolic interventricular septum thickness (IVS) and left ventricular posterior wall thickness (LVPW) were measured. Left ventricular fractional shortening (LVFS) was calculated from the M-mode echocardiogram. Data from three to five consecutive cardiac cycles were used for analysis.

2.4. Histopathology

On day 21 immediately following echocardiographic assessment, all animals were sacrificed. The hearts were removed, weighed and fixed using immersion in 10% phosphate-buffered formaldehyde before being embedded in paraffin. Thin tissue sections (5 μm in thickness) were stained with hematoxylin and eosin (H and E), or Masson’s trichrome using the standard protocols for histopathological analyses. Macroscopic findings were classified into five grades: 0, no inflammation; 1, presence of a small discolored focus; 2, presence of multiple small discolored foci; 3, diffuse discolored areas not exceeding a total of one-third of cardiac surface area and 4, diffuse discolored areas totaling more than one-third of cardiac surface area. Microscopic findings were assessed by myocardial infiltration and fibrosis scores. The severity of myocardial infiltration and fibrosis were graded into one of the five categories: 0, no involvement; 1, 25% involvement; 2, 25–50% involvement; 3, 50–75% involvement and 4, >75% involvement. All histological/macroscopic evaluations were performed in a double-blind manner by two professional staffs.

2.5. Lymphocyte proliferation assay

Proliferation of lymphocytes was determined as described previously (Wang et al., 2006). In brief, spleens were collected from rats upon sacrifice. Cell suspensions were prepared where lymphocytes were recovered by a Ficoll-Hypaque technique. A total of 2 × 10^6 cells/ml (in triplicates) were incubated in the presence or absence of 10 μg/ml porcine cardiac myosin (Sigma) for 72 h in 0.2 ml of RPMI 1640 ( Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum in 96-well microtiter plates. Cell proliferation was assessed by incorporation of 0.5 μCi [3H] thymidine into DNA during the final 12 h of incubation. Proliferation of lymphocytes was presented as counts per minute (cpm).

2.6. Cytokine assay

Enzyme-linked immunosorbent assay (ELISA) was used to measure serum concentrations of interferon (IFN)-γ, interleukin (IL)-2, -4 and -10 as per the instruction from manufacturer (BioSource International, Camarillo, CA, USA).

2.7. Statistical analysis

Data were presented as mean ± S.E.M. Differences among all groups were assessed using a one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. General features of experimental animals

Body weights were matched for all groups of rats at the time of immunization. Following 3 weeks of autoimmune disease, body weights were significantly lower in the myocarditis group compared with the group, the effect of which was ablated by Radix Astragali treatment. Hearts were significantly enlarged shown in
both absolute weight and cardiac size (normalized to body weight) in rats from myocarditis group compared with the control or Radix Astragali-treated myocarditis group. Heart rate was unaffected by either autoimmune myocarditis or Radix Astragali (Table 1).

### 3.2. Effect of Radix Astragali on autoimmunity-induced changes in myocardial contractile function

Transthoracic echocardiography was performed to assess heart function in all three rat groups. While there was no significant difference in LVDd among these three rat groups, the experimental autoimmune myocarditis significantly enhanced LVDs, LVPW and IVS while decreased left ventricular fraction shortening (LVFS), indicating severely altered cardiac structure and contractile function under autoimmune myocarditis. Radix Astragali treatment significantly attenuated autoimmune myocarditis-induced myocardial contractile dysfunction and left ventricular remodeling (Figs. 1 and 2).

### 3.3. Effect of Radix Astragali on autoimmune myocarditis-elicited inflammation and fibrosis

Representative H and E as well as Masson’s trichrome staining images are shown in Fig. 3. Autoimmune myocarditis elicited typical cardiac histopathological changes in myocardium which were manifested as presence of fragments of necrotic myocardial fibers, mononuclear cells, polymorphonuclear neutrophils, eosinophils and giant cells. Consistent with its beneficial effect on myocardial function and structure, Radix Astragali significantly protected the hearts from these pathological changes under autoimmune myocarditis. Our further quantitative evaluation revealed that the severity of EAM and affected area was significantly smaller in RA-treated group than in non-treated group (Fig. 4). These results suggest that treatment with Radix Astragali reduces the infiltration of inflammatory cells into myocardium.

### 3.4. Effect of Radix Astragali on lymphocyte proliferation

The effect of Radix Astragali treatment on lymphocyte proliferation in autoimmune myocarditis was shown in Fig. 5. There was no change in lymphocyte proliferation among three groups in the absence of antigen stimulation. Autoimmunity triggered by porcine cardiac myosin significantly facilitated lymphocyte proliferation, the effect of which was abrogated by Radix Astragali treatment. These data indicated that Radix Astragali is capable of suppressing lymphocyte proliferation in experimental autoimmune myocarditis.

### 3.5. Effect of Radix Astragali on Th1 and Th2 cytokines

Serum analysis revealed significantly elevated serum levels of the Th1 cytokines IFN-γ and IL-2 in rats from myocarditis group compared with the control group, the effect of which was abrogated by Radix Astragali treatment. Furthermore, treatment of Radix Astragali significantly upregulated the serum levels of the Th2 cytokine IL-4 and IL-10. Autoimmune myocarditis significantly elevated the levels of IL-10 but not IL-4 (Fig. 6). These results suggested that Radix Astragali treatment may shift the balance of helper T cells from Th1 to Th2.

### 4. Discussion

Our current study demonstrated, for the first time, the beneficial effect of Radix Astragali on cardiac dysfunction, morphological changes and cardiac remodeling elicited by an experimental model of autoimmune myocarditis. Radix Astragali treatment abrogated autoimmunity-induced body weight loss and cardiac hypertrophy (both absolute weight and cardiac size) induced by autoimmunity. Transthoracic echocardiography revealed that Radix Astragali abrogated autoimmunity-induced increase of LVDs, LVPW and IVS without affecting the LVDd. In addition to its beneficial effect on cardiac remodeling manifested by dilated cardiac chamber in experimental autoimmune myocarditis, Radix Astragali treatment reconciled the porcine cardiac myosin autoimmunity-induced decline in left ventricular contractile capacity following 3 weeks of treatment. Our data further revealed that Radix Astragali alleviated autoimmunity-induced distinct histopathological changes in myocardium including infiltration of inflammatory cells (neutrophils, lymphocytes and macrophages), interstitial edema and myocardial necrosis. These results have convincingly demonstrated the effectiveness of Radix Astragali in the treatment of autoimmune myocarditis.
Fig. 2. Summary of echocardiographic data: (A) LVDd: left ventricular end-diastolic dimension; (B) LVDs: left ventricular end-systolic dimension; (C) LVPW: left ventricular posterior wall thickness; (D) IVS: left ventricular diastolic interventricular septum thickness and (E) LVFS: left ventricular fractional shortening. Mean ± S.E.M., *p < 0.05 vs. control group, #p < 0.05 vs. myocarditis group, n = 8 rats per group.

Cardiac myosin-induced autoimmune myocarditis is a model of inflammatory heart disease initiated by CD4+T cells. It represents a paradigm of immunity-mediated cardiac damage in the pathogenesis of a subset of postinfectious human cardiomyopathies (Caforio and Iliceto, 2008; Afanasyeva et al., 2004). In our experimental model where myocarditis was triggered by immunization with purified cardiac myosin or specific peptides derived from cardiac myosin, myocarditis normally begins 12–14 days following the initial immunization and may become maximal after 21 days of immunization. Three weeks after immunization, the autoimmune myocarditis may transit into chronic course and the extent of inflammatory infiltrates may resolve slowly. Nevertheless, many animals develop dilated cardiomyopathy on follow-up examinations (Afanasyeva et al., 2004). In fact, the most recent WHO/WHF definition suggested inflammatory cardiomyopathy may be a distinct entity, defined as myocarditis in association with cardiac dysfunction. Myocarditis is the major cause of sudden death in young adults (Drory et al., 1991). Although most individuals recover well from
Fig. 3. Representative pathological images of H and E staining (×200, A–C) and Masson’s trichrome staining (×200, D–F). A and D: control group; B and E: myocarditis group; C and F: myocarditis with RA-treatment group.

acute infectious myocarditis, many patients may still go on to develop chronic autoimmune myocarditis and dilated cardiomyopathy leading to congestive heart failure.

Cytokines released by inflammatory cells during the autoimmune response are essential mediators of autoimmune disease. The acute inflammatory infiltrate is comprised of macrophages, neutrophils, CD4+ T cells and CD8+ T cells (Fairweather et al., 2005). T cells are important initiators and mediators of disease progression in autoimmune myocarditis. Th1 type cytokines (IFN-γ and IL-2) are often found in the inflammatory phase, whereas the production of Th2 (IL-4 and IL-10) cytokines can be found in the recovery phase of experimental autoimmune myocarditis (Hasegawa et al., 2005; Suzuki et al., 2007). IFN-γ-mediated Th1 responses are believed to be responsible for most autoimmune disease including myocarditis. During the innate immune response to infection, mice produce a mixed cytokine profile including IFN-γ and IL-4 production (Fairweather et al., 2004). On the other hand, IL-4-mediated Th2 responses activate B cells, induce MHC class II expression on B cells, promote allergic reactions, induce Ig class switching to IgG1 and IgE, and recruit eosinophils and Th2 cells to the site of inflammation (Galli et al., 2005). Data from our current study depicted that Th1 cytokine IFN-γ and IL-2 were suppressed and Th2 cytokine IL-4 and IL-10 was enhanced in hearts following Radix Astragali treatment, indicating potential beneficial effect of the herbal product on cytokine profile.

In summary, our results demonstrated that Radix Astragali inhibits the progression of myocardial function, remodeling and morphological alteration in a rat model of experimental autoimmune myocarditis. These data favor the notion that Radix Astragali

Fig. 4. Histopathological scores of three rat groups. Mean ± S.E.M., *p < 0.05 vs. control group, #p < 0.05 vs. myocarditis group, n = 8 rats per group.
Fig. 5. Lymphocyte proliferation in myocardia from three rat groups. Mean ± S.E.M., *p < 0.05 vs. control group, #p < 0.05 vs. myocarditis group, n = 8 rats per group.

Fig. 6. Levels of inflammatory cytokines from three rat groups. Mean ± S.E.M., *p < 0.05 vs. control group, #p < 0.05 vs. myocarditis group, n = 8 rats per group.

may be considered as a new therapeutic strategy for the treatment of autoimmune myocarditis. Nonetheless, further study is warranted to better understand the mechanism of action behind Radix Astragali-induced effect on autoimmunity and relationship between autoantibodies and myocardial performance.

Conflict of interest

None.

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