The protective effects of different compatibility proportions of the couplet medicines for Astragali Radix and Angelica sinensis Radix on myocardial infarction injury

Lu Chen,a,b,c,d*, Min Song,a,b,c,d, Lusha Zhang,a,b,c, Chunxia Li,a,b,c, Zhirui Fang,a,b,c, Joel Wake Coffie,a,b, Liyuan Zhang,a,b, Lulu Ma,a,b, Leyu Fang,a,b, Qianyi Wang,a,b, Wenjie Yang,a,b, Fanggang Li,e, Xiumei Gao,a,b,c and Hong Wang,a,b,c,f

*aTianjin State Key Laboratory of Modern Chinese Medicine, Tianjin, China; bKey Laboratory of Pharmacology of Traditional Chinese Medical Formulae, Ministry of Education, Tianjin University of Traditional Chinese Medicine, Tianjin, China; cTianjin Key Laboratory of Chinese Medicine Pharmacology, Tianjin University of Traditional Chinese Medicine, Tianjin, China; dInstitute of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China; eShandong Danhong Pharmaceutical Co., Ltd., Heze, China; fSchool of Integrative Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China

ABSTRACT

Context: Astragali Radix (AR) and Angelica sinensis Radix (ASR) combinations are used to treat cardiovascular disorders.

Objectives: This study investigates the protective effects of different compatibility proportions of AR and ASR on cardiac dysfunction in a C57BL/6 mouse model of myocardial infarction (MI).

Materials and methods: MI mice were induced by ligation of the left coronary artery and divided into six groups: sham, vehicle, 10 mg/kg/d simvastatin and combinations of AR and ASR at different ratios, including 1:1 (AR 2.5 g/kg + ASR 2.5 g/kg), 3:1 (AR 3.75 g/kg + ASR 1.25 g/kg) and 5:1 (AR 4.17 g/kg + ASR 0.83 g/kg). Both AR–ASR combinations and simvastatin were dissolved in saline solution and given daily by gavage. The left ventricle function, infarct size, heart tissue injury, apoptosis of cardiomyocytes, leukocyte infiltrates, capillary density and expression of cleaved caspase-3, cleaved caspase-9, Bcl-2, Bax, Bad, IL-1β, IL-6, VEGF, p-Akt and p-eNOS were analysed.

Results: Different combinations of AR and ASR improve cardiac function and reduce infarct size (61.15% vs. 39.3%, 42.65% and 45.5%) and tissue injury through different mechanisms. When AR was combined with ASR at ratio of 1:1, the inflammation and cardiomyocyte apoptosis were suppressed (p < 0.05, p < 0.01). The combination ratio of 3:1 exerted effect in promoting angiogenesis (p < 0.05). In the combination of AR and ASR at 5:1 ratio, angiogenesis was significantly improved (p < 0.01) and the apoptosis was inhibited (p < 0.05).

Conclusions: Our results reflect the regulation of multiple targets and links in herb pairs and provide an important basis for the use of AR and ASR combinations in the treatment of MI.

Introduction

Myocardial infarction (MI) and consequent heart failure are the most common types of cardiovascular disease and the leading causes of morbidity and mortality worldwide (Ibanez et al. 2018; Roth et al. 2018). MI develops when cholesterol particles accumulate on the walls of arteries due to various risk factors. The narrowed coronary arteries lead to decreased blood flow and eventually reduce the amount of oxygen supplied to heart muscle. The lack of oxygen can produce chest pain, angina pectoris or even a heart attack, which will impose a significant burden on quality of life (Heusch and Gersh 2017). The most effective treatment is to restore blood perfusion to relieve myocardial tissue hypoxia (Bagai et al. 2014). Current pharmacological treatments, such as inhibitors of the renin–angiotensin–aldosterone system (Kim et al. 2019) and β-adrenoreceptor blockers (Ibanez et al. 2013), prevent the expansion of myocardial necrosis to some extent; however, contraindications and adverse effects have limited their clinical application. To improve the quality of life and prognosis of patients with MI, it is critical to develop effective medicinal therapies which are both convenient to perform and well accepted. Moreover, as MI is a multifactorial disease with multiple mechanisms, as well as affecting various cell types, it is preferred to use the combination of additive or synergistic multitarget therapies for the optimal cardioprotection (Davidson et al. 2019).

Traditional Chinese medicine (TCM) herbs are widely used as medicines and daily dietary supplements in Asia on the mode action of ‘multi-component, multi-target, multi-pathway’ (Liu Z et al. 2016). According to Chinese medicine theory, promotion and activating blood circulation to remove blood stasis is one of
the main treatment strategies in ameliorating MI. Astragali Radix (AR), the dried radix of Astragalus membranaceus (Fisch.) Bge (Fabaceae) known as Huangqi in Chinese, is a major medicinal herb considered to enrich vital energy and treat stagnant blood flow. Angelica sinensis Radix (ASR), the dried radix of Angelica sinensis (Oliv.) Diels (Apiaceae) known as Dansgii in Chinese, has been used as a tonic agent to promote blood circulation for several years (Wei et al. 2016). To obtain synergistic effects and diminish possible adverse reactions, Chinese herbs are often used in formula. Herb pair is a centralized representative of herb compatibility. The AR in formula. Herb pair is a centralized representative of herb compatibility proportions of AR and ASR in mice MI model and ASR may be considered as a complementary therapeutic agents. The two herbs were identified by Dr. Li Tianxiang (Experiment Market) in Anguo, China on 21 March 2017 to 23 March 2017. All crude herbs were purchased from the specialized market of Tianjin University of Traditional Chinese Medicine of Tianjin University (Tianjin, China). Avertin and simvastatin were purchased from Beijing Weitong Lihua Biologica Products (Beijing, China). Avertin and simvastatin were purchased from Sigma-Aldrich (St. Louis, MO). The anti-CD45, anti-CD31, β-tubulin, VEGF, p-eNOS, eNOS and biotin-conjugated goat anti-rabbit IgG polyclonal antibodies were obtained from Abcam (Cambridge, UK). Caspase-3, cleaved caspase-9, Bcl-2, Bax, Bad, p-Akt, Akt and GAPDH antibodies were bought from Cell Signalling Technology (Boston, MA). Interleukin 6 (IL-6) and interleukin 1 β (IL-1β) Mouse ELISA kits were obtained from R&D Systems (Minneapolis, MN). Alex Fluor 594 Goat Anti-Rabbit IgG (H + L) was from Life Technologies (Waltham, MA).

Materials and methods

Reagents

The reference compounds of calycosin, formononetin, astragalside IV (AS-IV), ferulic acid and ligustilide were purchased from Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Avertin and simvastatin were purchased from Sigma-Aldrich (St. Louis, MO). The anti-CD45, anti-CD31, β-tubulin, VEGF, p-eNOS, eNOS and biotin-conjugated goat anti-rabbit IgG polyclonal antibodies were obtained from Abcam (Cambridge, UK). Caspase-3, cleaved caspase-9, Bcl-2, Bax, Bad, p-Akt, Akt and GAPDH antibodies were purchased from Cell Signalling Technology (Boston, MA). The in situ cell death detection kit and protease and phosphatase inhibitor cocktails were purchased from Roche Diagnostics (Mannheim, Germany). Interleukin 6 (IL-6) and interleukin 1 β (IL-1β) Mouse ELISA kits were obtained from R&D Systems (Minneapolis, MN). Alex Fluor 594 Goat Anti-Rabbit IgG (H + L) was from Life Technologies (Waltham, MA).

Preparation of Chinese herbal decoction

All crude herbs were purchased from the specialized market of Chinese herbal medicines (Anguo Traditional Chinese Medicine Market) in Anguo, China on 21 March 2017 to 23 March 2017. The two herbs were identified by Dr. Li Tianxiang (Experiment Teaching Department, Tianjin University of Traditional Chinese Medicine). Each voucher specimen was deposited at the Academy of Traditional Chinese Medicine of Tianjin University of Traditional Chinese Medicine. The voucher specimen numbers of AR and ASR are no. 20170527 and no. 20170516, respectively.

AR, ASR and different ratio combinations of AR–ASR (1:1, 3:1 and 5:1) were prepared. According to different formulations, the appropriate amounts of crude herbs were weighed separately to form a combined weight of 360 g (300 g AR and 60 g ASR for the 5:1 formulation, 270 g AR and 90 g ASR for the 3:1 formulation, 180 g AR and 180 g ASR for the 1:1 formulation). Before decocting, the medicinal materials were soaked in pure water for 1 h. The mixture was boiled in six volumes of water (v/w) (2160 mL) for 40 min the first time and four volumes of water (1440 mL) for the next two times. All the filtrates were mixed together and condensed to a total volume of 360 mL using a rotary vacuum centrifuge. The final concentration of AR, ASR and the combinations were 1 g/mL. The concentrate was sub-packaged and stored at −4 °C.

Calycosin, formononetin and AS-IV act as effective compounds in AR (Shi et al. 2014). Ferulic acid and ligustilide are the characteristic components of ASR (Shi et al. 2014). The chemical structures of these five compounds are shown in Figure 1(A). The components were measured to identify AR, ASR and the combinations by high-performance liquid chromatography (HPLC). The final concentrations of the reference compounds of calycosin, formononetin, AS-IV, ferulic acid and ligustilide were 61.75, 59.50, 272, 6.25 and 118.75 μg/mL, respectively. HPLC was performed by an Agilent 1260 Infinity High Performance Liquid Chromatography (Agilent Technologies, Palo Alto, CA). Solutions (10 mL) of each group were precisely measured and extracted by water-saturated n-butanol for four times. The extract was dried and dissolved by methanol. After filtering through a 0.22 μm filter membrane, each filtered sample (10 μL) was separated on an Inertsil ODS-3 column (4.6 mm × 250 mm, 5 μm). Gradient elution was carried out with acetonitrile (A)–0.2% formic acid solution (B) as follows: 0–25 min, 15 → 25% A, 85 → 75% B; 25–50 min, 25 → 40% A, 75 → 60% B; 50–65 min, 40 → 90% A, 60 → 10% B; 65–75 min, 90 → 90% A, 10 → 10% B; 75–76 min, 90 → 15% A, 10 → 85% B; 76–88 min, 15 → 15% A, 85 → 85% B. The flow rate was 1.0 mL/min and the column temperature was 35 °C. Typical HPLC fingerprints of the five main components in AR, ASR and different combinations of AR and ASR are shown in Figure 1(B).

Animals

Due to the presumption that cyclic hormonal changes of female mice across the ovulatory cycle introduce excess variability to measures of interest, male C57BL/6 mice of specific pathogen free (SPF), 22–25 g, were purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. (Beijing, China) and maintained at the Animal Center of Institute of Biomedical Engineering, Chinese Academy of Medical Sciences (Tianjin, China). All animals were kept under 22–25 °C and a 12 h light/dark cycle with standard food pellets and free access to tap water. All animal care and experimental procedures were approved by the Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine (TUTCM20170628) and performed in accordance with the approved guidelines on the use of laboratory animals. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health (Garber et al. 2011) and the Declaration of Helsinki.

Myocardial infarction model and drug treatments

After one week of adaptation, 12-week old experimental mice underwent surgery to induce acute MI by ligation of the left anterior descending (LAD) coronary artery, as described before (Kolk et al. 2009; Reichert et al. 2017). In brief, after anaesthesia
with injection of Avertin (0.33 mL/20 g) into the abdominal cavity, mice were orally intubated and artificially ventilated on a rodent respirator. Hearts were then exposed through the left lateral thoracotomy. The LAD coronary artery was visualized and ligated with an 8-0 suture line. The occlusion of coronary was confirmed by pallor and regional wall motion abnormality of the left ventricle (LV). Mice in the sham group underwent the same time-matched surgical procedure without ligation. All the operations were performed by an experienced surgeon who was blinded to the experimental groups.

Post-operatively, the mice were randomly divided into six groups: (1) sham group (n = 12). Mice had no ligation and they were orally administered with saline solution; (2) vehicle group (n = 15). The mice with surgery received saline solution orally; (3) simvastatin group (as a positive control drug) (n = 12). The mice were treated with 10 mg/kg/d simvastatin by gavage; 4–6. AR–ASR combination groups with different ratios (1:1, 3:1 and 5:1) (n = 15 for each group). Before drug treatments, the combinations were diluted with saline solution into 0.5 g/mL. The mice were administered with 10 mL/kg/d plant extracts based on the doses used in clinical practice. Both the AR–ASR combinations and simvastatin were dissolved in normal saline solution and given daily by gavage starting on day 0. Drugs were given for four consecutive weeks. At 7 and 28 days after MI surgery, mice were sacrificed. Hearts were perfused with cold phosphate buffered solution (PBS) and a portion of the LV was either frozen.
in liquid nitrogen for biochemical analysis or fixed in 4% paraformaldehyde for histological analysis.

**Echocardiography**

Cardiac function was examined by transthoracic echocardiography with a Vevo 2100 high-resolution ultrasound biomicroscope (VisualSonics, Toronto, Canada) and Vevo analysis software at days 7, 14 and 28 post-MI. The investigator was blinded to group assignment. Mice were anaesthetized with 2% isoflurane inhalation and placed in supine position. Heart rates were maintained at 400–500 beats per minute for measurements. Images were obtained from the B-mode parasternal long axis view and M-mode of the parasternal short-axis view to calculate the cardiac diastolic and systolic functions. Left-ventricular end-systolic volumes (LVEDVs) and left-ventricular end-diastolic volumes (LVEDVs) were measured through parasternal long-axis scans. The left-ventricular fractional shortening (LVFS) and left-ventricular ejection fraction (LVEF) were calculated in accordance with modified American Society of Echocardiography recommendations.

**Histological and immunofluorescent assessments**

Mice received drug once a day for 4 weeks after surgery. Hearts were harvested after the 28-day echocardiographic analysis. Heart tissue was fixed with 4% paraformaldehyde, dehydrated, embedded in paraffin, and transversely sectioned into 5 μm pieces. Masson’s trichrome staining was performed to evaluate the degree of fibrosis. Fibrotic tissue was stained to blue-grey and viable myocardium to red. The fibrosis area was calculated as the ratio of the length of fibrotic area to the length of left-ventricular inner circumference with Image J software. The haematoxylin and eosin (H&E) staining was performed to detect tissue recovery after ischaemia injury, as described previously (Baudouy et al. 2017).

For immunohistochemical labelling, paraffin sections were warmed to 60°C for 5 min and then rehydrated through xylene and a series of graded ethanol solutions. Sections were incubated with preheated antigen retrieval buffer (0.1 M sodium citrate buffer, pH 6.0) for 10 min. After washing in PBS 3 times, blocking was done by 5% foetal bovine serum in PBS for 1 h at room temperature.

To assess post-MI leukocyte infiltrates, mouse hearts were immunostained for CD45 at seven days post-MI and CD45 positive cells were quantified in the peri-infarct zone. Anti-CD45 antibody (ab10558, Abcam, Cambridge, UK, diluted 1:100 with milk) was used for immunohistochemistry of leukocytes. Samples were incubated with primary antibody for 24 h at 4°C. A biotin-conjugated goat anti-rabbit IgG polyclonal (ab205718, Abcam, Cambridge, UK, 1:500) was used as the secondary antibody.

Primary anti-CD31 antibody (ab28364, Abcam, Cambridge, UK, diluted 1:20 with 1% foetal bovine serum in PBS) was incubated with the sections overnight at 4°C. Negative controls were without primary antibody. Secondary antibody (Alex Fluor 594 Goat Anti-Rabbit IgG (H+L), diluted 1:200 with 1% foetal bovine serum in PBS) was then incubated with the sections for 1 h in the dark at room temperature. Finally, the sections were viewed and photographed using a Nikon Ti-U fluorescence microscope (Tokyo, Japan).

**Terminal deoxynucleotidyl transferase UTP nick end labelling (TUNEL) assay**

For this parameter, mice received drug once a day for 4 weeks after surgery and were sacrificed 28 days after surgery. Apoptosis of cardiomyocytes was detected by TUNEL assay which was performed on paraffin-embedded sections using the in situ cell death detection kit at 28 days after treatment with different combinations of AR and ASR. According to manufacturer’s instructions, the dewaxed and rehydrated slides were incubated with 0.1% Triton X-100 for 8 min, rinsed twice with PBS, and then incubated in TUNEL reaction mixture for 60 min at 37°C. After washed with PBS, samples were analysed by fluorescence microscopy in the range of 520–560 nm and detected in 570–620 nm. The number of TUNEL-positive nuclei was counted at ×200 magnification, 10 fields per section blinded to the study group. TUNEL+ cell density (%) is expressed as the ratio of TUNEL-positive nuclei to the total number of nuclei.

**Western blotting analysis**

Western blotting analyses were performed to detect the cleaved caspase-3, cleaved caspase-9, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), Bcl-2-associated death promoter (Bad), p-Akt, Akt, p-eNOS, eNOS, vascular endothelial growth factor (VEGF) protein expressions at day 28 after surgery. The heart tissues were homogenized in RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM sodium vanadate, 1 mM PMSF, 10μg/mL aprotinin, 10μg/mL leupeptin, 10 mM sodium fluoride) with a cocktail of protease and phosphatase inhibitors using tissue homogenizers. The mixture was spun 5000×g for 10 min to pellet unresolved fragments. Supernatants were collected and the protein concentration was measured by BCA method. After diluting in sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 0.1% bromophenol blue), the protein samples were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Membranes were blocked with blocking buffer (5% skim milk or 5% BSA in TBS with 0.1% Tween 20) for 1 h. Membranes were then incubated with primary antibodies at 4°C overnight. Twelve primary antibodies were used in the experiments: caspase-3 (9662, Cell Signalling Technology, Boston, MA), cleaved caspase-9 (9509, Cell Signalling Technology, Boston, MA), Bcl-2 (3498, Cell Signalling Technology, Boston, MA), Bax (2772, Cell Signalling Technology, Boston, MA), Akt (4691S, Cell Signalling Technology, Boston, MA), p-Akt (4060S, Cell Signalling Technology, Boston, MA), α-tubulin (ab52866, Abcam, Cambridge, UK), VEGF (ab51745, Abcam, Cambridge, UK), p-eNOS (ab76199, Abcam, Cambridge, UK), eNOS (ab66127, Abcam, Cambridge, UK) and GAPDH (21185, Cell Signalling Technology, Boston, MA). After washing, appropriate secondary antibodies were applied to the membranes for 1 h at room temperature. The reactive bands were detected using chemiluminescence according to the manufacturer’s instruction. Images were scanned and band intensities were analysed with photoshop CS5 software (Adobe Systems, San Jose, CA).

**Enzyme-linked immunosorbent assay (ELISA) analysis**

Levels of IL-1β and IL-6 in heart tissues were detected by mouse ELISA kits. Heart tissues were harvested at 7 days after surgery and were frozen in liquid nitrogen. The extracted tissues were homogenized in liquid nitrogen for biochemical analysis or fixed in 4% paraformaldehyde for histological analysis.
were homogenized in cold PBS, containing EDTA-free protease inhibitor, phosphatase inhibitor and PMSF mixture. The concentrations of IL-1β and IL-6 in the myocardium were determined by ELISA according to the manufacturer’s instructions.

**Statistical analysis**

Statistical analysis was performed using SPSS software (version 17.0, SPSS, Chicago, IL). Data were expressed as mean ± standard deviation (S.D.). Differences between two groups were estimated with Student’s t-test. The results were analysed by one-way analysis of variance (ANOVA) for comparison of each parameter among two or more independent groups. Values of $p < 0.05$ were considered statistically significant.

**Results**

**Different combinations of AR and ASR improve cardiac function and reduce infarct size after MI**

To test the therapeutic effects of different combinations of AR and ASR, a mouse model of acute MI was established via permanent ligation at the middle of the LAD coronary artery, as previously described (Reichert et al. 2017). Cardiac function was examined by performing echocardiographic measurements of LVEF and LVFS at 0, 7, 14 and 28 days after MI. After that, mice were sacrificed, and infarct size and extent of fibrosis were evaluated by Masson’s trichrome staining of heart tissue sections. Echocardiographic results are shown in Figure 2. The results show that the combinations of AR and ASR at 1:1 ratio significantly elevated LVEF at 28 days (Figure 2(A)), and LVFS at 7 and 28 days (Figure 2(B)) post-MI ($p < 0.01$). AR–ASR mixtures of 3:1 significantly increased the LVEF at 7 and 14 days (Figure 2(A)), and LVFS at 14 days (Figure 2(B)) post-MI ($p < 0.05$ or $p < 0.01$). LVEF (Figure 2(A)) and LVFS (Figure 2(B)) were also significantly higher at 14 and 28 days after AR–ASR mixed at 5:1 treatment, compared with those of untreated MI hearts ($p < 0.01$).

Infarct size is shown in Figure 3. Masson’s trichrome-stained myocardial sections at 28 days suggest the area of fibrosis was significantly reduced in different combinations of AR–ASR treated mice than that in vehicle mice on day 28 ($p < 0.05$) (Figure 3(A,B)). These results suggest that AR–ASR combinations at 1:1, 3:1 and 5:1 all improved cardiac performance by

![Figure 2](image1.png)

**Figure 2.** Treatments of different ratios of AR and ASR improve cardiac function after MI. (A, B) Echocardiographic measurements were performed on days 0, 7, 14 and 28 to detect the cardiac protective effects of different compatibility proportion of the couplet medicines for AR and ASR. Different ratios of AR and ASR improved cardiac function following MI in terms of LVEF (A) and LVFS (B) after post-infarction. $n = 12$ in each group. Scale bar, 1 mm. $n = 6$ in each group. The data are expressed as mean ± S.D. **$p < 0.01$ vs. sham mice treated with saline; *$p < 0.05$, **$p < 0.01$ vs. vehicle mice treated with saline. Sim: simvastatin.

![Figure 3](image2.png)

**Figure 3.** Treatments of different ratios of AR and ASR reduce infarct size after MI. (A) Infarct size was evaluated on Masson’s trichrome staining at 28 days after MI. The myocardial fibrotic tissue is defined on the basis of the difference in signal intensity between fibrotic tissue and viable myocardium and this difference generates the image contrast. (B) Quantitative analysis of fibrosis area at 28 days after MI. The fibrosis area was calculated as the ratio of the length of fibrotic area to the length of left-ventricular inner circumference with Image J software. Scale bar = 1 mm. $n = 6$ in each group. The data are expressed as mean ± S.D. $p < 0.05$ vs. vehicle mice treated with saline. Sim: simvastatin.
enhancing the preservation and/or recovery of functional myocardial tissues.

**Different combinations of AR and ASR reduce tissue injury in mice heart tissue**

The border zone tissue injuries in hearts of mice are shown in Figure 4. Haematoxylin–eosin staining was performed to observe the morphology and arrangement of cardiomyocytes in border zone of infarction. Histological assessment confirmed the improvement by different combinations of AR and ASR treatment when compared with that of vehicle (Figure 4). In the sham group, the morphology of the myocardial cells was uniform and the myofilaments were arranged neatly. In the model mice treated with saline, myocardial tissues were ruptured and curled with disorderly arranged myocardial fibres and infiltration of a large number of lymphocytes. However, in the AR–ASR combinations-treated groups, the myocardial fibres arranged orderly with less infiltration of inflammatory cells (Figure 4), indicating tissue injury in mice heart tissue was reduced in groups receiving combinations of AR–ASR at 1:1, 3:1 and 5:1 ratios.

**The combinations between AR and ASR at 1:1 and 5:1 inhibit apoptosis after MI**

To determine the mechanisms responsible for the protective effects against cardiac dysfunction and remodelling of AR–ASR combinations, apoptosis, inflammation and angiogenesis were respectively assessed at 1 week or 4 weeks post MI. We first examined the cardiomyocytes apoptosis by TUNEL staining in the infarct border zone of mice treated with saline or different combinations of AR and ASR. Nuclei were stained blue with DAPI. As shown in Figure 5(A,B), there was little staining in the sham tissue. In vehicle group, the number of TUNEL-positive nuclei was significantly increased. Mice receiving combinations of AR–ASR at 1:1 and 5:1 had a lower number of apoptotic cells compared with vehicle treatment ($p < 0.05$). AR–ASR mixed at 3:1 had no significant effect on apoptosis after MI. Consistent with this, AR mixed with ASR at 1:1 ratio decreased activation of caspase-3 and caspase-9 ($p < 0.05$ or $p < 0.01$), increased Bcl-2 (protein promoting cellular survival and inhibiting the activities of pro-apoptotic proteins) expression ($p < 0.01$) and decreased Bad (pro-apoptotic protein) expression ($p < 0.01$) in response to MI. AR–ASR mixture at 5:1 ratio also decreased activation of caspase-3 and caspase-9 ($p < 0.01$), and downregulated Bax (pro-apoptotic protein) expression significantly ($p < 0.01$) (Figure 5(C,D)). These results suggested that the combinations of AR and ASR at 1:1 and 5:1 reduced tissue injury partially by inhibiting the apoptosis in ischaemic heart tissue.

**The combination between AR and ASR at 1:1 reduces inflammation after MI**

The infarct expansion will be promoted by recruitment and activation of white blood cells. To assess leukocyte infiltrates post-
MI, heart tissues in the peri-infarct zone were immunostained for CD45 at seven days post-MI. The results showed that leukocyte infiltrates were significantly decreased in group receiving combination of AR–ASR at 1:1 ratio \((p < 0.05)\) (Figure 6(A,B)). Moreover, ELISA was performed to detect the pro-inflammatory cytokines release in heart tissue. IL-1β and IL-6 were increased in mice after MI induced by LAD ligation \((p < 0.05)\). Treatment of AR–ASR mixed at 1:1 significantly reduced the expression levels of IL-1β and IL-6 in the infarcted myocardium compared with those in the vehicle group \((p < 0.05)\) (Figure 6(C)). These results suggested that the combination between AR and ASR at 1:1 exerted an anti-inflammatory effect in MI mice.

The combinations between AR and ASR at 3:1 and 5:1 enhance angiogenesis after MI

To further detect the effect of different combinations of AR and ASR on MI-induced angiogenesis, the histological assessment was performed. At the border zone of the ischaemic region, capillary density was evaluated by identifying CD31⁺ vessels. Remarkably, the overall capillary density at the border zone of ischaemic myocardium 4 weeks after MI was significantly higher in the AR–ASR mixtures of 3:1 and 5:1-treated mice compared with the saline-treated animals \((p < 0.05\) or \(p < 0.01)\) (Figure 7(A,B)). These findings show that the combinations between AR
and ASR at 3:1 and 5:1, especially the ratio of 5:1, increased neovascularization after MI.

To elucidate the molecular mechanism, western blot analyses were performed to detect the VEGF, p-Akt and p-eNOS protein expressions at day 28 after surgery. VEGF is an important cytokine involved in creating new blood vessels in hearts of patients with ischaemic heart disease (Crafts et al. 2015). Akt/eNOS pathway can regulate cell survival, migration, tube formation and NO release, which is essential for the repair of ischaemic tissue damage (Shiojima and Walsh 2002; Amano et al. 2003; Chen et al. 2005). As shown in Figure 7(C,D), treatment of combinations of AR and ASR at 3:1 significantly augmented VEGF protein expression (p < 0.01) and increased Akt phosphorylation (p < 0.05). AR mixed with ASR at 5:1 significantly increased Akt and eNOS phosphorylation (p < 0.05). The above results indicated that the acceleration of improved angiogenesis in AR–ASR combinations at 3:1 and 5:1-treated hindlimb ischaemic mice may be explained, at least in part, by the activation of pro-angiogenic factors.

Discussion

In this study, the effects of different combinations of AR and ASR on cardiac protection were investigated in a mouse model of MI. The results suggested that different ratio combinations of AR and ASR improve cardiac function and reduce infarct size and tissue injury through different mechanisms. The combination at 1:1 inhibited apoptosis and reduced inflammation after MI. The combination ratio of 3:1 exerted effect in promoting angiogenesis. The 5:1 ratio of AR–ASR not only reduced apoptosis, but also promoted angiogenesis post-MI significantly.

Together, these observations demonstrated that different combinations of AR–ASR exerted protective effects against cardiac dysfunction. Although the mechanisms are diverse among different ratios, the herb pairs of AR and ASR could be exploited as a promising strategy for the prevention and treatment of MI.

Compatibility of AR and ASR is commonly utilized in Traditional Chinese Medicine to treat cardiovascular diseases. The most popular combination is 'Dang-Gui Decoction for Enriching the Blood', which is a traditional Chinese formulation comprising AR and ASR at a weight ratio of 5:1. It is widely used for promoting haematopoiesis as well as enhancing cardiovascular function. Modern pharmacological studies showed that the AR–ASR combinations can promote haematopoiesis (Li et al. 2017), stimulate NO production in endothelial cells (Gong et al. 2016), protect the cardiomyocytes from H2O2 injury through improving cell antioxidant ability (Li et al. 2011) and ameliorate coronary artery ligation-induced myocardial ischaemia associated with MAPK/NF-κB pathway (Ma et al. 2017). In the formula, AR is sweet and tepid and acts as the Emperor role. AR is considered to be the best herb to treat the ‘Qi’ (vital energy) deficiency and showed promising effects in improving biochemical and histological changes of heart failure (Yang et al. 2012; Gong et al. 2018). Astragalus injection made from AR has been widely used clinically for the treatment of heart disease (Ren et al. 2016). ASR is sweet and warm and acts as the Minister role in the combinations. It is widely used in clinic to invigorate blood circulation and regulate menstruation. Experimental and clinical studies have highlighted that ASR could reduce ischaemic injury and improve cardiac function after MI (Ma et al. 2015; Wei et al. 2016). The major bioactive constituents of AR and ASR show myocardioprotective effects against MI injury. For example,
AS-IV from AR can significantly reduce the myocardial infarct size and increase shortening fraction (Zheng et al. 2018) by a variety of mechanisms including promoting angiogenesis (Yu et al. 2015), regulating energy metabolism (Tu et al. 2013), anti-oxidant (Zhang et al. 2006), anti-inflammation (Lu et al. 2015; Cheng et al. 2016) and anti-apoptosis (Si et al. 2014; Yin et al. 2019). Astragalus polysaccharides from AR improve cardiac function via ROS-p38 and PI3K/AKT signalling pathways and suppress cardiomyocyte apoptosis (Liu et al. 2018). Angelica sinensis polysaccharides from ASR can attenuate hypoxia-induced cardiomyocyte cell injury by down-regulation of miR-22 (Pan and Zhu 2018) and protect cardiomyocytes against oxidative injury and endoplasmic reticulum stress by activating ATF6 pathway (Niu et al. 2018). In the present study, we compared the effects of the compatibility of AR and ASR in different proportion on cardioprotection in a mouse MI model. AR–ASR combinations treatment significantly promoted cardiac function and reduced infarct size following a 28-day therapy, emerging as an effective approach in the treatment of MI patients.

The underlying mechanisms that different combinations of AR and ASR protect heart from coronary artery ligation-induced myocardial ischaemia are essential to be illustrated. The variation in the contents of bioactive components may account for the different effects of AR–ASR combinations at different ratios. Our preliminary studies (data not shown) demonstrated that AR-derived AS-IV, calycosin, formononetin, ASR-derived ferulic acid and total polysaccharides from AR and ASR are higher in combinations of AR–ASR at 5:1 ratios. However, ASR-derived ligustilide is higher in the 1:1 ratio. At 3:1 ratio, the contents of the above active substances are all at the intermediate level. This is consistent with previous reports on chemical assessment of the herbal decoction containing AR and ASR (Dong et al. 2006; Gao et al. 2007; Zheng et al. 2010; Zhang et al. 2012). The exact mechanisms underlying the differences remain to be elucidated by further studies.

In the clinical practice of herbal practitioners, the compatibility amongst all the herbs within a decoction will be revised according to the syndrome of the patients. Thus, the investigation of the underlying mechanisms on cardioprotective effects of different AR–ASR combinations is necessary. When AR was combined with ASR at the ratio of 1:1, leukocyte infiltrates were decreased and the inflammatory cytokine secretion was inhibited in the heart tissue compared with the vehicle group after seven-day treatment. At the meantime, the cardiomyocyte apoptosis was suppressed, partially through inhibiting activation of caspase-3 and caspase-9 and decreasing the ratio of Bad to Bcl-2. However, in the combination of AR and ASR at 5:1 ratio, the angiogenesis was significantly improved along with enhanced...
Akt-eNOS phosphorylation. After MI, a step-by-step myocardial remodelling takes place involving the inflammatory phase and the reparative phase. During inflammatory phase, the milieu in the heart is highly inflammatory. Sequential infiltration of the injured myocardium with neutrophils, monocytes and their descendant macrophages contribute to the initiation of inflammation (Soehnlein and Lindbom 2010; Mantovani et al. 2011; Liu et al. 2016). The excessive inflammatory response will result in the necrotic area being replaced with granulation tissue and eventually a collagen-rich scar (Prabhu and Frangogiannis 2016). It suggests that the combination of AR–ASR at 1:1 can be applied as an adjuvant therapeutic agent during the early stages post-MI to promote effective resolution of inflammation, and eventually facilitate cardiac repair and improve LV function. In the reparative or proliferative phase, neovascularization of viable myocardium in the infarct border zone is crucial during the process of tissue remodelling (Cochain et al. 2013). The efficient perfusion is required to prevent cardiomyocyte death, which can lead to infarct expansion, left ventricular dilation and heart failure (Shiojima et al. 2005). As the combination ratio of 5:1 exerted the strongest effects in promoting angiogenesis and inhibiting cardiomyocyte apoptosis, it can serve as a late-stage therapeutic agent for acute MI.

In this study, we found that treatments with different combinations of AR and ASR dramatically improved cardiac function, decreased myocardial infarct size and reduced tissue injury in MI mice. Moreover, the myocardial protective effects of AR combined with ASR at 1:1, 3:1 and 5:1 treatment were respectively achieved by inhibiting inflammation and apoptosis, promoting angiogenesis, and regulating angiogenesis and inhibiting cardiomyocyte apoptosis. These results suggest that the treatment with AR–ASR combinations during different infarct periods might be useful approach to heart protection.

Author contributions
Hong Wang designed the study. Lu Chen analyzed the data and wrote the manuscript. Min Song, Lusha Zhang, Chunxiao Li and Zhirui Fang carried out experiments. Joel Wake Coffie, Liyuan Zhang, Lulu Ma, Qianyi Wang, Wenjie Yang, Leyu Fang and Fanggang Li analyzed data. Xiumei Gao designed the study and wrote the manuscript. Min Song, Lusha Zhang, Chunxiao Li and Hong Wang designed the study. Lu Chen analyzed the data and wrote the manuscript. Min Song, Lusha Zhang, Chunxiao Li and Min Song analyzed the data. Xiumei Gao reviewed the manuscript. All authors made the final approval of the version to be published. All authors reviewed the manuscript.

Disclosure statement
The authors report no declarations of interest.

Funding
This study was supported by the National Natural Science Foundation of China [Grant number: 81603329], Program of International S&T Cooperation Project of China [Grant number: 2015DFA30430] and Natural Science Foundation of Tianjin Municipal Government [Grant number: 16JCZDJC6300].

References
Amano K, Matsubara H, Iba O, Okigaki M, Fujiyama S, Imada T, Kojima H, Nozawa Y, Kawashima S, Yokoyama M, et al. 2003. Enhancement of ischemia-induced angiogenesis by eNOS overexpression. Hypertension. 41(1):156–162.
Bagal A, Dangas GD, Stone GW, Granger CB. 2014. Reperfusion strategies in acute coronary syndromes. Circ Res. 114(12):1918–1928.
Baudouy D, Michiels JF, Vukolic A, Wagner KD, Wagner N. 2017. Echocardiographic and histological examination of cardiac morphology in the mouse. J Vis Exp. 128:e55843.
Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, Byzova TV. 2005. Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. Nat Med. 11(11):1188–1196.
Cheng S, Yu P, Yang L, Shi H, He A, Chen H, Han J, Xie L, Chen J, Chen X. 2016. Atragaloside IV enhances cardioprotection of remote ischemic conditioning after acute myocardial infarction in rats. Am J Transl Res. 8(11):4657–4669.
Chiu PY, Leung HY, Siu AH, Poon MK, Dong TT, Tsim KW, Ko KM. 2007. Dang-Gui Buxue Tang protects against oxidant injury by enhancing cellular glutathione in H9c2 cells: role of glutathione synthesis and regeneration. Planta Med. 73(2):134–141.
Cochain C, Channon KM, Silvestre JS. 2013. Angiogenesis in the infarcted myocardium. Antioxid Redox Signal. 18(9):1100–1113.
Crafts TD, Jensen AR, Blocher-Smith EC, Markel TA. 2015. Vascular endothelial growth factor: therapeutic possibilities and challenges for the treatment of ischemia. Cytokine. 71(2):385–393.
Davidson SM, Ferdinandy P, Andreadiou I, Botker HE, Heusch G, Ibanez B, Owize M, Schulz R, Yellon DM, Hausenloy DJ, et al. 2019. Multitrigger strategies to reduce myocardial ischemia/reperfusion injury: JACC Review Topic of the Week. J Am Coll Cardiol. 73(1):89–99.
Dong TT, Zhao KJ, Gao QT, Ji ZN, Zhu TT, Li J, Duan R, Cheung AW, Tsim KW. 2006. Chemical and biological assessment of a Chinese herbal decoction containing Radix Astragali and Radix Angelicae sinensis: determination of drug ratio in having optimized properties. J Agric Food Chem. 54(7):2767–2774.
Gong AG, Liu J, Cheung JK, Duan D, Cheung AW, Zhao K, Li WZ, Dong TT, Tsim KW. 2007. Verification of the formulation and efficacy of Danggui Buxue Tang (a decoction of Radix Astragali and Radix Angelicae sinensis): an exemplifying systematic approach to revealing the complexity of Chinese herbal medicine formulae. Chin Med. 21(1):12.
Garber JC, Barbee RW, Bielitzki JT, Clayton LA, Donovan JC, Hendriksen CF, Kohn DF, Lipman NS, Locke PA, Melcher, et al. 2011. Guide for the care and use of laboratory animals. 8th ed. Washington (DC): National Academy Press (US).
Gong AG, Lau KM, Zhang LM, Lin HQ, Dong TT, Tsim KW. 2016. Danggui Buxue Tang, Chinese herbal decoction containing Astragali Radix and Angelicae Sinensis Radix, induces production of nitric oxide in endothelial cells: signaling mediated by phosphorylation of endothelial nitric oxide synthase. Planta Med. 82:418–423.
Gong AGW, Duan R, Wang HY, Kong XP, Dong TTX, Tsim KWK, Chan K. 2018. Evaluation of the pharmaceutical properties and value of Astragali Radix Medicines (Basel). 5:1–16.
Heusch G, Gersh BJ. 2017. The pathophysiology of acute myocardial infarction and strategies of protection beyond reperfusion: a continual challenge. Eur Heart J. 38(11):774–784.
Ibanez B, James S, Agewall S, Antunes MI, Bucciarelli-Ducci C, Bueno H, Caforio ALP, Crea F, Gotthuvenis JA, Halvorsen S, et al. 2018. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: the task force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J. 39:119–177.
Ibanez B, Macaya C, Sanchez-Brunete V, Pizarro G, Fernandez-Frieria L, Mateos A, Fernandez-Ortiz A, Garcia-Ruiz JM, Garcia-Alvarez A, Iniguez A, et al. 2013. Effect of early metoprolol on infarct size in ST-segment-elevation myocardial infarction patients undergoing primary percutaneous coronary intervention: The Effect of Metoprolol in Cardioprotection During an Acute Myocardial Infarction (METOCARD-CNIC) trial. Circulation. 128:1495–1503.
Kim YH, Her AY, Jeong MH, Kim BK, Lee SY, Hong SJ, Shin DH, Kim JS, Ko YG, Choi D, et al. 2019. Impact of renin-angiotensin system inhibitors on long-term clinical outcomes in patients with acute myocardial infarction treated with successful percutaneous coronary intervention with drug-eluting stents: comparison between STERNI and NSTEMI. Atherosclerosis. 280:166–173.
Kolk MV, Meyberg D, Deuse T, Tang-Quan KR, Robbins RG, Rechenspurner H, Schreper S. 2009. LAD-ligation: a murine model of myocardial infarction. J Vis Exp. 32:e14383.
Lei Y, Gao Q, Li YS. 2003. Study on effects of Astragalus, Angelica and their combination on vascular endothelial cell proliferation in vitro. Zhongguo Zhong Xi Yi Jie He Za Zhi. 23(10):753–756.

Lei Y, Wang JH, Chen KJ. 2003. Comparative study on angiogenesis effect of Astragalus membranaceus and Angelica sinensis in chick embryo choriolantoic membrane. Zhongguo Zhong Yao Za Zhi. 28(9):876–878.

Li F, Tang R, Chen LB, Zhang KS, Huang XP, Deng CQ. 2017. Effects of Astragalus combined with Angelica on bone marrow hematopoiesis suppression induced by cyclophosphamide in mice. Biol Pharm Bull. 40(5):598–609.

Li YD, Ma YH, Zhao JX, Zhao XK. 2011. Protection of ultra-filtration extract of Astragalus polysaccharide in vivo and in vitro. Int J Biol Macromol. 111:947–952.

Liu J, Wang H, Li J. 2016. Inflammation and inflammatory cells in myocardial infarction and reperfusion injury: a double-edged sword. Clin Med Insights Cardioli. 10:79–84.

Liu Z, Guo F, Wang Y, Li C, Zhang X, Li H, Diao L, Gu J, Wang W, Li D, et al. 2016. BATMAN-TCM: a Bioinformatics Analysis Tool for Molecular mechanism of Traditional Chinese Medicine. Sci Rep. 6(1):21146.

Lu M, Tang F, Zhang J, Luan A, Mei M, Xu C, Zhang S, Wang H, Maslov LN. 2015. Astragaloside IV attenuates injury caused by myocardial ischemia/reperfusion in rats via regulation of toll-like receptor 4/nuclear factor-kappaB signaling pathway. Pthotherapy Res. 29(4):599–606.

Ma CH, Long HY, Zhu WN, He XL, Zhang TJ, Ruan J. 2017. Dong Gui Bu Xue Tangameliorates coronary artery ligation-induced myocardial ischemia in rats. Biomed Pharmacother. 88:617–624.

Ma JP, Guo ZB, Jin L, Li YD. 2015. Phytochemical progress made in investigations of Angelica sinensis (Oliv.) Diels. Chin. J Nat Med. 13:241–249.

Mantovani A, Cassatella MA, Costantini C, Jaillon S. 2011. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol. 11(8):519–531.

Niu X, Zhang J, Ling C, Bai M, Peng Y, Sun S, Li Y, Zhang Z. 2018. Polysaccharide from Angelica sinensis protects H9c2 cells against oxidative injury and endoplasmic reticulum stress by activating the ATF6 pathway. J Int Med Res. 46(5):1717–1733.

Pan H, Zhu L. 2018. Angelica sinensis polysaccharide protects rat cardiomyocytes H9c2 from hypoxia-induced injury by down-regulation of microRNA-22. Biomed Pharmacother. 106:225–231.

Prabhu SD, Frangogiannis NG. 2016. The biological basis for cardiac repair after myocardial infarction: from inflammation to fibrosis. Circ Res. 119(1):91–112.

Reichert K, Colantuono B, McCormack I, Rodrigues F, Pavlov V, Abid MR. 2017. Murine left anterior descending (LAD) coronary artery ligation: an improved and simplified model for myocardial infarction. J Vis Exp. 122: e55353.

Ren Y, Chen ZQ, Zhang MZ, Guo LH, He DY. 2016. Cluster analysis of medication laws for treating coronary heart disease by distinguished veteran doctors of traditional Chinese medicine. Zhongguo Zhong Xi Yi Jie He Za Zhi. 36(4):411–414.

Roth GA, Abate D, Abate KH, Ayas SM, Abbafati C, Abbasi N, Abbastabar H, Abd-Allah F, Abdela J, Abdelalim A, et al. 2018. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet. 392(10159):1736–1788.

Shi X, Tang Y, Zhu H, Li W, Li W, Li Z, Luo N, Duan JA. 2014. Pharmacokinetic comparison of seven major bio-active components in normal and blood deficiency rats after oral administration of Danggui Buxue decoction by UPLC-TQ/MS. J Ethnopharmacol. 153(1):169–177.

Shiojima I, Sato K, Izuimiy Y, Schieker S, Ito M, Liao R, Colucci WS, Walsh K. 2005. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. J Clin Invest. 115(8):2108–2118.

Shiojima I, Walsh K. 2002. Role of Akt signaling in vascular homeostasis and angiogenesis. Circ Res. 90(12):1243–1250.

Si J, Wang N, Wang H, Xie J, Yang J, Yi H, Shi Z, Ma J, Wang W, Yang L, et al. 2014. HIF-1alpha signaling activation by post-ischemia treatment with astragaloside IV attenuates myocardial ischemia-reperfusion injury. PLoS One. 9(9):e107832.

Soehnlein O, Lindbom L. 2010. Phagocyte partnership during the onset and resolution of inflammation. Nat Rev Immunol. 10(6):427–439.

Tu L, Pan CS, Wei XH, Yan L, Liu YY, Fan JY, Mu HN, Li Q, Li L, Zhang Y, et al. 2013. Astragaloside IV protects heart from ischemia and reperfusion injury via energy regulation mechanisms. Microcirculation. 20:736–747.

Wei WL, Zeng R, Gu CM, Qu Y, Huang LF. 2016. Angelica sinensis in China—a review of botanical profile, ethnopharmacology, phytochemistry and chemical analysis. J Ethnopharmacol. 190:116–141.

Yang Y, Peng X, Xu H, Zhu J, Deng C. 2018. Inhibition of aortic intimal hyperplasia and vascular smooth muscle proliferation and extracellular matrix protein expressions by Astragalus-Angelica combination. Evid Based Complement Alternat Med. 2018:1–15.

Yang QY, Chen KJ, Lu S, Sun HR. 2012. Research progress on mechanism of action of Radix Astragali in the treatment of heart failure. Chin J Integr Med. 18(3):235–240.

Yin B, Hou XW, Lu ML. 2019. Astragaloside IV attenuates myocardial ischemia/reperfusion injury in rats via inhibition of calcium-sensing receptor-mediated apoptotic signaling pathways. Acta Pharmacol Sin. 40(5):599–607.

Yu JM, Zhang XB, Jiang W, Wang HD, Zhang YN. 2015. Astragalosides promote angiogenesis via vascular endothelial growth factor and basic fibroblast growth factor in a rat model of myocardial infarction. Mol Med Rep. 12(5):6718–6726.

Zhang WD, Chen H, Zhang C, Liu RH, Li HL, Chen HZ. 2006. Astragaloside IV from Astragalus membranaceus shows cardioprotection during myocardial ischemia in vivo and in vitro. Planta Med. 72(1):4–8.

Zhang WL, Zheng KY, Zhu KY, Zhan JY, Bi CW, Chen JP, Du CY, Zhao KJ, Lau DT, Dong TT, et al. 2012. Chemical and biological assessment of Angelica herbal decoction: comparison of different preparations during historical applications. Phytochemistry. 19(11):1042–1048.

Zheng Q, Zhu JZ, Bao XY, Zhu PC, Tong Q, Huang YZ, Zhang QH, Zhang KJ, Zheng GQ, Wang Y. 2018. A preclinical systematic review and meta-analysis of astragaloside IV for myocardial ischemia/reperfusion injury. Front Physiol. 9:795–810.

Zheng YZ, Choi RC, Li J, Xie HQ, Cheung AW, Duan R, Guo AJ, Zhu JT, Chen VP, Bi CW, et al. 2010. Ligustilide suppresses the biological properties of Danggui Buxue Tang: a Chinese herbal decoction composed of radix astragali and radix Angelica sinensis. Planta Med. 76(5):439–443.