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Original Article

Pretreatment with a combination of ligustrazine and berberine improves cardiac function in rats with coronary microembolization

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Aim: We have shown that a combination of ligustrazine and berberine produces more effective inhibition on platelet activation and inflammatory reactions in rat acute myocardial infarction compared with either agent alone. In this study we evaluated the beneficial effects of a combination of ligustrazine and berberine in a rat model of coronary microembolization (CME).
Methods: SD rats were treated with ligustrazine, berberine, ligustrazine+berberine, or clopidogrel for 2 weeks. When the treatment completed, CME was induced by injection of sodium laurate into the left ventricular, while obstructing the ascending aorta. All rats were intubated for hemodynamic measurements. Blood samples were collected for biochemical analyses, flow cytometry, and ELISAs. Heart tissues were isolated for histopathology and subsequent protein analyses.
Results: Pretreatment with the combination of ligustrazine (27 mg·kg^{-1}·d^{-1}) and berberine (90 mg·kg^{-1}·d^{-1}) significantly improved cardiac function, and decreased myocardial necrosis, inflammatory cell infiltration, microthrombosis and serum CK-MB levels in CME rats. In addition, this combination significantly decreased plasma ET-1 levels and von Willebrand factor, inhibited ADP-induced platelet activation, and reduced TNFα, IL-1β, ICAM-1 and RANTES levels in serum and heart tissues. The protective effects of this combination were more prominent than those of ligustrazine or berberine alone, but comparable to those of a positive control clopidogrel (6.75 mg·kg^{-1}·d^{-1}).
Conclusion: The combination of ligustrazine and berberine significantly improved cardiac function in rat CME model via a mechanism involving antiplatelet and anti-inflammatory effects.

Keywords: coronary microembolization; ligustrazine; berberine; clopidogrel; cardioprotection; platelet activation; inflammatory reactions

Introduction

Coronary microembolization (CME) is a potential cause of cardiac dysfunction in the absence of atherosclerotic obstruction of the epicardial coronary artery. Decades ago, CME was first identified at autopsy of patients dying from a sudden cardiac event[1]. However, CME is now more frequently observed following thrombolytic therapy and coronary interventions for an *in situ* coronary thrombus, or the spontaneous lysis of a remote arteriole thrombus; these may result in myocardial

micronecrosis, cardiac dysfunction, arrhythmias, and coronary reserve reduction[2]. CME is a main cause of non-obstructive coronary artery disease. One study[3] showed that patients with non-obstructive coronary artery disease were also at an elevated risk for cardiovascular events. An increasing number of studies[4-5] have shown that platelet activation is critical in the formation of *in situ* coronary thrombi and that the subsequent inflammatory reaction is a main contributor to contractile dysfunction.

Ligustrazine is extracted from the roots and stems of *Ligusticum chuanxiong* Hort (Chuan Xiong). Previous studies demonstrated that ligustrazine inhibited platelet activation induced by adenosine diphosphate (ADP)[6-7]. However, these studies did not explore this inhibitory effect *in vivo*. *In vivo* research

suggested that ligustrazine improved cardiac function in rats with heart failure by inhibiting myocardial cell apoptosis and cardiomyocyte hypertrophy[8-9]. Berberine is an important alkaloid derived from *Coptis chinensis* Franch (Huang Lian). Previous studies[10-11] indicated that berberine had cardioprotective effects that included lowering lipids, reducing ischemia/reperfusion-induced myocardial apoptosis, and attenuating adverse left ventricular remodeling[12-14]. Our previous study in rats with acute myocardial infarction[15] showed that combined ligustrazine and berberine produced more effective inhibition of platelet activation and inflammatory reactions than did either agent separately. However, the effects of ligustrazine plus berberine have not been examined in rats with CME.

In the present study, we established a rat model of CME and hypothesized that treatment with ligustrazine plus berberine would improve cardiac function by inhibiting platelet activation and the consequent inflammatory cascade.

Materials and methods
Drugs and reagents
Ligustrazine was provided by the Beijing Yan Jing Pharmaceutical Co Ltd (Beijing, China). Berberine was purchased from the Beijing Cheng Ji Pharmaceutical Co Ltd (Beijing, China). Clopidogrel sulfate tablets were purchased from Sanofi-Aventis (Hangzhou, China). Sodium laurate was obtained from Sigma-Aldrich (St Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for endothelin-1 (ET-1), von Willebrand factor (vWF), P-selectin, cyclic AMP (cAMP), tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), intracellular adhesion molecule-1 (ICAM-1), and regulated on activation, normal T cell-expressed and secreted (RANTES) were obtained from R&D Systems (Minneapolis, MN, USA). Vasodilator-stimulated phosphoprotein phosphorylation (VASP-P) kits (PLT VASP/P2Y$_{12}$) were purchased from Biocytex (Marseille, France). Antibodies against TNFα, IL-1β, ICAM-1, RANTES, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Abcam (Cambridge, MA, USA).

Animals
Adult male Sprague-Dawley rats (approximately 300–350 g) were provided by the Academy of Military Medical Sciences Laboratory Animal Center (certificate number SCXK-(Jun) 2012–0004). Animals were acclimated for at least one week in standard conditions and given free access to a standard diet and distilled water. All procedures were approved by the Animal Care and Use Committee of Xiyuan Hospital, China Academy of Chinese Medical Sciences, and conformed to the Animal Management Rule of the Ministry of Health, People's Republic of China (Document 55, 2001).

CME rat model and treatments
Experimental CME can be induced by injecting homologous microthrombotic particles based on embolization from a distant source following spontaneous lysis[16-17]. The present study established a CME model by left ventricle (LV) injection of sodium laurate, as previously described[18-19]. This model was characterized by the formation of *in situ* coronary thrombi. Ninety male rats were allocated randomly to six groups: sham, CME model, ligustrazine (27 mg·kg^{-1}·d^{-1}), berberine (90 mg·kg^{-1}·d^{-1}), ligustrazine + berberine (L+B; 27 mg·kg^{-1}·d^{-1} + 90 mg·kg^{-1}·d^{-1}, respectively), and clopidogrel (positive control; 6.75 mg·kg^{-1}·d^{-1}). The indicated drugs were administered daily by oral gavage for two weeks, with the sham and CME model groups receiving vehicle. Twelve hours after the last treatment, the animals were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally) and ventilated with a small animal ventilator (Chengdu Taimeng Software Co Ltd, Chengdu, China). A thoracotomy was performed at the midline of the chest, followed by a sternotomy between the second and third intercostal space. The pericardium was opened and the ascending aorta exposed fully. Sodium laurate (2 g/L, 0.2 mL) was injected as a bolus into the LV using a 29-gauge needle during a 30 s occlusion of the ascending aorta. For the sham group, an identical procedure was performed and 0.2 mL physiological saline was injected, instead of sodium laurate. The thoracic cavity and the skin incision were then closed with sutures. All rats were intubated 24 h after the operation to carry out hemodynamic measurements. Blood samples were collected for biochemical analyses, flow cytometry, and ELISAs. Hearts from five rats in each group were isolated for histopathology. The remaining hearts were frozen quickly for subsequent protein analyses.

Hemodynamics
Animals were anesthetized with pentobarbital sodium and arterial pressure was recorded by a polyethylene catheter (PE-50), positioned in the right carotid artery. The catheter was then inserted into the LV to record the ventricular pressure. These data were collected using a pressure transducer coupled to a pressure amplifier. Pressure analog signals were digitized by a data acquisition and analysis system, with a sampling rate of 2000 Hz. All equipment was from Chengdu Taimeng Software Company. These data were used to determine heart rate (HR), LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and LV maximum positive and negative pressure changes over time (+dp/dt_{max} and -dp/dt_{max}, respectively).

Histopathological changes
Hearts were harvested, fixed in 4% paraformaldehyde, embedded, and cut into 4-µm sections from the injection point downwards, in parallel with the coronary ditch. Sections were stained with hematoxylin and eosin for the evaluation of inflammatory cell infiltration. Micronecrotic foci were identified with Heidenhain staining. The sections were scored for myocardial necrosis and cellular infiltration by a skilled and blinded observer. The scores were: 0, no myocardial lesions; 1+, lesions involving < 25% of the myocardium; 2+, lesions involving 25%–50% of the myocardium; 3+, lesions involving 50%–75% of the myocardium; 4+, lesions involving > 75% of the myocardium. Microthrombosis was evaluated in sections

using Carstairs staining, as described previously[18]. The number of microthrombi/100 microarteries (diameter ≤ 100 µm) was calculated for each group.

Creatine kinase MB fraction (CK-MB)
Blood was obtained from the abdominal aorta of all rats prior to sacrifice, centrifuged at $3500 \times g$ for 10 min, and stored at -80°C. The serum levels of CK-MB were determined using an automatic biochemical analyzer (Cobas8000, Roche).

Flow cytometry analysis of VASP-P
To measure ADP-induced platelet activation, the VASP-239 phosphorylation state was determined in citrate-anticoagulated blood samples using the PLT VASP/P2Y$_{12}$ kit, as described previously[20]. Samples were treated in accordance with the manufacturer's protocol and were analyzed within 4 h of collection. In brief, whole blood was incubated for 10 min at room temperature with either the solutions of PGE$_1$ or PGE$_1$ plus 10 µmol/L ADP that were provided with the kit. These blood samples were then treated with fixative containing paraformaldehyde. Platelets were permeabilized and incubated with either an anti-VASP-P mouse monoclonal antibody (16C2) or the negative isotype control provided with the kit. Samples were then incubated with a polyclonal anti-mouse IgG antibody conjugated to fluorescein isothiocyanate and a platelet counterstaining reagent-PE prior to immediate analysis by flow cytometry using a FACS Calibur (Becton Dickinson, San Jose, CA, USA). The platelet population was identified using the forward and side scatter distributions, and 5000 platelets were gated and collected. Data from the negative isotype control was used to correct the mean fluorescent intensities (MFIcs) of PGE$_1$ (MFIc$_{PGE}$) and PGE$_1$ plus ADP (MFIc$_{PGE+ADP}$) samples that were incubated with anti-VASP-P. The platelet reactivity index (PRI) was calculated as outlined in the kit using the mean corrected fluorescence intensities and the following formula: PRI=[(MFIc$_{PGE}$ - MFIc$_{PGE+ADP}$)/MFIc$_{PGE}$] ×100.

ELISA determinations of ET-1 and vWF
Plasma ET-1 and vWF levels were measured using an antigen-based sandwich ELISA, according to the manufacturer's instructions. For ET-1 measurements, 96-well plates were coated with 100 µL of an anti-ET-1 monoclonal antibody per well. Between subsequent steps in the assay, the coated plates were washed three times in washing buffer. After a 30-min preincubation with washing buffer at 37 °C, 100 µL of standard ET-1, control solution, or rat plasma were added and incubated for 2 h at 37 °C. A goat polyclonal anti-murine ET-1 antibody was then added for 90 min, followed by a horseradish peroxidase (HRP)-conjugated donkey anti-goat antibody for 1 h, at room temperature. Diluted *o*-phenylenediamine (100 µL) was then added. The enzymatic reaction was stopped by adding 100 µL 1 mol/L sulfuric acid, and the absorbance was then measured at 492 nm using a microplate spectrophotometer.

P-selectin and cAMP determinations
Plasma P-selectin and cAMP levels were determined using an antigen-based sandwich ELISA, according to the manufacturer's instructions.

Inflammatory mediator levels
Serum levels of TNFα, IL-1β, ICAM-1, and RANTES were determined using an antigen-based sandwich ELISA, according to the manufacturer's instructions.

Western blotting
Frozen heart tissues were homogenized in cold RIPA buffer (50 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton, 0.5% deoxycholic acid, 0.1% SDS, plus protease inhibitor cocktail) using a glass homogenizer. The homogenized tissues were centrifuged at $14\,000 \times g$ for 30 min at 4°C and the protein concentrations of the supernatants were determined by Bradford assay. Samples were then mixed with 4×SDS buffer to a final dilution of 1 ×, and heated to 95°C for 5 min. Proteins were separated by electrophoresis using SDS-polyacrylamide gels and blotted onto P^{sq} (RANTES, TNFα, and IL-1β) or polyvinylidine fluoride (ICAM-1) membranes. Proteins were detected using rabbit polyclonal anti-RANTES (Abcam, ab9783), rabbit polyclonal anti-IL-1β (Abcam, ab9722), rabbit polyclonal anti-TNFα (Abcam, ab9755), or mouse monoclonal anti-ICAM-1 (Abcam, ab2213) antibodies. HRP-goat anti-mouse IgG (Jackson, 115-035-003) or HRP-goat anti-rabbit IgG (Pregene #GR-100) were used as secondary antibodies, as appropriate. ImageJ software was used to analyze the intensity of the protein bands.

Statistical analysis
Data are expressed as mean±standard error of the mean (SEM). Significant differences were determined using one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test. A *P* value less than 0.05 was considered significant. Statistical analyses were performed by SPSS 20.0 software (IBM, Chicago, IL, USA).

Results
Histopathological findings
The histopathological findings are listed in Table 1. In contrast to the sham group, the CME model group showed significant myocardial necrosis, with inflammatory cell infiltration and *in situ* coronary microthrombi (*P*<0.01, Figures 1–3). Myocardial necrosis, inflammatory cell infiltration, and *in situ* coronary microthrombi were decreased in the L+B group, as compared with the CME model group (*P*<0.01). Clopidogrel was used as a positive control and this compound also reduced inflammatory cell infiltration (*P*<0.05) and suppressed formation of microthrombi (*P*<0.01), as compared with the CME model group. Ligustrazine alone exerted a preventative effect against microthrombi (*P*<0.01), but had no significant effect on myocardial necrosis and inflammatory cell infiltration, as compared with the CME model group (*P*>0.05). Berberine
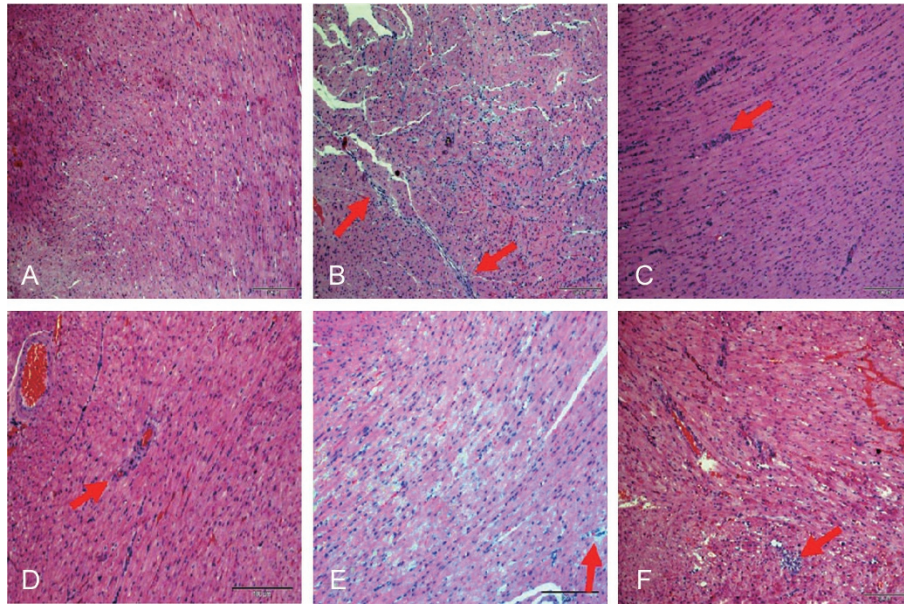
Figure 1. Visualization of inflammatory cellular infiltrates (red arrows) by hematoxylin and eosin staining (×200; bar=100 μm). Rat heart tissue sections are shown from the sham (A), coronary microembolization (CME) model (B), ligustrazine (C), berberine (D), L+B (E), clopidogrel treatment groups (F). L, Ligustrazine; B, Berberine.
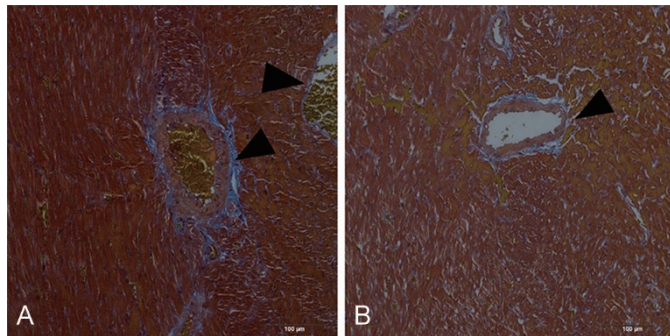

Figure 2. *In situ* coronary microthrombi were visualized by Carstairs staining. Rat heart tissue sections are shown from the: coronary microembolization (CME) model (A) and sham treatment (B) groups. Multiple arteriolar thrombi (arrow heads) were detected in the CME model group, but not in the sham group. (×200; bar=100 μm).

Table 1. Treatment with ligustrazine and berberine on histology in a rat coronary microembolization model, 24 h after surgery. Data represent the mean±SEM tissue scores in sections that were Heidenhain-stained (for necrosis), hematoxylin and eosin-stained (for infiltration), or Carstairs-stained (for microthrombosis). [c]$P<0.01$ *vs* sham. [e]$P<0.05$, [f]$P<0.01$ *vs* the coronary microembolization model. $n=5$.

	Necrosis	Infiltration	Microthrombosis (%)
Sham	0.0±0.0[f]	0.0±0.0[f]	0.0±0.0[f]
Model	3.2±0.2[c]	2.8±0.2[c]	50.0±3.2[c]
Ligustrazine (L)	2.4±0.2[c]	2.0±0.3[c]	30.8±3.1[cf]
Berberine (B)	2.8±0.4[c]	2.2±0.4[c]	40.6±3.1[c]
L+B	1.4±0.2[cf]	1.4±0.2[cf]	27.6±2.3[cf]
Clopidogrel	2.2±0.2[cf]	1.6±0.2[bf]	29.4±2.4[cf]

produced no significant reductions of myocardial necrosis, inflammatory cell infiltration, or microthrombi ($P>0.05$).

Cardiac function

The effects of treatment with L+B on the cardiovascular physiology of rats subjected to CME were examined by measuring various hemodynamic parameters. The results are summarized in Table 2. We found no significant change in HR between the study groups ($P>0.05$). Compared to the sham group, rats in the CME model group displayed a marked increase in LVEDP ($P<0.01$) and a decrease in LVSP ($P<0.01$), $+dp/dt_{max}$ ($P<0.01$), and $-dp/dt_{max}$ ($P<0.05$). Pretreatment with L+B significantly decreased LVEDP ($P<0.01$) and increased LVSP ($P<0.01$) and $\pm dp/dt_{max}$ ($P<0.05$), as compared to the CME model group. Similar changes were observed in the clopidogrel group, with no significant difference between the sham and the L+B groups ($P>0.05$). CME rats pretreated with ligustrazine alone exhibited a significant improvement in $+dp/dt_{max}$ ($P<0.05$) and a reduction in LVEDP ($P<0.05$), and the CME rats pretreated with berberine alone had a decreased LVEDP ($P<0.05$), as compared with untreated CME model rats. These results suggest that pretreatment with L+B improves cardiac function in this rat model of CME.

Levels of serum CK-MB

To further evaluate the cardioprotective effect of L+B in this CME model, the level of CK-MB in serum was measured after 24 h. This analysis showed that the level of CK-MB was significantly higher in the CME model group than in the sham group ($P<0.01$). As compared with the CME model group, L+B treatment reduced the serum CK-MB level significantly ($P<0.01$). CME rats pretreated with ligustrazine alone also
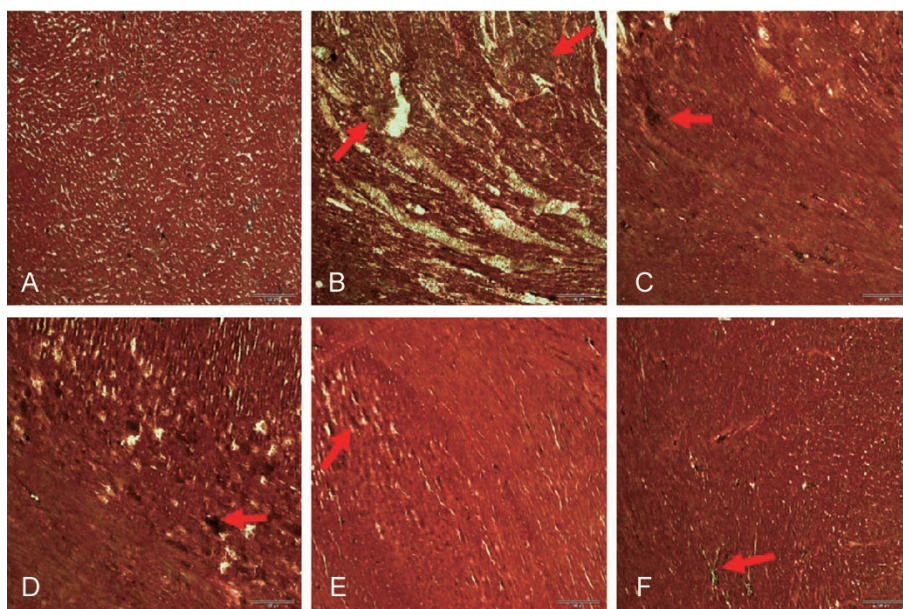
Figure 3. Myocardial necrosis (red arrows) was visualized by Heidenhain staining. Rat heart tissue sections are shown from the the sham (A), coronary microembolization (CME) model (B), ligustrazine (C), berberine (D), L+B (E), clopidogrel treatment groups (F). L, Ligustrazine; B, Berberine. Necrotic myocardium was stained brown to black. (×200; bar=100 μm).

Table 2. Treatment effects on cardiac function, assessed hemodynamically 24 h after surgery in a rat coronary microembolization model. Data are expressed as mean±SEM. n=6. [b]$P<0.05$, [c]$P<0.01$ *vs* sham. [e]$P<0.05$, [f]$P<0.01$ *vs* the coronary microembolization model.

	LVSP (mmHg)	LVEDP (mmHg)	HR (beat/min)	+dp/dt_{max} (mmHg/s)	-dp/dt_{max} (mmHg/s)
Sham	134±15[e]	-9.4±5.7[f]	508±17	10188±994[f]	-7327±1380[e]
Model	100±20[c]	7.7±5.4[c]	495±24	6510±1919[c]	-4639±1761[b]
Ligustrazine (L)	125±16	-2.1±3.9[e]	485±19	9935±3078[e]	-6375±1608
Berberine (B)	118±16	-1.7±2.6[e]	504±16	8602±779	-6395±1307
L+B	134±8[f]	-3.6±6.6[f]	509±12	10110±445[e]	-7194±1076[e]
Clopidogrel	129±14[f]	-5.5±1.6[f]	502±7.4	10190±913[f]	-7306±1329[e]

LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; +dp/dt_{max}, the maximum rate of increase of the LVSP; -dp/dt_{max}, the maximum rate of decrease of the LVSP.

Table 3. Effects of ligustrazine and berberine on the serum concentration of creatine kinase MB fraction in a rat coronary microembolization model, 24 h after surgery. Data are expressed as mean±SEM. n=8. [c]$P<0.01$ *vs* sham. [e]$P<0.05$, [f]$P<0.01$ *vs* the coronary microembolization model.

Groups	Creatine kinase MB fraction (IU/L)
Sham	674±114[f]
Model	1545±229[c]
Ligustrazine (L)	1022±188[e]
Berberine (B)	1348±223
L+B	867±223[f]
Clopidogrel	1012±210[e]

exhibited a significant reduction in CK-MB ($P<0.05$), while there was no significant change in CK-MB in the group treated with berberine only ($P>0.05$). The results from the clopidogrel group were similar to those of the ligustrazine group.

Plasma levels of vWF and ET-1

To evaluate the protective function of L+B on the endothelium, plasma levels of vWF and ET-1 were measured one day after exposure to sodium laurate. The CME model group exhibited a significant increase in vWF and ET-1 levels, as compared to the sham group ($P<0.05$; Figure 4). This increase in vWF and ET-1 was significantly attenuated in the clopidogrel, L+B, ligustrazine, and berberine treatment groups ($P<0.05$). vWF and ET-1 levels were similar after clopidogrel and L+B treatments ($P>0.05$). However, the vWF levels in rats treated with clopidogrel, L+B, ligustrazine, and berberine were still significantly higher than those observed in the sham group ($P<0.05$).

Platelet activation

Plasma P-selectin and cAMP levels, as well as PRI, were examined to investigate the effect of L+B on ADP-induced platelet
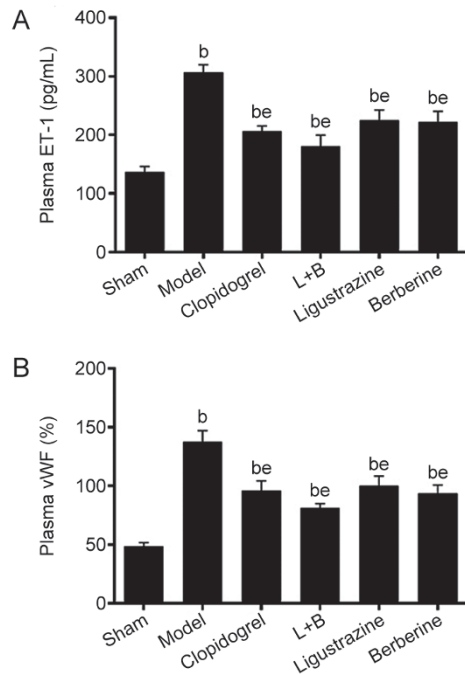
Figure 4. Plasma levels of von Willebrand factor (vWF) and endothelin-1 (ET-1) after ligustrazine and berberine treatment. Data are expressed as mean±SEM. *n*=6. [b]*P*<0.05 *vs* sham. [e]*P*<0.05 *vs* coronary microembolization (CME) model.

activation. As shown in Figure 5, PRI was significantly lower in the L+B, ligustrazine, and clopidogrel groups (*P*<0.05) than in the CME model group. The plasma P-selectin level was significantly higher in the CME model group, as compared with the sham group (*P*<0.05). Treatment with L+B reduced the plasma P-selectin level significantly (*P*<0.05), as did clopidogrel. Furthermore, the CME model group had a lower plasma cAMP level than the sham group (*P*<0.05) and cAMP levels were significantly increased in the L+B and clopidogrel groups (*P*<0.05).

Serum inflammatory mediator levels

Serum TNFα, IL-1β, ICAM-1, and RANTES levels were measured as indices of a systemic inflammatory response. Serum levels of these mediators were increased significantly in the CME model group, as compared with the sham group (*P*<0.05, Figure 6). Treatment with L+B significantly attenuated this increase in TNFα, IL-1β, ICAM-1, and RANTES levels (*P*< 0.05). Clopidogrel decreased TNFα, ICAM-1, and RANTES, but not IL-1β, levels, as compared with the CME model group (*P*<0.05). Ligustrazine alone also decreased serum TNFα, and berberine alone significantly reduced RANTES, as compared with the CME model group (*P*<0.05 for both comparisons). The level of RANTES in the L+B group was significantly lower than that of the ligustrazine group (*P*<0.05).

Heart expression of TNFα, IL-1β, ICAM-1, and RANTES proteins

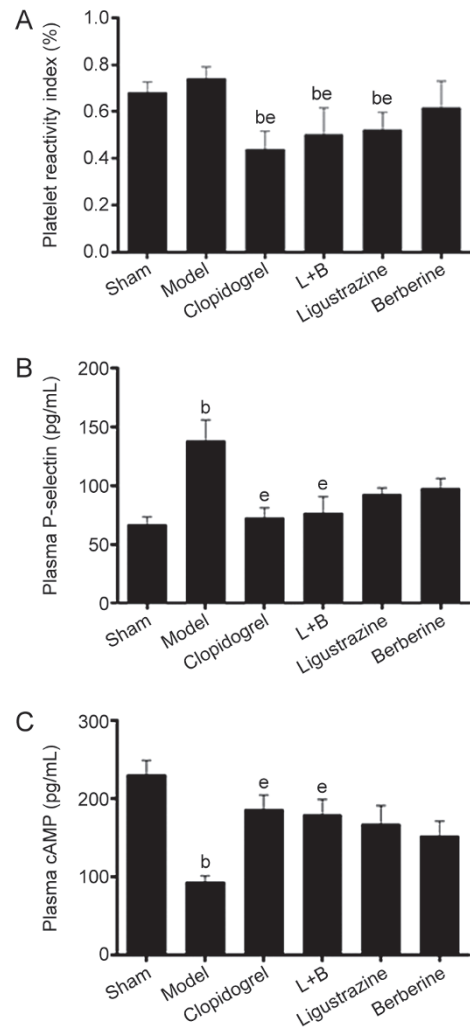Levels of TNFα, IL-1β, ICAM-1, and RANTES proteins in rat hearts were calculated in relation to the constitutively

Figure 5. Effect of treatment with ligustrazine and berberine (L+B) on platelet activation. Data are expressed as mean±SEM. *n*=6. [b]*P*<0.05 *vs* sham. [e]*P*<0.05 *vs* coronary microembolization (CME) model.

expressed GAPDH protein as indicators of the local cardiac inflammatory response. As expected, their expression was increased in the CME model group, as compared with the sham group (*P*<0.05; Figure 7). Pretreatment with clopidogrel or L+B significantly attenuated the increased expression of all four proteins (*P*<0.05), although the expression of TNFα, IL-1β, and ICAM-1 in the clopidogrel group remained higher than that observed in the sham group (*P*<0.05). Meanwhile, the expression of TNFα, IL-1β, ICAM-1, and RANTES in the L+B group did not differ significantly from that of the sham group (*P*>0.05). Interestingly, the level of IL-1β in the L+B group was significantly lower than the levels observed in the groups treated with ligustrazine or berberine only (*P*<0.05).

Discussion

Over the past three decades, an increasing number of studies have identified disorders in the function and structure of the coronary microcirculation in many clinical conditions. These disorders represent critical contributors to myocardial
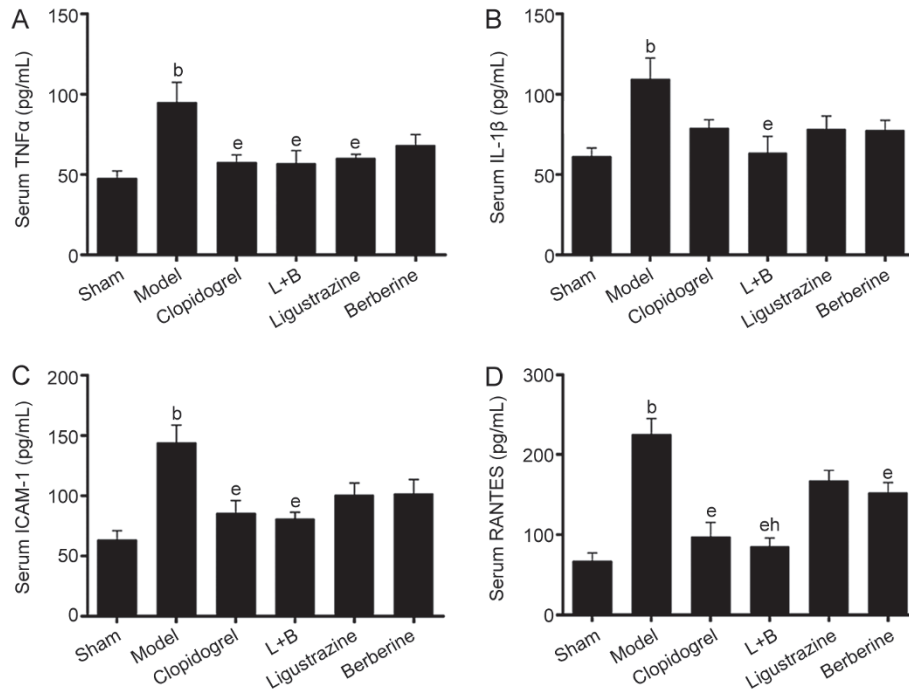
Figure 6. Effect of treatment with ligustrazine and berberine (L+B) on serum inflammatory mediators. Data are expressed as mean±SEM. $n=6$. [b]$P<0.05$ *vs* sham. [e]$P<0.05$ *vs* coronary microembolization (CME) model. [h]$P<0.05$ *vs* ligustrazine or berberine alone.
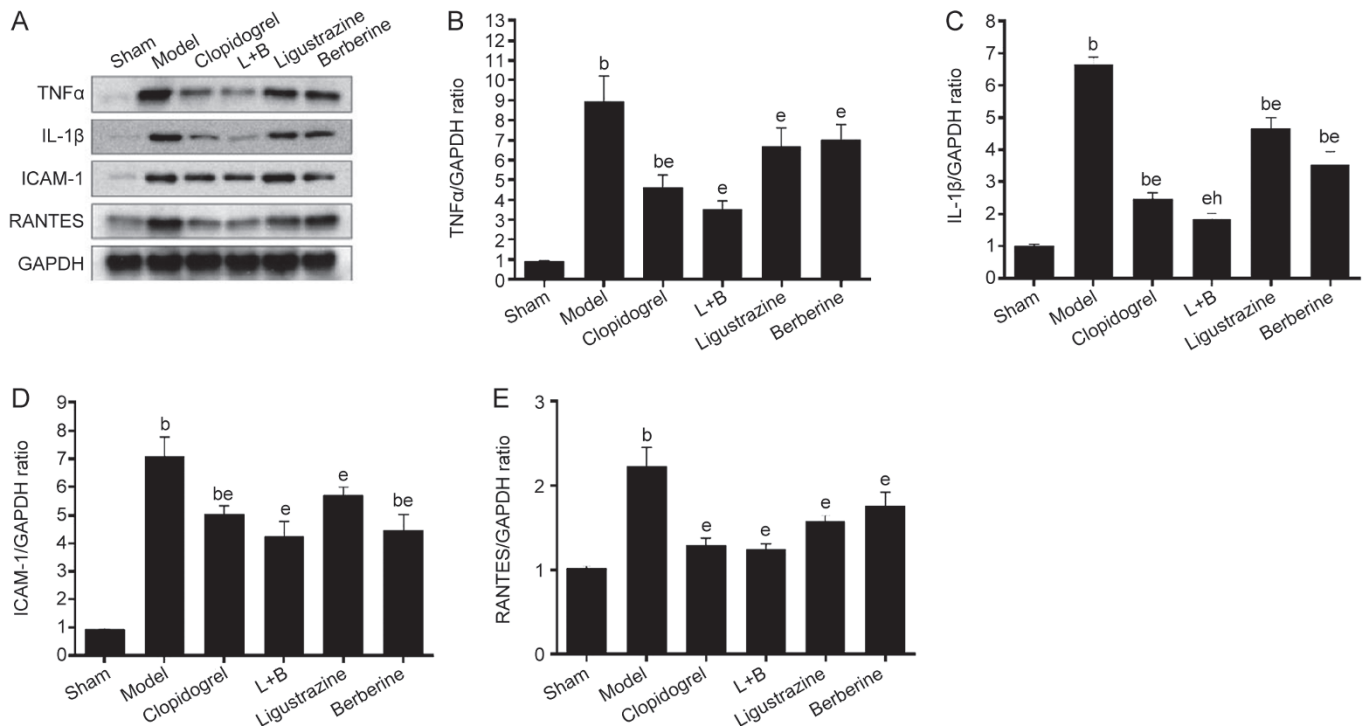

Figure 7. Western blot images of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, intracellular adhesion molecule-1 (ICAM-1), and regulated on activation, normal T cell-expressed and secreted (RANTES) protein levels in myocardial cells. Densitometric data are expressed as mean±SEM. $n=6$. [b]$P<0.05$ *vs* sham. [e]$P<0.05$ *vs* coronary microembolization (CME) model. [h]$P<0.05$ *vs* ligustrazine or berberine alone.

ischemia and have become important therapeutic targets[21]. Therefore, the establishment of an animal model of CME that

mimics the clinical pathology is important for studies of conditions where coronary microvascular dysfunction occurs, such

as microvascular angina.

In the present study, a CME model was successfully established in rats by the LV injection of sodium laurate, as evidenced by endothelial injury, *in situ* coronary thrombi, myocardial necrosis with obvious inflammatory cell infiltration, and LV dysfunction. Compared with previous CME models induced by the LV injection of homologous microthrombotic particles[16-17], the endothelial injury in the present model was induced first by sodium laurate, triggering platelet adhesion and aggregation that resulted in *in situ* thrombosis within coronary arterioles. In addition, endothelial injury or thrombi formation were not observed in the large coronary arteries. These animal pathologies were therefore more similar to those observed in patients, as compared with the previously reported models. Our findings also indicated that sodium laurate had dose-dependent effects on the endothelium. In a preliminary experiment, 0.2 mL of 1 g/L sodium laurate was injected into the LV and no thrombi were observed in the coronary arterioles (diameter ≤ 100 μm). However, the dose of 0.2 mL of 2 g/L sodium laurate used in the present study caused endothelial injury and thrombotic occlusion of the coronary arterioles.

Clopidogrel inhibits platelet activation by blocking the $P2Y_{12}$ platelet receptor and it is one of the most widely used antiplatelet drugs for patients with coronary artery disease. The present study showed that, in addition to its antiplatelet effects, clopidogrel showed anti-inflammatory activity; this was evidenced by the decreased serum levels of TNFα, ICAM-1, and RANTES, and heart tissue expression of TNFα, ICAM-1, IL-1β, and RANTES proteins. These findings were consistent with previous studies[22-25] and indicated that the cardioprotective effects of clopidogrel observed in the present study may be, in part, attributed to these anti-inflammatory effects.

Both ligustrazine and berberine are the component of Chinese herbs that have been used for more than two decades for the treatment of atherosclerotic and inflammatory diseases[26-29]. Our study demonstrated that ligustrazine alone improved cardiac function in CME rats, as evidenced by an increased +dp/dt_{max}, decreased LVEDP, and decreased serum CK-MB level. Berberine also decreased the LVEDP, but had no obvious impact on CK-MB levels. Both the histopathological findings and hemodynamic results indicated that use of ligustrazine and berberine in combination produced greater effects than either agent alone. Our study identified a unique antiplatelet effect of ligustrazine (reduced PRI), while the marked anti-inflammatory effect of berberine may contribute to its cardioprotective activity. Interestingly, the use of ligustrazine and berberine in combination enhanced these antiplatelet and anti-inflammatory effects. A series of previous studies by Shang *et al*[30-31] showed that Chinese herbs that activated blood circulation produced synergistic effects when administered with detoxifying herbs. The findings of the present study indicated that ligustrazine and berberine may also have synergistic effects, although the mechanism involved remains to be elucidated.

$P2Y_{12}$ inhibitors such as clopidogrel are associated with increased survival rates in patients with coronary microvascular dysfunction[32-33]. The present study showed that treatment with the combination of ligustrazine and berberine produced a similar effect as clopidogrel, blocking the $P2Y_{12}$ platelet receptor and increasing cAMP and VASP-239 phosphorylation. Although ligustrazine alone also inhibited the $P2Y_{12}$ platelet receptor, this effect was enhanced when it was administered with berberine.

Previous studies[34-35] have demonstrated the pivotal role of inflammation in adverse LV function. Patients with heart failure often have elevated levels of pro-inflammatory cytokines and chemokines. Among the cytokines, TNFα has been implicated in the development of LV dysfunction[36]. IL-1β increases dynamically following CME and is associated with impaired cardiac function[37]. RANTES has been identified as a specific marker for unstable angina pectoris by several studies[38-39]. The adhesion molecule, ICAM-1, also represents a marker of slow coronary flow caused by microvascular dysfunction[40-41]. Therefore, we chose these four molecular factors for the present evaluation of CME-related inflammatory responses. Although only some of these markers were reduced in rats treated with ligustrazine or berberine separately, all four were significantly affected in animals treated with these compounds in combination. These effects were greater than those of clopidogrel, suggesting that the inflammatory response reflected not only platelet activation, but also vascular endothelial injury. Plasma levels of both ET-1 and vWF, which are markers of vascular endothelial injury in the cardiovascular system, were reduced in rats treated with the combination of ligustrazine and berberine.

Coronary microvascular dysfunction can present clinically as chest pain in individuals with normal coronary arteriograms (*ie*, microvascular angina) or in the context of other cardiac disease processes. The assessment and visualization of human coronary microcirculation anatomy is clearly challenging and this creates difficulties in relation to diagnosis and treatment. Therefore, the present study is of great significance for clinical practice and complements the increasing body of clinical research demonstrating the cardioprotective effects of ligustrazine and berberine[42-43].

The present study had some limitations. First, we only investigated the short-term protective effects of the test compounds. Thus, future studies should investigate whether this protective effect is sustained over the longer term. Second, the present study did not explore whether the mechanisms underlying the action of the combination of ligustrazine and berberine were associated with other factors.

In conclusion, our study found that a rat CME model showed cardiac dysfunction, which was alleviated by combined treatment with ligustrazine and berberine. The mechanism underlying this protective effect appeared to involve inhibition of endothelial injury, reduced ADP-mediated platelet activation, and anti-inflammatory effects. The combined use of ligustrazine and berberine might provide a reasonable phytomedicine for the treatment of patients with diseases

involving coronary microvascular dysfunction.

Author contribution

Da-zhuo SHI and Xiao-juan MA designed the study and revised the manuscript; Ying ZHANG wrote the manuscript and performed the animal experiments; Chun-yu GUO, Ming-ming WANG, Na KOU, Hua QU, and Hui-min MAO contributed to the molecular biology experiments.

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