ORIGINAL ARTICLE

Salvianolate Reduces Murine Myocardial Ischemia and Reperfusion Injury via ERK1/2 Signaling Pathways *in vivo*[*]

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ABSTRACT   **Objective:** To analyze the effects of salvianolate on myocardial infarction in a murine *in vivo* model of ischemia and reperfusion (I/R) injury. **Methods:** Myocardial I/R injury model was constructed in mice by 30 min of coronary occlusion followed by 24 h of reperfusion and pretreated with salvianolate 30 min before I/R (SAL group). The SAL group was compared with SHAM (no I/R and no salvianolate), I/R (no salvianolate), and ischemia preconditioning (IPC) groups. Furthermore, an ERK1/2 inhibitor PD98059 (1 mg/kg), and a phosphatidylinositol-3-kinase (PI3-K) inhibitor, LY294002 (7.5 mg/kg), were administered intraperitoneal injection (i.p) for 30 min prior to salvianolate, followed by I/R surgery in LY and PD groups. By using a double staining method, the ratio of the infarct size (IS) to left ventricle (LV) and of risk region (RR) to LV were compared among the groups. Correlations between IS and RR were analyzed. Western-blot was used to detect the extracellular signal-regulated kinase 1/2 (ERK1/2) and protein kinase B (AKT) phosphorylation changes. **Results:** There were no significant differences between RR to LV ratio among the SHAM, I/R, IPC and SAL groups ($P>0.05$). The SAL and IPC groups had IS of 26.1%±1.4% and 22.3%±2.9% of RR, respectively, both of which were significantly smaller than the I/R group (38.5%±2.9% of RR, $P<0.05$, $P<0.01$, respectively). Moreover, the phosphorylation of ERK1/2 was increased in SAL group ($P<0.05$), while AKT had no significant change. LY294002 further reduced IS, whereas the protective role of salvianolate could be attenuated by PD98059, which increased the IS. Additionally, the IS was not linearly related to the RR ($r=0.23$, 0.45, 0.62, 0.17, and 0.52 in the SHAM, I/R, SAL, LY and PD groups, respectively). **Conclusion:** Salvianolate could reduce myocardial I/R injury in mice *in vivo*, which involves an ERK1/2 pathway, but not a PI3-K signaling pathway.
KEYWORDS   ischemia and reperfusion injury, salvianolate, extracellular signal-regulated kinase 1/2, protein kinase B, Chinese medicine

Early reperfusion after coronary occlusion is the most important strategy for treating the patients with acute myocardial infarction. However, myocardial ischemia-reperfusion (I/R) can induce lethal ventricular arrhythmia and myocardial infarction.[1-3] Nearly 50% myocardial infarction occur following I/R,[4] and 20%–35% of patients had severely reduced tissue-level perfusion,[5] associated with progressive myocardial infarct, cardiac arrhythmia, leading to I/R injury.[6] Despite the different etiologies that lead to partial or complete arrest of cardiac circulation, both patient groups share myocardial I/R injury as a common pathophysiological process.[7]

Salvia miltiorrhiza has been widely used for treating cardiovascular diseases in China for more than 2000 years. It has also been indicated for chronic hepatitis and liver fibrosis treatment,[8] as well as inflammatory diseases such as lethal sepsis.[9] Salvianolate, as a highly purified water-soluble component extracted from *Salvia miltiorrhiza*, containing magnesium lithospermate B (≥85%), rosmarinic acid (≥10.1%) and lithospermic acid (≥1.9%),[10] as shown in Figure 1. Clinical trials demonstrated that salvianolate is effective and safe for treating acute coronary syndrome.[11]

Studies showed that magnesium lithospermate B, an important component of salvianolate, demonstrated multiple biological activities, such as improving microcirculation, suppressing the formation of reactive oxygen species, inhibiting platelet adhesion, and protecting myocardium against ischemia.[12-16]

Although the roles of *Salvia miltiorrhiza* and magnesium lithospermate B have been well reported, few report is available for the protective effect and underlying mechanisms of salvionolate B on I/R-induced injury in mice. In the present study, we evaluated a hypothesis that salvionolate could protect heart from myocardial I/R injury in an *in vivo* murine model, which, if proven, can lead to further investigations of the therapeutic effect of salvionolate B and its mechanisms against I/R injury *in vivo*.
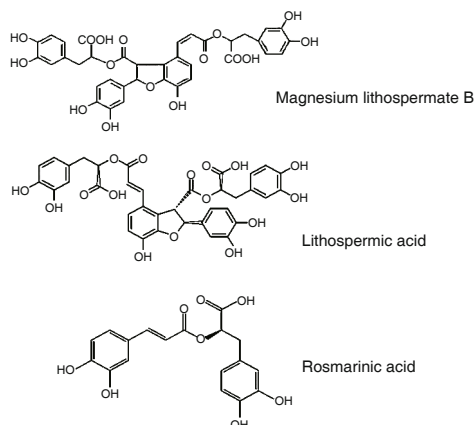

Figure 1.   Three Components of Salvianolate

METHODS

Animals and Reagents

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication No. 85–23, revised 1996) and approved by the Institutional Animal Care and Use Committee of Guangdong Province Hospital of Chinese Medicine, Guangzhou University of Chinese Medicine. Male wild-type C57BL/6J mice (10–12 weeks, 25 ± 5 g body weight) were obtained from the Experimental Animal Center of Guangdong Province, China. Triphenyltetrazoliumchloride (TTC) and Evans blue were purchased from Dingguo Biotechnology Co. (Beijing, China). Neutral buffered formalin (10%) was purchased from Wexis Biotechnology Ltd., Co. (Guangzhou, China). Pentobarbital sodium, verapamil, LY294002 and PD98059 were purchased from Sigma Chemical (St

Louis, MO, USA), salvianolate was purchase from Green Valley Co. (Shanghai, China).

In Vivo Myocardial I/R Model

The murine model of I/R has been previously described in detail.[17,18] Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg), intraperitoneal injection (i.p.), intubated, and ventilated with room air at a rate of 110 strokes/min and with a tidal volume of 0.25 mL using a mouse ventilator (Inspira, Harvard Apparatus, Holliston, MS, USA). The chest was opened through a left thoracotomy with the aid of a dissecting microscope. A 6–0 nylon suture was passed under the mid-left anterior descending (LAD) coronary artery (2–3 mm inferior to the left auricle) and a nontraumatic occluder was applied on the artery. Ischemia was elicited by a 30-min coronary occlusion followed by 24-h reperfusion (Figure 2). Significant changes, including widening of the QRS complex and elevation of ST segment in electrocardiography, were indicators of successful coronary occlusion. The chest was closed in layers, and animals were weaned from the ventilator when they resumed spontaneous breathing.[19, 20]
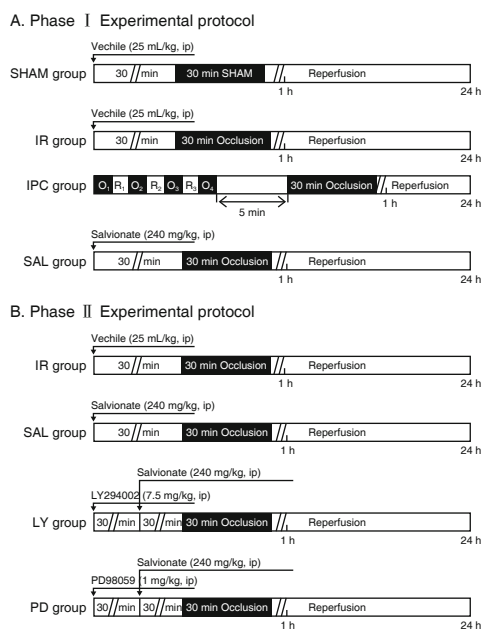

Figure 2.   Experimental Coronary Occlusion/Reperfusion Protocols

Experimental Protocol

Figure 2 shows the coronary occlusion/reperfusion protocols. Based on literature and clinical usage in patients (with dose conversion between human oral usage and animals), salvianolate at a dose

of 240 mg/kg body weight mixed with 0.5 mL saline was administered via direct i.p for 30 min prior to surgery. In the phase Ⅰ protocol, mice were assigned to four groups: SHAM, I/R, ischemia preconditioning (IPC), and SAL groups (Figure 2A). All mice, except in the SHAM group, were subjected to 30 min of coronary occlusion followed by 24 h of reperfusion. IPC group served as a positive control with protective effects of IPC, elicited by a sequence of 3 min coronary occlusion (O)/3 min reperfusion (R) cycles (Figure 2A) prior to I/R. During the 30 min period before I/R, the I/R and SHAM groups received saline (0.5 mL) and the SAL group received salvianolate. Based on literature and clinical usage in patients (with dose conversion between human oral usage and animal), salvianolate at a dose of 240 mg/kg body weight mixed with 0.5 mL saline was administered i.p. In phase Ⅱ protocol, mice were assigned to four groups: I/R group, SAL group, LY group, PD group (Figure 2B). In the LY group, LY294002, a phosphatidylinositol-3-kinase (PI3-K) inhibitor, was administered 7.5 mg/kg via direct intraperitoneal injection, for 30 min prior to salvianolate i.p., followed by I/R surgery. In the PD group (PD98059 group, 1 mg/kg, i.p), an extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor, was administered in the same way as the LY group.

In Vitro Tissue Staining

At the end of 24-h reperfusion, the heart was perfused with $1 \times$ phosphate buffer solution (PBS, pH 7.4) through an aortic cannula. The ligature around the LAD was retied. Evans blue dye 1% 1–2 mL was injected into the left coronary artery by reversing perfusion through the aorta, and the dye was circulated and uniformly distributed, except in the portion of the heart previously perfused by the occluded coronary artery. The heart was quickly excised and both atria and the right ventricle were removed. The left ventricle was weighed and sliced horizontally to yield six slices. After being weighted individually, the slices were incubated in 1% TTC prepared with $1 \times$ PBS for 8–15 min at 37 ℃, fixed in 10% neutral buffered formaldehyde for 24–48 h, and then photographed under a microscope with a digital camera.

Infarct Size Measurement

The areas stained with Evans blue [blue area, normal zone (NZ)], TTC [red staining, risk region (RR)],

and TTC-negative area [white area, infarct size (IS)] were measured digitally using Image Pro-plus (Version 6.0). The myocardial IS was measured and expressed as a percentage of infarct size over the total RR. We identified infarct, at-risk, and non-ischemic areas based on tissue staining and measured infarct sizes by computerized video planimetry. The intra-observer and inter-observer variability in the measurements of IS were also evaluated. When two different observers (Yu J and Qi J) calculated IS without communicating with each other, the intra-observer and inter-observer variability in the measurements of IS were carefully evaluated.

Western Blot Analysis

Western blot was performed as previously described.[10] Briefly, samples were lysed in 100 μL buffer containing 20 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 5 mmol/L ethylene diamine tetraacetic acid (EDTA), 50 mmol/L NaF, 1 mmol/L sodium vanadate, 0.1% sodium dodecyl sulfonate (SDS), 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 1 mmol/L leupeptin, 0.1 mmol/L aprotinin, and 1 mmol/L phenylmethanesulfonyl fluoride. Protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA), and proteins were separated on a 10% SDS-polyacrylamide gel and then electrophoretically transferred to nitrocellulose membranes (Pall Corporation, East Hill, NY, USA). Results are expressed as the changes over SHAM group. Following antibodies were used in this study: anti-phospho-ERK1/2 (Thr202/Tyr204, Cell Signaling Technology, Beverly, MA, USA), anti-phospho-PKB (Ser473, Cell Signaling Technology), anti-ERK1/2 (Santa Cruz Technology, Delaware, CA, USA), the sheets were analyzed with antibodies according to the supplier's protocol and peroxidase was visualized using an enhanced-chemiluminescence system (ECL kit, Pierce Biotechnology, Inc.). Bands were visualized by use of a super western sensitivity chemiluminescence detection system (Pierce, IL). Autoradiographs were quantitated by a densitometry Science Imaging system (Bio-Rad, Hercules, CA).

Statistical Analysis

Data were expressed as means ± standard error of mean ($\bar{x} \pm$ SEM). Bonferroni's post hoc method was used to assess the significance of differences using GraphPad Prism version 4.0. A *P*-value of <0.05 was

considered statistically significant.

RESULTS

A total of 83 mice were used for the experiment. Twenty-eight mice were excluded because of death in 12 mice, severe bleeding during surgery in 2, technical problems in 10 or inadequate postmortem staining in 4. As for the surgery, the most difficult process was to ligate the LAD accurately and pressure stably. Death and technical problems were the main reasons leading to exclusion. Fifty-five mice (66%) successfully completed the entire protocol and were included in the results (Table 1).

Salvionate Reduces Myocardial I/R Injury in Mice

To explore the effect of salvianolate on myocardial I/R injury, the weight of the RR and left ventricle (LV) was compared among the 4 groups (Figure 3). There were no significant differences in the weight ratio of RR to LV among the four groups (P>0.05, Figure 4, Table 2). In the I/R group, I/R resulted in the IS of 38.5%±2.9% of the region at risk. In contrast, the same I/R treatment resulted in IS of 26.1%±1.4% and 22.3%±2.9% of the region at risk in the SAL and IPC groups, repectively, both of which were significantly smaller than in the I/R group (Table 2). Consistent with IS to RR ratio, the IS to LV ratio showed the same results (Figure 4).

ERK1/2 Phosphorylation Was Increased after Salvionate Treatment by Western Blot

The ERK1/2 phosphorylations of threonines at 202th and 204th sites were enhanced in IPC group,
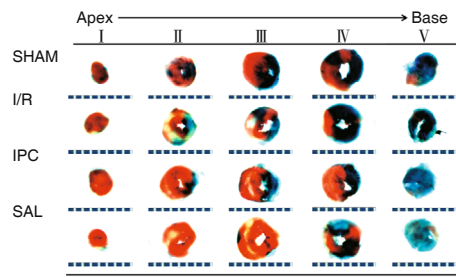

Figure 3.   Example Dye Staining of the Normal, Risk, and Infarcted Regions in Phase Ⅰ Study ($\times$10)
Notes: Blue-stained portion: normal region; red-stained portion: ischemia region; unstained portion (white area): infarcted region. Scale at bottom is in mm.
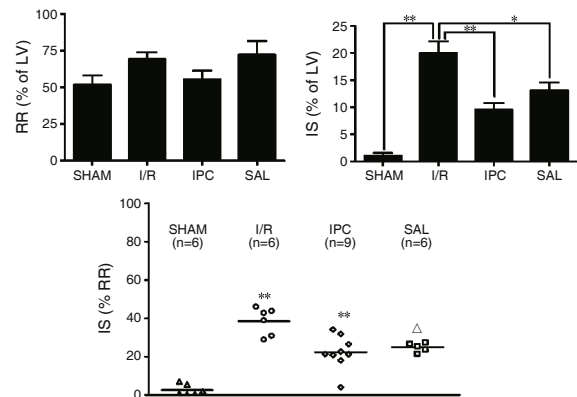

Figure 4.   RR and IS to LV Ratio, and IS to RR Ratio
Notes: *P<0.05, **P<0.01, compared with I/R group; △P<0.01, compared with SHAM group

compared with the SHAM group (P<0.05, Figures 5A–5B). Interestingly, the phosphorylations of threonines at 202th and 204th sites were also increased after salvianolate stimulation, compared with the SHAM group. Moreover, the AKT phosphorylation of tyrosine

Table 1.   Reasons for Excluding Mice from Study [n (%)]

Group	SHAM	IR	IPC	SAL	LY	PD	Total
Bleeding	1 (14)	0	1 (8)	0	0	0	2 (2)
Death	0	2 (11)	2 (15)	1 (7)	4 (25)	3 (33)	12 (15)
Technical problems	0	4 (2)	0	1 (7)	1 (6)	4 (3)	10 (12)
Poor postmorten staining	0	0	1 (8)	1 (7)	1 (6)	1 (7)	4 (5)
Mice instrumented	7 (100)	18 (100)	13 (100)	15 (100)	16 (100)	14 (100)	83 (100)
Mice excluded	1 (14)	6 (33)	4 (31)	3 (20)	6 (37)	8 (57)	28 (34)
Mice included in study	6 (86)	12 (67)	9 (69)	12 (80)	10 (63)	6 (43)	55 (66)

Table 2.   Weight of LV, RR, and IS in Phase Ⅰ Study ($\bar{x}\pm$SEM)

Group	n	LV (mg)	RR (mg)	IS (mg)	RR (% of LV)	IS (% of RR)	IS (% of LV)
SHAM	7	99.0±4.6	41.5±4.6	0.90±0.5	51.9±6.4	2.7±1.2	1.1±0.5
IR	7	85.8±4.7	59.8±5.5	21.2±3.2△	69.3±4.5	38.5±2.9△	20.0±2.2△
IPC	10	79.8±1.6	47.4±3.8	12.1±1.6*	59.6±4.7	22.3±2.9**	9.6±1.2*
SAL	6	72.8±2.3	52.7±6.7	15.0±2.8*	72.3±9.4	26.1±1.4*	14.8±1.7*

Notes: *P<0.05, **P<0.01, vs. the I/R group; △P<0.01, vs. the SHAM group; the same below

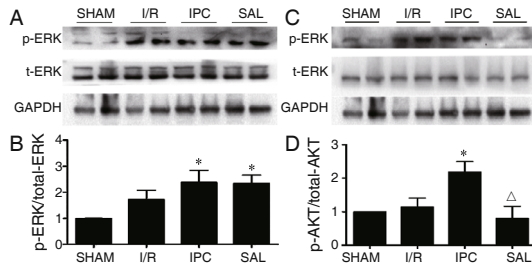at 473th site was increased in IPC group ($P<0.05$, *vs.* SHAM group, Figures 6), but not in SAL group.

Figure 5.   Phosphorylation of ERK1/2, Was Increased in Response to Salvionate
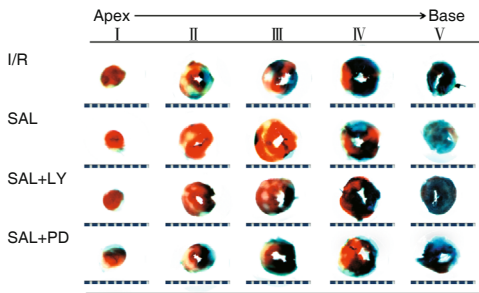Notes: *$P<0.05$ *vs.* SHAM; △$P<0.01$ *vs.* IPC

Figure 6.   Example Dye Staining of the Normal, Risk, and Infracted Regions (×10)
Note: Blue-stained portion: normal region; red-stained portion: ischemia region; unstained portion (white area): infarcted region. Scale at bottom is in mm.

PD98059, An ERK1/2 Inhibitor, Could Inhibit the Myocardial I/R Protection by Salvionate

To further confirm the potential roles of the ERK1/2 and PI3-K/AKT pathway in the protective role of salvionolate on myocardial I/R injury, pharmacological intervention was taken in I/R model using phase Ⅱ experimental protocol (Figure 2B). As shown in Figure 5 and Table 3, by the double staining method, we found that LY294002 (7.5 mg/kg), the PI3-K inhibitor, further reduced IS when administered 7.5 mg/kg i.p. for 30 min prior to salvianolate, followed by I/R surgery. However, the protective role of salvianolate on I/R injury disappeared when PD98059, an ERK 1/2 inhibitor, was administered 1 mg/kg i.p. to the I/R mice (Figures 6 and 7). The correlation coefficient was ≥0.90 between the intra-doserver and
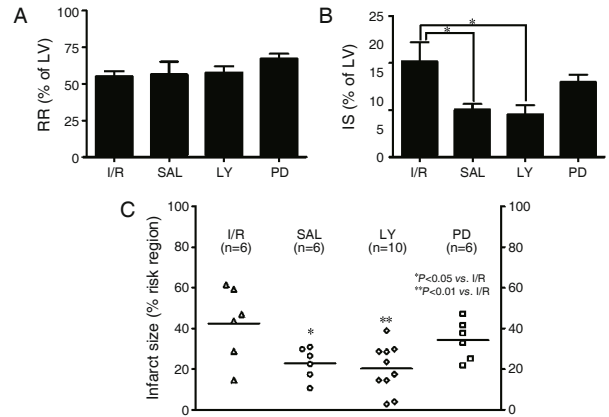

Figure 7.   RR and Infarct Sizes Compared to Left Ventricle, and IS to RR Ratio in Pase Ⅱ Study
Note: *$P<0.05$, **$P<0.01$, *vs.* I/R in the same group

inter-observer variability and the differences <5%.

Correlations between IS and RR

To evaluate the relations between IS and RR, linear analysis was performed among the six groups, the ratios between the IS to RR were 0.23, 0.45, 0.06, 0.62, 0.17, and 0.52 in the SHAM, I/R, IPC, SAL, LY and PD groups, respectively (Figure 8). All the groups showed no tendency of IS increase with RR except the SAL group ($P>0.05$).
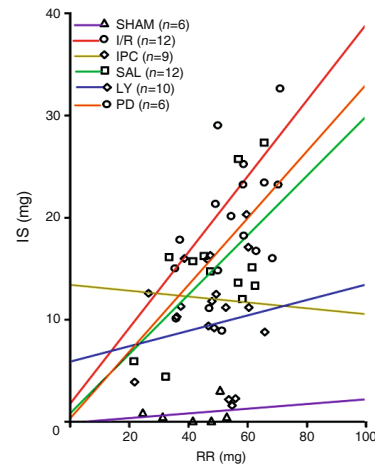

Figure 8.   Relationships between the RR and IS
Notes: Linear regression equations: SHAM group, y=0.02301x−0.08, $r=0.23$; I/R group, y=0.3711x+1.79, $r=0.45$; IPC group, y=−0.02867x+13.42, $r=0.06$; SAL group, y=0.2907x+0.8454, $r=0.62$; LY group, y=0.0756+5.893, $r=0.17$, $P>0.05$; PD group, y=0.3269x+0.3391, $r=0.52$

Table 3.   Weight of LV, RR, and IS in Phase Ⅱ Study ($\bar{x} \pm$ SEM)

Group	n	LV (mg)	RR (mg)	IS (mg)	RR (% of LV)	IS (% of RR)	IS (% of LV)
I/R	6	84.8±2.1	47.0±3.6	22.0±5.6	55.4±3.4	45.8±9.5	18.7±4.7
SAL	6	82.4±5.5	44.7±4.5	15.0±2.8*	56.6±8.7	29.5±5.7*	13.4±3.9*
LY	10	83.3±3.0	48.3±3.9	9.5±1.8*	57.8±4.3	20.3±3.7**	9.1±1.9*
PD	6	84.8±2.3	57.0±3.0	19.0±1.9	67.4±3.4	34.3±4.0	16.0±1.5

DISCUSSION

This study was to illustrate the protective role of salvianolate on myocardial ischemia and reperfusion injury in mice. Our results can be summarized as follows: (1) salvianolate could alleviate myocardial I/R injury in vivo mouse; (2) the effects of salvianolate could be via an ERK 1/2 signaling pathway; (3) PI3-K signaling pathway might not participate in the protective role of salvianolate on myocardial I/R injury.

Salvianolate, a highly purified aqueous extract from Salvia miltiorrhiza, is considered to "invigorate" the blood and reduce blood stasis. In the present study, we investigated the cardio-protective effects of salvianolate i.p 30 min before I/R surgery in mice. The protection provided by salvianolate, evidenced by an instant decrease in infarct size, was present at a high dose of salvianolate, consistent with previous report.[21] From this data, we can explicit not only why salvianolate alleviate angina pectoris from patients of long-time coronary artery diseases, but also explain the rapid protective effect of salvianolate on acute coronary syndrome,[11] just like clopidogrel, which could be given loading dosage before percutaneous coronary intervention, can reduce I/R injury and reopen the coronary artery instantly.

Myocardial I/R injury involve a complex network of MAPKs, PI3-K and AKT signaling pathways. The classical MAPK signaling pathway can be divided into three branches, named ERK, JNK, and p38.[22] All three cascades have been implicated in regulating cardiomyocytes apoptosis.[23,24] Magnesium lithospermate B, one of the most important component of salvianolate, was reported to play protective role through Akt, p38, NF-KB, TGF-beta signaling pathway.[25-28] However, most of the studies were based on H9C2 cell line in vitro; it cannot really reflect the mechanisms in vivo. Furthermore, there is no any paper reported the mechanisms of salvianote effect on murine myocardial I/R injury in vivo. To identify potential target proteins with which salvianolate interacts during I/R injury, two key protein kinases were chosen, namely PI3-K and ERK1/2, both of which were indicated to participate in the pathway of reperfusion injury salvage kinase (RISK). [29] We investigated both ERK and PI3-K because they are critical components in the RISK pathway.We found that salvianolate significantly reduced myocardium IS,

and the Western-blot data showed that salvianolate could reverse myocardial I/R injury through the ERK1/2 phosphorylations of threonines at the 202th and 204th sites. Furthermore, the protective effects of salvianolate were partially inhibited by the ERK1/2 specific inhibitor PD98059, not by the PI-3K inhibitor LY294002. Therefore, both the molecular and pharmacologic methods demonstrated that salvianolate reduced murine myocardium I/R injury via an ERK1/2-dependent, but not a PI3-K dependent signaling pathway. Although PI3-K and AKT signaling activation were reported to have cardioprotective effects in myocardial I/R,[30] our present results revealed that salvianolate treatment had little influence on PI3-K, consistent with a previous report.[26] Therefore, our data revealed that ERK1/2 phosphorylation of threonines at the 202th and 204th sites, not AKT phosphorylation of tyrosine at the 473th site was increased in myocardial I/R injury in response to salvianolate stimulation.

The reliability of the measurements of infarct size was of paramount importance in the outcome of the present investigation. Previously, double staining was considered impractical for quantify IS at the late phase of reperfusion.[21] Because of the high quality of staining, the measurements of IS in this study were accurate and reproducible. Previous studies have revealed the size of the myocardial infarction were linearly related to the IS of the region at risk, whereas other researchers reported there were no tendency of IS increase with RR.[17,18] The current study observed an average infarct size of 38.5%–45.8% of the region at risk (I/R group), similar to the reporte by Guo Y, et al.[18]

This study used PD98059 and LY294002 to inhibit ERK1/2 and PI-3K pathways in studying the protective effects of salvianolate against I/R injury. In addition to ERK1/2 and PI-3K, these inhibitors may also affect other unintended targets. Further study with pharmacological and genetic evidence will be needed to supports our hypothesis. Previous reports showed that both PKGI (cGMP-dependent protein kinase type Ⅰ) and AMPK (AMP-activated protein kinase) are involved in the ischemic heart.[31,32] Whether salvianolate modulates PKGI and/or AMPK requires further investigation.

In conclusion, the present results enhanced our understanding of the role of salvianolate on I/R injury.

We have shown that selective ERK1/2 modulation for cardioprotection is feasible, suggesting that it has the potential to be exploited as a therapeutic target. A high loading dose of salvianolate 30 min prior to LAD ligation could lessen IS by I/R injury. This data experimentally provided the evidence that salvianolate decreasing I/R injury at an early stage as a clinical therapy, which helps to elucidate the effectiveness of salvianolate in treating patients with acute coronary syndrome.

Conflict of Interests

None of authors received funding or research grants from the relevant drug manufacturers in this research. The authors declared no conflict of interests.

Author Contributions

Zhang MZ, Guo LH conceived and designed the experiment; Qi JY, Wang L, Yu J, and Huang X performed the experiments; Huang DH, and Zhou M analyzed the data; Qi JY wrote the paper; Wu JS revised the paper. All authors approved the manuscript submission.

Acknowledgments

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