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Original Article

# Protective Effects of Danlou Tablet (丹蒌片) against Murine Myocardial Ischemia and Reperfusion Injury *In Vivo**

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**ABSTRACT** **Objective:** To observe the *in vivo* effect of Danlou Tablet (丹蒌片, DLT) on myocardial ischemia and reperfusion (I/R) injury. **Methods:** DLT effects were evaluated in mouse heart preparation using 30-min coronary occlusion followed by 24-h reperfusion and compared among sham group ($n$=6), I/R group ($n$=8), IPC group (ischemia preconditioning, $n$=6) and DLT group (I/R with DLT pretreatment for 3 days, 750 mg·kg$^{-1}$·day$^{-1}$, $n$=8). The effects of DLT were characterized in infarction size (IS) compared with risk region (RR) and left ventricle using the Evans blue/triphenyltetrazolium chloride double dye staining method *in vivo*. Furthermore, the dose-dependent effect of DLT on I/R injury was evaluated by double staining method. Five different concentrations of DLT (0.625, 1.25, 2.5, 5 and 10 g·kg$^{-1}$·day$^{-1}$) were chosen in this study, and dose-response curve of DLT was obtained on these data. **Results:** The ratio of IS to left ventricle was significantly smaller in the DLT and IPC groups than the I/R group ($P$<0.05 or $P$<0.01), the ratio of IS to RR was also reduced in the DLT and IPC groups ($P$<0.01), while there were no differences in RR among the four groups ($P$>0.05). Experiments showed incidence of arrhythmias was reduced in the DLT group ($P$<0.01). Furthermore, DLT produced a dose-dependent inhibitory effect with a half maximal inhibitory concentration of 1.225 g·kg$^{-1}$·day$^{-1}$. **Conclusions:** Our research concluded that DLT was effective in reducing I/R injury in mice, and provided experimental supports for the clinical use of DLT.

**KEYWORDS** ischemia and reperfusion, Chinese medicine, Danlou Tablet

Early reperfusion is effective in rescuing the ischemic myocardium and reducing mortality, thus interventional therapies aimed at ameliorating the myocardial consequences of ischemia-reperfusion (I/R) are being intensively explored and have been the main treatment for acute coronary syndrome. However, delayed reperfusion can paradoxically cause injury to the ischemic myocardium,[1,2] and not decrease the risk of serious heart events.[3] Myocardial I/R injury is evoked by ischemic hypoxia and reperfusion-induced peroxide. Injury of myocardium due to I/R includes cardiac contractile dysfunction, arrhythmias, as well as acute myocardial infarction (AMI).[4] In the United States, about 1 million people suffered from myocardial infarction (MI) and 0.45 million died from MI every year.[5]

Recently there is a surge of interest in using complementary therapies such as herbal remedies in treating AMI diseases. Many traditional herbal medicines have been claimed to be useful for controlling I/R injury. Chinese patent medicine, Danlou Tablet (丹蒌片, DLT) has been widely used in China to treat patients with angina pectoris and acute coronary syndrome.[6] It was reported that DLT could alleviate unstable angina pectoris, hyperlipidemia,[7] and improve vascular endothelial function in patients with metabolic syndrome.[8] Furthermore, experiments revealed that DLT could protect rats from MI and cardiac remodeling induced by isoproterenol intraperitoneal injection (i.p),[9] and reduce hyperlipidemia and vascular endothelial injury.[10]

While the efficacy of DLT is well documented, the underlying mechanisms remain elusive. In the present study, we evaluated a hypothesis that DLT could protect heart against I/R injury by examining the


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myocardial injury after I/R in mouse hearts pretreated with DLT. We also investigated the role of the DLT on myocardial infarct size in an *in vivo* model in mice.

## METHODS

### Preparation of DLT

DLT were gifted and authenticated by Prof. PANG Yu-hua, Solid Production Laboratory, Jilin Connell Pharmaceutical Corp. (Jilin, China; batch No. 20111103). Detailed information could be attained in the previous report.[11] In brief, the standards of tanshinol, 5-hydroxymethyl furfural, puerarin, daidzin, salvianolic acid B, salvianolic acid A, tanshinone Ⅱ A were purchased from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Among DLT, *Rhizoma chuanxiong*, *Rhizoma Alismatis*, *Rhizoma Curcumae Longae* were pulverized into ultra fine powder by pulverizing mill (No. TWF450, Beijing Guoyao Longli Technology Co., Ltd.). *Radix Paeoniae Rubra*, *Pericarpium trichosanthis*, and *Bulbus Allii macrostemi* were extracted with ethanol, filtrated and concentrated. *Rhizoma chuanxiong* and *Salvia miltiorrhiza* were extracted with 75% ethanol, filtrated and concentrated. After ethanol extraction, *Radix Astragali*, *Rhizoma Drynariae* and *Salvia miltiorrhiza* were decocted twice, filtrated and concentrated. The concentrated liquids and super fine powder obtained all above were combined, and the drug of DLT were then formed through a series of steps, including vacuum drying, pulverizing, granulating, tabletting and coating.

### Animals and Modeling

A total of 120 male wild-type C57BL/6J mice (10 to 12 weeks old, 25±5 g body weight) were obtained from the Experimental Animal Center of Guangdong Province, China (certification No. SCXK(Yue)2013-0002, batch Nos. 44007200008769 and 44007200009246). To evaluate the cardio-protective effects of DLT on I/R injury, firstly a murine I/R model was created as described earlier.[12-14] Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg, 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. An 6–0 nylon suture was passed under the mid-left anterior descending coronary artery (LAD, 2–3 mm from the tip of 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. Significant changes, including widening of the QRS complex and elevation of ST segment in electrocardiography (ECG), were indicators of successful coronary occlusion. The chest was closed in layers, and animals were weaned from the ventilator when they resumed spontaneous breathing.

This study was performed in accordance with the guidelines and with approval from the Institutional Animal Care and Use Committee of Guangdong Province Hospital of Chinese Medicine, Guangzhou University of Chinese Medicine, and with the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (8th ed, Washington DC, 2011).

### Experimental Protocols

Two phase protocols of pilot studies were performed to verify the effectiveness and dosage of DLT on I/R injury. Phase Ⅰ protocol was based on our previous study, literature, and clinical usage in patients (with dose conversion between human oral usage and animals). DLT powder at a dose of 750 mg/kg body weight mixed with 0.5 mL saline was administered daily via direct gastric gavage, for 3 days prior to surgery. Forty-four mice were randomized into 4 groups using a random number table: sham, I/R, ischemic preconditioning (IPC) and DLT groups (Figure 1). I/R mice were subjected to 30-min coronary occlusion followed by 24-h reperfusion. IPC group served as a positive control of the protective effects of IPC (preconditioned with a sequence of 5 cycles of 4-min occlusion and 4-min reperfusion). The I/R group and sham group received saline (0.5 mL/day) for the same 3 days.

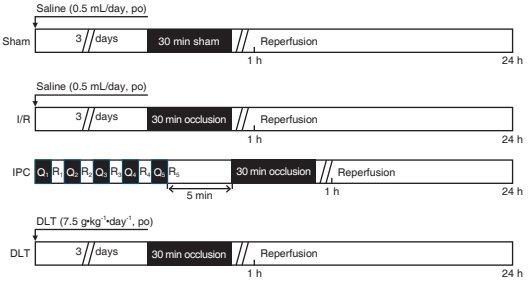


**Figure 1. Experimental Protocol**

Notes: Four groups of mice were studied for infarct size analysis. Days 1–3: mice were subjected to either saline (sham and I/R groups) or DLT via direct gastric gavage daily. Day 4: mice in sham, I/R, DLT, and IPC (preconditioned with a sequence of 5 cycles of 4-min occlusion and 4-min reperfusion) groups were subjected to a 30-min LAD occlusion. Day 5: after 24-h reperfusion following LAD occlusion, all animals were sacrificed for subsequent measurement of infarct size.

Phase Ⅱ protocol was to evaluate the dose-dependent effect of DLT on I/R injury. Five different concentrations of DLT (0.625, 1.25, 2.5, 5 and 10 $g \cdot kg^{-1} \cdot day^{-1}$, respectively) were chosen in this study, DLT 0.625 group ($n$=7), DLT 1.25 group ($n$=8), DLT 2.5 group ($n$=10), DLT 5 group ($n$=10), DLT 10 group ($n$=11), and DLT 0 group (I/R control, $n$=8) were obtained and dose-response curve of DLT was analyzed on these data.

## Heart Rate and Temperature

To keep the I/R process in a physiological state, rectal temperature was controlled strictly throughout the experiment with the use of heat lamps and a heating pad. Heart rate and rectal temperature before the 30-min coronary occlusion (pre-occlusion), at 5, 15 and 30 min into the occlusion, and at 5, 10 min after reperfusion in all groups were observed and compared with the average heart rate reported in conscious mice (668 ± 31 beats/min).[13,17]

## Postmortem Tissue Analysis

At the end of 24-h reperfusion, the heart was perfused with 1 × phosphate buffered solution (PBS, pH 7.4) through an aortic cannula. The ligature around the left anterior descending artery (LAD) was retied. Then 2 mL of 1% Evans blue dye 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 (risk region, RR). The heart was quickly excised and both atria and the right ventricle were removed. The left ventricle (LV) was weighed and sliced horizontally to yield 5 to 6 slices. After being weighted individually, the slices were incubated in 1% triphenyltetrazolium chloride (TTC) prepared with 1 × PBS for 8–15 min at 37 ℃, fixed in 10% neutral buffered formaldehyde for 24 h, and then photographed under a microscope with a digital camera. TTC and Evans blue were purchased from Ding-Guo Biotechnology Corp. (Beijing, China); 10% neutral buffered formalin was purchased from WEX Corp. (Guangzhou, China); pentobarbital sodium was purchased from Sigma-Aldrich Corp. (Guangzhou, China).

## Infarct Size Measurement and Heart Rhythm Analysis

Infarct size (IS) is well known as the standard method to evaluate I/R injury. To determine whether DLT produces a protected cardiac effect, IS was measured in the sham, I/R, IPC and DLT mice after subjecting them to a 30-min coronary occlusion and 24-h reperfusion. IS (white area) from RR (red area) and RR from normal zone (dark blue) were compared among these four groups (Figure 2). The areas stained with Evans blue (blue area), TTC (red staining), and TTC-negative area (white area) were measured digitally using Image Pro-plus (Version 6.0). The myocardial infarct size was measured and expressed as a percentage of infarct size over the total RR. Infarct, at risk, and non-ischemic areas were identified based on tissue staining and measured IS by computerized videoplanimetry.

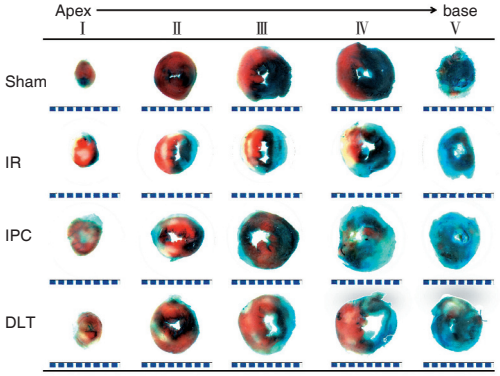


**Figure 2. Example Dye Staining of the Normal, Risk, and Infracted Regions of Mice**

Notes: Photomicrographs (× 10) of heart sections obtained from mice subjected to myocardial I/R (30 min/24 h) treated with sham, I/R, IPC, and DLT. Blue-stained portion: nonischemic, normal region; red-stained portion: I/R, risk but not infarcted region; unstained portion (white area): I/R, infarcted region. Scale at bottom is in mm.

To evaluate the relations between the IS and RR, linear analysis of their correlation were performed. The concentration-dependence of I/R injury inhibition induced by DLT was quantified by fitting the experimental data to a one-binding site model: Y=Bottom+[Top–Bottom]/(1+10^[Log $IC_{50}$–X] × Hillslope). The values of IS/RR were normalized and data were fitted with a non-linear regression fitting of inhibition.

Continuous electrocardiographic monitoring (RM6240; Chengdu Instruments, China) was performed during *in vivo* myocardial I/R with LAD ligation. Heart rate and rhythm were analyzed throughout the experiment. The incidence and type of arrhythmias, including premature ventricular contractions (PVCs), ventricular tachycardia (VT), and atrioventricular blocks (AVBs), were evaluated during I/R based on limb lead recordings of

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electrocardiogram (ECG).

### Statistical Analysis

Data were expressed as mean ± standard error of mean ($\bar{x} \pm$SEM). All data were analyzed with one-way ANOVA for normally distributed data or Kruskal-Wallis one-way ANOVA on ranks for data that are not normally distributed, as appropriate, followed by unpaired Student's *t* test with the Bonferroni correction. A *P*-value less than 0.05 was considered statistically significant. All statistical analyses were performed using the GraphPad Prism version 4.0 software.[15,16]

## RESULTS

### Animal Exclusion

In phase Ⅰ study, 8 mice died (18% total mortality), this mortality was consistent with reported for open-chest LAD ligation surgeries in mice.[13] Totally, 16 mice (36%) were excluded because of death (8 mice), severe bleeding during surgery (1 mouse), technical problems (6 mouse, malfunction of the ventilation system, and damage to the coronary vessels) or inadequate postmortem staining (1 mice). Totally 28 mice (64%) successfully completed the entire protocol and were included in the results (6, 8, 6, 8 in the sham, IR, IPC and DLT groups, respectively).

### Heart Rate and Temperature

As shown in Table 1, heart rate remained stable throughout the protocol in each group. Although in some groups the heart rate was 10%–20% lower than the average heart rate reported in conscious mice (668 ± 31 beats/min),[13,17] it was still within the range of measurements obtained in these pilot studies (440–560 beats/min). Heart rate and temperature did not differ significantly among the four groups in which the 30-min coronary occlusion was performed.

### Myocardial RR and IS, and Their Relationship

As illustrated in Figure 3A, there were no significant differences among the four groups in their LV weights and in their RR weights (Table 2). In the I/R group, the 30-min coronary occlusion followed by 24-h reperfusion resulted in an IS of 44.7% ± 6.0% of RR. In contrast, the DLT group had an IS of 20.8% ± 4.4% of RR (10.7% ± 2.2% of LV, Figures 3B and 3C). The IPC group, which was the positive control group of protective effect of I/R injury, had an IS of 18.8% ± 3.8% of RR. Large, confluent areas of infarction spanned most of the thickness of the LV wall. Assessment of cell death at 24 h represented

**Table 1. Comparison of Heart Rate and Rectal Temperature in Mice among Groups ($\bar{x} \pm$SEM)**

| Group | *n* | Pre-occlusion | Occlusion | | | Reperfusion | |
|---|---|---|---|---|---|---|---|
| | | | 5 min | 15 min | 30 min | 5 min | 10 min |
| Heart rate (beats/min) | | | | | | | |
| Sham | 6 | 444 ± 19 | 531 ± 14 | 501 ± 17 | 483 ± 21 | 507 ± 35 | 482 ± 23 |
| I/R | 8 | 474 ± 22 | 541 ± 31 | 502 ± 32 | 526 ± 39 | 527 ± 38 | 520 ± 38 |
| IPC | 6 | 476 ± 16 | 534 ± 10 | 513 ± 20 | 533 ± 23 | 532 ± 17 | 523 ± 23 |
| DLT | 8 | 481 ± 12 | 560 ± 12 | 564 ± 18 | 560 ± 21 | 552 ± 19 | 547 ± 24 |
| Temperature (℃) | | | | | | | |
| Sham | 6 | 36.9 ± 0.06 | 37.0 ± 0.05 | 37.1 ± 0.03 | 37.0 ± 0.04 | 37.1 ± 0.07 | 37.0 ± 0.05 |
| I/R | 8 | 36.9 ± 0.04 | 37.0 ± 0.04 | 37.0 ± 0.03 | 37.0 ± 0.03 | 37.0 ± 0.04 | 37.0 ± 0.02 |
| IPC | 6 | 37.0 ± 0.07 | 37.0 ± 0.05 | 37.0 ± 0.04 | 37.0 ± 0.02 | 37.0 ± 0.03 | 37.1 ± 0.03 |
| DLT | 8 | 37.0 ± 0.07 | 36.9 ± 0.05 | 37.1 ± 0.04 | 37.0 ± 0.04 | 37.1 ± 0.05 | 37.0 ± 0.04 |

**Table 2. Comparison of LV, Risk Region and Infarction Weight in Mice among Groups ($\bar{x} \pm$SEM)**

| Group | *n* | BW (g) | LV Wt (mg) | RR Wt (mg) | IS Wt (mg) | RR/LV (%) | IS/RR (%) | IS/LV Wt (%) |
|---|---|---|---|---|---|---|---|---|
| Sham | 6 | 25.0 ± 0.8 | 99.0 ± 4.6 | 51.5 ± 4.6 | 0.9 ± 0.5 | 51.9 ± 6.4 | 2.7 ± 1.2 | 1.1 ± 0.5 |
| I/R | 8 | 26.0 ± 1.4 | 101.0 ± 6.6 | 46.3 ± 4.0 | 21.8 ± 4.4 | 48.4 ± 2.0 | 44.7 ± 6.0 | 14.4 ± 2.0 |
| IPC | 6 | 28.0 ± 0.9 | 104.0 ± 4.0 | 52.2 ± 5.6 | 9.7 ± 1.8$^{*}$ | 49.9 ± 4.3 | 18.8 ± 3.8$^{**}$ | 5.3 ± 1.1$^{**}$ |
| DLT | 8 | 24.0 ± 0.8 | 85.6 ± 3.2 | 52.6 ± 3.3 | 9.3 ± 1.5$^{**}$ | 62.0 ± 2.8 | 20.8 ± 4.4$^{**}$ | 10.7 ± 2.2$^{*}$ |

Notes: Wt: weight; RR: risk region. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* I/R group

the final extent of myocardial infarction in this model (see the example of the DLT group in Figure 2). The small S.E.M. (Figure 3C) and similar IS in both groups indicated stability of this I/R model.

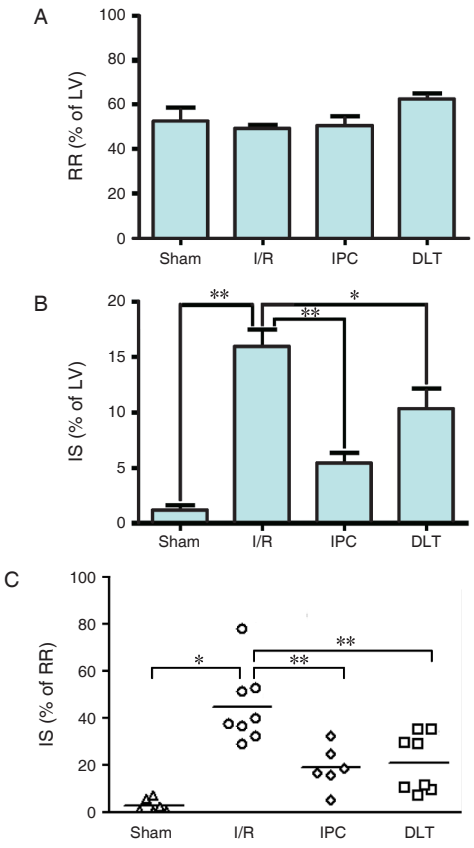


**Figure 3. RR and IS of Mice ($\bar{x} \pm$SEM)**

Nots: Myocardial RR expressed as percent of LV (A). Myocardial IS expressed as percent of total LV (B) and RR (C). $^{*}P<0.05$, $^{**}P<0.01$

Results of linear analysis showed that the size of the infarction was not linearly related to the size of the region at risk ($r$=0.16, 0.63, 0.39 and 0.55 in the sham, I/R, IPC and DLT groups, respectively, Figure 4). There were no significantly tendency of IS increase with RR ($P$>0.05). Considering similar RR in all I/R hearts, which was about 50% and independent from other variables, our data suggest that IS was a property of mice type and surgical interventions.

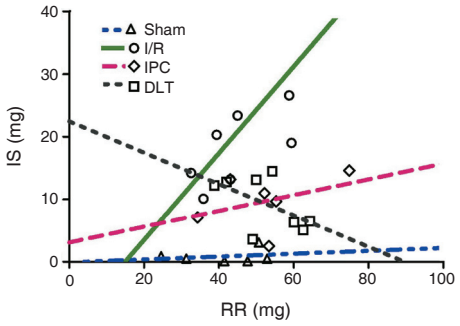


**Figure 4. Relationships between the Size of RR and IS of Mice**

Notes: Linear regression analysis showed that the IS was unrelated to the size of RR in all groups. Linear regression equations: sham group, y=0.01x+0.3517, $r$=0.16, $P$>0.05; I/R group, y=0.6924x-10.34, $r$=0.63, $P$>0.05; IPC group, y=0.1262x+3.097, $r$=0.39, $P$>0.05; DLT group, y=–0.2508x+22.47, $r$=0.55, $P$>0.05.

## Arrhythmia

As shown in Figure 5, PVCs, VT and AVBs occurred frequently in untreated hearts, but much less frequently in hearts pretreated with DLT ($P$<0.01), demonstrating that DLT was effective in reducing I/R induced arrhythmias.

## Dose-Dependent Relationship of DLT on I/R

Table 3 and Figure 6 illustrate the inhibitory effects of 6 DLT doses (0, 0.625, 1.25, 2.5, 5, and 10 g•kg$^{-1}$•day$^{-1}$) on I/R injury in heart slices treated with the classic double staining. As shown in Table 3, there were no significant differences among the six groups in their LV weight and RR/LV. However, IS/RR in DLT 5 and DLT 10 groups were significant reduced compared with DLT 0 group (DLT 5 *vs*. DLT 0, $P$<0.05; DLT 10 *vs*. DLT 0, $P$<0.01, respectively). Moreover, the ratio of IS to LV were significant reduced in the DLT 10 group, compared with the DLT 0.625 group ($P$<0.05).

Curves followed the sigmoidal shape (Figure 7) and the concentration of DLT that gave a half-maximal response of 1.225 g•kg$^{-1}$•day$^{-1}$. Therefore, there was a

**Table 3. Effects of DLT on LV, RR and Infarction Weights in Mice among Groups ($\bar{x} \pm$SEM)**

| DLT (g•kg$^{-1}$•day$^{-1}$) | $n$ | LV Wt (mg) | RR Wt (mg) | Infarct Wt (mg) | RR/LV (%) | Infarct/RR (%) | Infarct/LV (%) |
|---|---|---|---|---|---|---|---|
| 0 | 8 | 100.80 ± 6.64 | 46.35 ± 4.01 | 21.77 ± 4.39 | 48.41 ± 1.96 | 44.67 ± 6.01 | 14.38 ± 2.00 |
| 0.625 | 7 | 99.46 ± 5.78 | 46.25 ± 2.76 | 18.29 ± 2.86 | 56.12 ± 4.35 | 37.63 ± 7.11 | 14.32 ± 2.88 |
| 1.25 | 8 | 92.31 ± 2.33 | 49.26 ± 4.04 | 15.11 ± 1.54 | 61.73 ± 5.55 | 28.46 ± 4.39 | 11.25 ± 1.25 |
| 2.5 | 10 | 102.90 ± 1.84 | 47.57 ± 4.11 | 12.64 ± 2.62 | 51.77 ± 4.80 | 24.03 ± 4.43 | 7.95 ± 1.84 |
| 5 | 10 | 98.02 ± 2.71 | 46.51 ± 4.57 | 11.15 ± 1.84 | 55.32 ± 4.94 | 23.41 ± 5.26$^{*}$ | 8.04 ± 1.41 |
| 10 | 11 | 99.86 ± 2.45 | 41.90 ± 3.54 | 8.53 ± 1.27 | 48.42 ± 3.86 | 18.20 ± 3.01$^{**}$ | 5.81 ± 1.23$^{*\triangle}$ |

Notes: Wt: weight. $^{*}P$<0.05, $^{**}P$<0.01 *vs*. DLT 0 group; $^{\triangle}P$<0.05 *vs*. DLT 0.625 group

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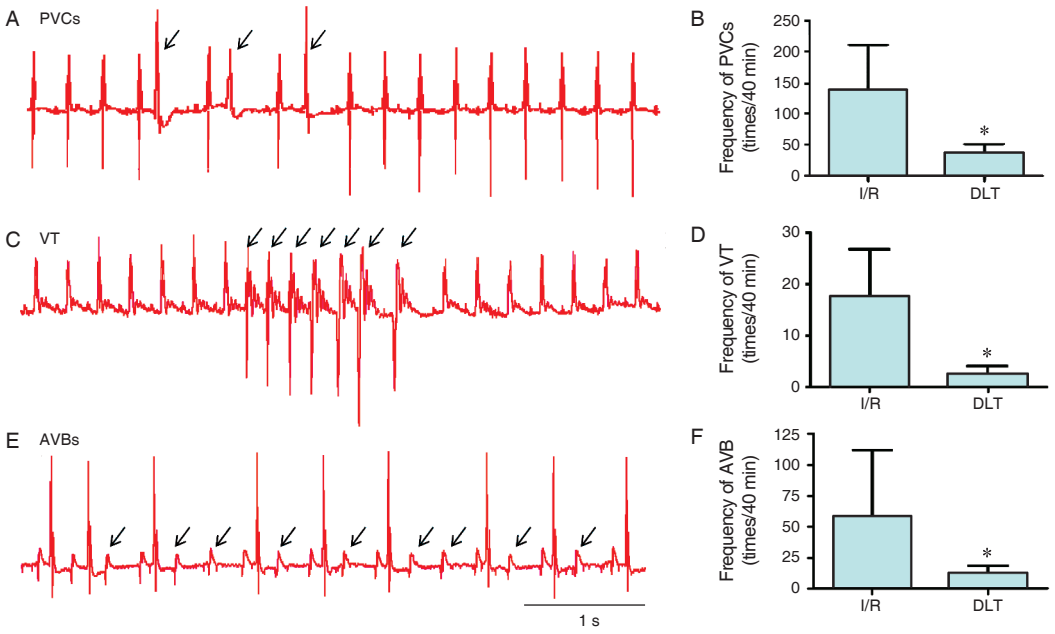


**Figure 5. Reduced Incidence of Arrhythmia in Mice of the DLT Group**

Notes: Various types of arrhythmia, including PVCs (A), VT (C) and AVBs (E) were observed during I/R injury. Incidence of arrhythmia of PVCs (B), VT (D) and AVBs (F) were reduced in the DLT group, **P*<0.01 *vs*. I/R group ( $\chi^2$ test).

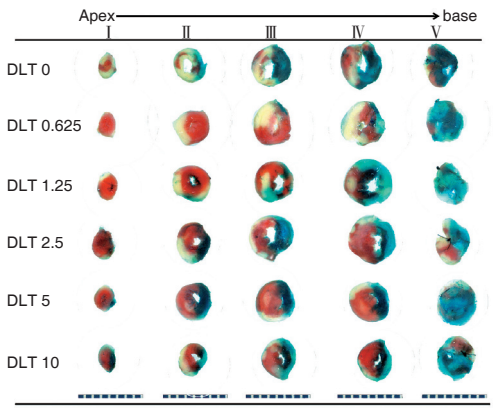


**Figure 6. DLT Dose-Dependently Staining of Normal, Risk, and Infracted Regions of Mice**

Notes: Photomicrographs ( × 10) of heart sections obtained from mice subjected to myocardial ischemia/reperfusion (30 min/24 h) treated with I/R, DLT 0.625, 1.25, 2.5, 5, and 10 g•kg$^{-1}$•day$^{-1}$. Scale at bottom is in mm.

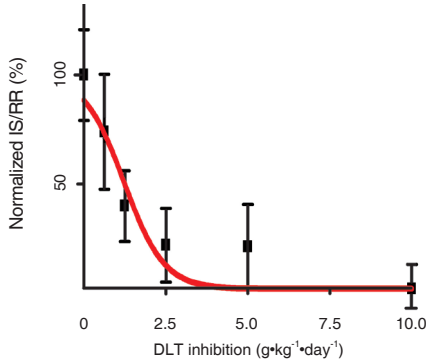


**Figure 7. Dose-Dependent Curve of DLT Effect on Normalized Inhibition of IS in Mice ($\bar{x} \pm$ SEM)**

Notes: $IC_{50}$ = 1.225 g/(kg•day)

dose-dependent manner of DLT inhibiting myocardial I/R injury in mice.

## DISCUSSION

Our results demonstrated that DLT could reduced IS and arrhythmia in mice, so as to alleviate myocardial ischemia and reperfusion injury *in vivo*. Many cardio-protective drugs failed to treat myocardial I/R injury effectively in clinical trials, which might be due to the complex I/R mechanisms.[18,19] Increasing evidence has suggested that the reperfusion injury salvage kinase (RISK) pathway is a key regulator of mechanisms underlying cardioprotection in I/R injury.[20,21] Various pharmacological agents (such as insulin and adenosine) and factors (such as pre- and post-conditioning) have been demonstrated to protect the heart from myocardial I/R injury by activating Akt and extracellular signal-regulated kinase 1 and 2 (ERK1/2) signals.[22] Thus enhancing the RISK pathway appears to be an excellent strategy for protecting the heart from injury during I/R events.[23]

Considering DLT was a complex herbal formulation with multiple active ingredients, there should be multiple mechanisms. It was reported that DLT could up-regulate the expression of myocardial Bcl-2 protein expression, down-regulate caspase-3 protein expression, reduce myocardial cell apoptosis

and the myocardial infarct size. Moreover, experiment showed DLT could inhibit endothelin, decreasing nitric oxide synthase, and reducing the Ang Ⅱ content, therefore DLT has certain protective effect on vascular endothelial injury. Furthermore, *Salvia miltiorrhiza*, one major component of DLT, could protects myocardial mitochondrial membrane from I/R injury.[24,25] Tanshinol, the water-soluble component of *Salvia miltiorrhiza*, could protect against myocardial I/R injury via Akt and ERK1/2 phosphorylation pathways.[26] *Radix Astragali*, the major component of DLT, might be protect against myocardial infarction by increasing the adenosine triphosphate-sensitive potassium current and improving intracellular calcium handling.[25,27] Thus, the cardio-protective effects of DLT could be mediated by multiple signaling pathways.

In the present study, we found 90% arrhythmia occurred in ischemia state. Moreover, we found that many mice died from electro-mechanic dissociation, showing completely AVBs, ventricular fibrillation occurred little in mice in reperfusion state. Compared with previous study,[28] we found that the I/R model was more stable with temperature controlling, and IS was bigger with temperature controlling. The effects of 4-h reperfusion are similar to that of 24-h reperfusion (data not shown), which were consistent with Bolli's reported.[12]

Despite the many facets of I/R physiology that are not included in our study, we believe that it is an important step towards a comprehensive I/R pathway. This study is still insufficient in providing evidence-based experimental verifications of the effectiveness of DLT and its mechanism. Further studies with both pharmacological and genetic evidences are needed to support our hypothesis.

In conclusion, the present study showed that a high loading dose of DLT 3 days before LAD ligation reduced IS by I/R injury. This result experimentally proved the effectiveness of DLT as a clinical therapy to protect against I/R injury at an early stage. Chinese medicine has been of great benefit to Asian people for centuries. However, evidence-based experimental verifications of their effectiveness and mechanistic studies have been insufficient. Experimental evidences of the DLT-induced cardio-protection can help explain the improved outcomes after the immediate administration of DLT to patients with acute coronary syndrome and may lead to the development of better drugs and/or new therapeutic applications of DLT.

## Conflict of Interest

No conflict of interest, financial or otherwise, are declared.

## Author Contributions

Zhang MZ conceived and designed the experiment; Qi JY and Wang L performed the experiments; Gu DS analyzed the data; Guo LH wrote the paper; Zhu W revised the paper. All authors approved the manuscript submission.

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