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Original Article

Tetrandrine reduces infarct size and preserves cardiac function in rat myocardial ischemia/reperfusion injury through activation of Akt/GSK-3b signaling

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Abstract: Objective: This study aims to determine the effect of TTD on myocardial ischemia/reperfusion (MI/R) injury (a pathological condition where ROS production is significantly increased) and to investigate the underlying mechanisms. Methods: This study utilized an in vivo rat model of MI/R injury and an in vitro neonatal rat cardiomyocyte (NRC) model of simulated ischemia/reperfusion (SI/R) injury. Infarct size was measured by Evans blue/TTC double staining. NRC injury was determined by MTT and lactate dehydrogenase (LDH) leakage assay. ROS accumulation and apoptosis were assessed by flow cytometry. Mitochondrial membrane potential (MMP) was determined by 5, 59, 6, 69-tetrachloro-1, 19, 3, 39-tetrathylbenzimidazol carbocyanine iodide (JC-1). Cytosolic translocation of mitochondrial cytochrome c and expression of caspase-9, caspase-3, Bcl-2 family proteins, and phosphorylated Akt and GSK-3b were determined by western blot. Results: Pretreatment with TTD (50 mg/kg) significantly augmented rat cardiac function, as evidenced by increased left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP) and LV dP/dt. TTD reduced myocardial infarct size, apoptotic cell death, and blood creatine kinase/lactate dehydrogenase levels after MI/R. In NRCs, TTD (10 mM) inhibited SI/R-induced ROS generation ($P < 0.01$), decreased cellular apoptosis, stabilized the mitochondrial membrane potential (MMP), and attenuated cytosolic translocation of mitochondrial cytochrome c. TTD inhibited activation of caspase-9 and caspase-3, increased the phosphorylated Akt and GSK-3b, and increased the Bcl-2/Bax ratio. Conclusions: These data demonstrate that TTD mediated cardioprotective effect against MI/R-induced apoptosis via a mitochondrial-dependent apoptotic pathway.

Keywords: Tetrandrine, myocardium, apoptosis, ROS, mitochondria

Introduction

Tetrandrine (TTD) is a bisbenzylisoquinoline alkaloid extracted from the root of Stephania tetrandra S. Moore [1]. Although the mechanisms responsible for Stephaniatetrandra S. Moore's various effects remain largely unknown, several active ingredients termed TTD have been isolated from the plant. An increasing number of studies have focused on the antioxidation properties of TTD. It is reported that TTD could reverses human cardiac myofibroblast activation and myocardial fibrosis, independent of calcium channel blockade [2]. In animal studies, TTD has shown beneficial effects on limiting cardiac myocardial remodeling in response to both pressure overload and coronary ischemia [3, 4]. Furthermore reports of the effects of TTD on myocardial ischemia/reperfusion (MI/R) injury are limited. However, it has not been investigated whether TTD exerts protective effect against myocardial ischemia-reperfusion (MI/R) injury, or by what potential mechanisms.

Toxic reactive oxygen species (ROS) generated during MI/R both directly and indirectly affect cardiomyocyte function, promoting apoptosis and necrosis [5]. Mitochondrial dysfunction increases ROS production, exacerbating oxidant-induced apoptosis [6]. During early reperfusion, ROS burst alters intracellular redox states, modifies the inner mitochondrial membrane potential (MMP), and releases mitochondrial-

-cytochrome c into the cytosol, ultimately activating caspase-3 in the final apoptotic pathway [7]. It is necessary to preventing ROS production and preserve mitochondrial integrity in order to protect myocardium against MI/R injury. Although TTD appears to be involved in anti-apoptotic activities via targeting of specific regulatory signals, the molecular mechanism that underlies these activities is not fully understood.

In this study, we would investigate: 1) whether TTD exerts any cardioprotective effect against MI/R injury, 2) to determine whether TTD may decrease oxidative stress in rats subjected to MI/R; and if so, 3) further examining the responsible underlying mechanisms.

Materials and methods

Ethics statement and study approval

All animal care and experiments were performed with the consent of the Animal Ethics Committee at Provincial People's Hospital of Henan, University of Zhengzhou and all mice were maintained in a specific pathogen-free environment and fed a standard diet.

Myocardial ischemia/reperfusion (MI/R) model in rats

Adult male Sprague-Dawley rats were anesthetized with 2% isoflurane, monitoring heart rates. MI was induced by tying a 6-0 silk slipknot around the left anterior descending coronary artery [8]. Ischemia was monitored and confirmed by ST segment elevation upon electrocardiogram (ECG). After 30 minutes ischemia, the slipknot was released for 3 hours. Rats were randomly assigned to one of the following treatments (n=10/group): A, Sham operation group, receiving vehicle IP injection (10 ml/kg saline) and operative procedures without coronary slipknot; B, MI/R group, receiving vehicle IP injection (10 ml/kg saline) 30 minutes prior to coronary I/R; and C, MI/R+TTD group, receiving TTD IP injection (50 mg/kg) 30 minutes prior to coronary I/R.

In vitro neonatal rat cardiomyocyte (NRC) model of simulated ischemia/reperfusion (SI/R) injury

Neonatal rat cardiomyocytes (NRCs) were isolated from 1-2 day old Sprague-Dawley rats. Briefly, excised hearts were washed in Hanks balanced salt solution. Ventricles were minced and subjected to 0.125% trypsin, filtered, and centrifuged (1000 rpm, 15 min). Resuspended cells in a humidified incubator (5% CO_2, 37°C) for 90 minutes. Cells were harvested and seeded on 60-mm culture dishes. 5-Bromo-29-deoxyuridine (100 mM) was added during the first 48 hours to inhibit non-myocyte proliferation. Simulated I/R (SI/R) was employed as the protocol [9]. Confluent-beating cells in 6-well plates were subjected to medium replacement with simulated ischemia buffer, incubated for 3 hours, and then re-oxygenated in a standard incubator for 2 hours with medium replacement with re-oxygenation buffer. Cells subjected to control conditions were cultured with normal Tyrode solution (pH 7.4) in a humidified atmosphere of 5% CO_2/21% O_2 balanced with N_2 at 37°C for 5 hours. Four separate NRC groups were tested: A, Control group, incubated with Tyrode solution for the entire experimental period; B, SI/R group, incubated with simulated ischemia buffer for 3 hours hypoxia, followed by 2 hours re-oxygenation; C, Vehicle group, subjected to 0.2% (v/v) DMSO administration 30 minutes prior to SI/R; D, SI/R+TTD group, subjected to TTD (10 mM) administration 30 minutes prior to SI/R.

Assessment of area at risk and infarct size

After reperfusion conclusion, ligate LAD permanently at the same position and inject 4 ml of 2% Evans blue dye through the jugular vein. The heart was then removed and frozen at -20°C and sliced into 1-mm sections perpendicular to the base-apex. All heart slices were incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma) for 10 min at 37°C. By plan emery (Image-Pro plus; Media Cybernetics, Bethesda, MA), the total transverse areas, the area at risk, and the infarct size were calculated from all the slices. An independent person blinded to the experiments performed the polarimetry and calculations.

Assessment of NRC injury

Methyl thiazolyl tetrazolium (MTT) assay and lactate dehydrogenase (LDH) leakage assay was employed to assess myocardial cellular damage. In brief, 20 μl/well of MTT solution (5 mg/ml phosphate buffered saline) was added and incubated for 5 h. The medium was aspi-

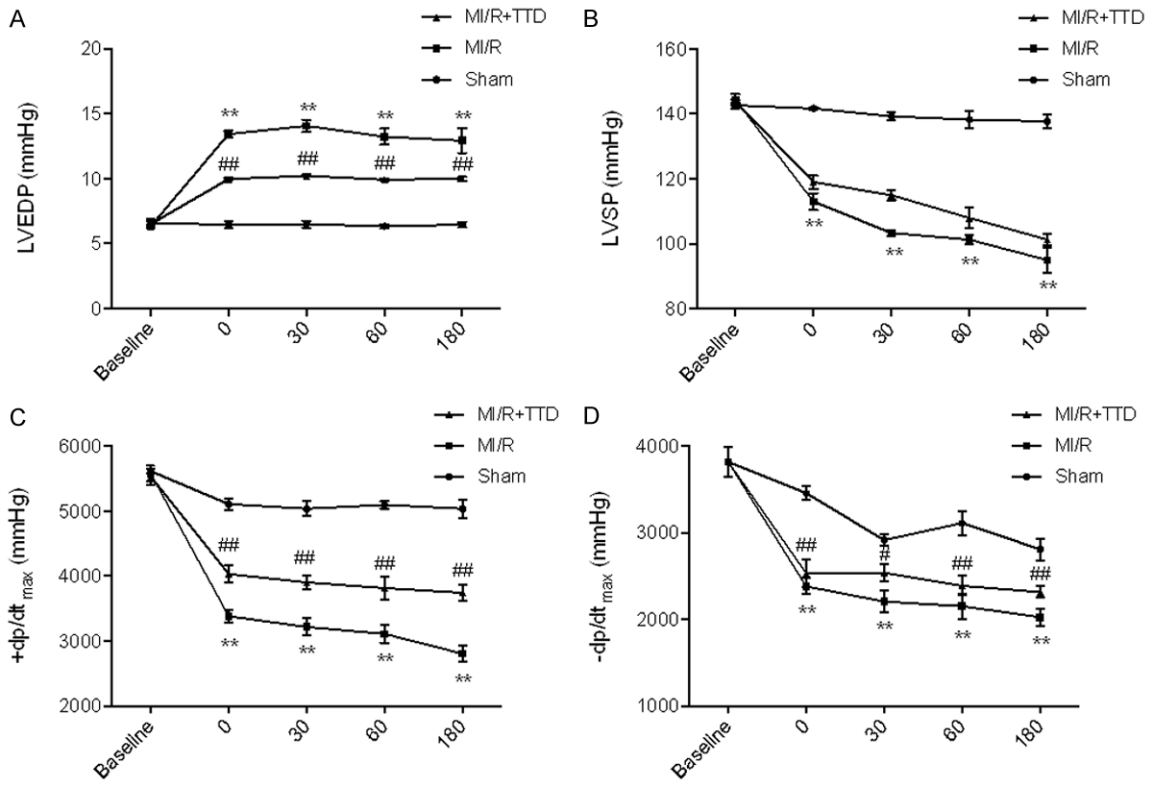

Figure 1. Tetrandrine improves rat cardiac function after ischemia and reperfusion. Values presented are mean ± SEM. LVSP: left ventricular systolic pressure; LVEDP: left ventricular end diastolic pressure; dp/dtmax, the instantaneous first derivation of left ventricle pressure; MI/R, myocardial ischemia/reperfusion (0 minute, 30 minutes/1 hour, 3 hours). n=10/group. **P < 0.01 vs. Sham, #P < 0.05, ##P < 0.01 vs. MI/R.

rated and replaced with 150 μl/well of acidic isopropanol (0.04 NHCl in isopropanol). The color intensity of the formazan solution was measured at 570 nm using a microplate spectrophotometer (ELx800 Biotech Instruments, USA). In the LDH assay, leakage of the cytoplasm-located enzyme LDH into the extracellular medium was measured. 0.2 mL of culture medium from NRCs post H/R treatment was analyzed by spectrophotometry via commercial assay kit (UV-120-02, Shanghai, China), per manufacturer's protocol.

Assessment of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was assessed using the fluorescent dye JC-1 (Invitrogen, USA). Suspension cardiomyocytes were incubated with JC-1 (200 μM) at 37°C for 20 min. It followed by twice ice-cold PBS washing in order to remove remaining reagents. Fluorescence was measured by Flow Cytometer (Beckman Coulter, USA) at excitation and emission wave lengths (ex/em) of 530 and 580 nm (red), and then at ex/em of 485/530 nm (green) respectively.

Measurement of intracellular reactive oxygen species (ROS)

The assay was carried out as the previously mentioned in manufacturer. Wash the cardiomyocytes twice with ice-cold PBS, incubate them in DMEM solution containing 10 μM DCFH-DA (Invitrogen, USA). Centrifuge the cells at 800 g for 5 min, washed twice with ice-cold PBS, the fluorescence intensity of each group was determined by Flow Cytometer (Beckman Coulter, USA) at ex/em wave lengths of 485 and 528 nm, respectively.

Western blot analysis

Whole cell extracts were prepared by lysis/extraction reagent (Sigma-Aldrich, St. Louis, MO) was used to generate total cell lysates. Protein concentrations in the supernatants

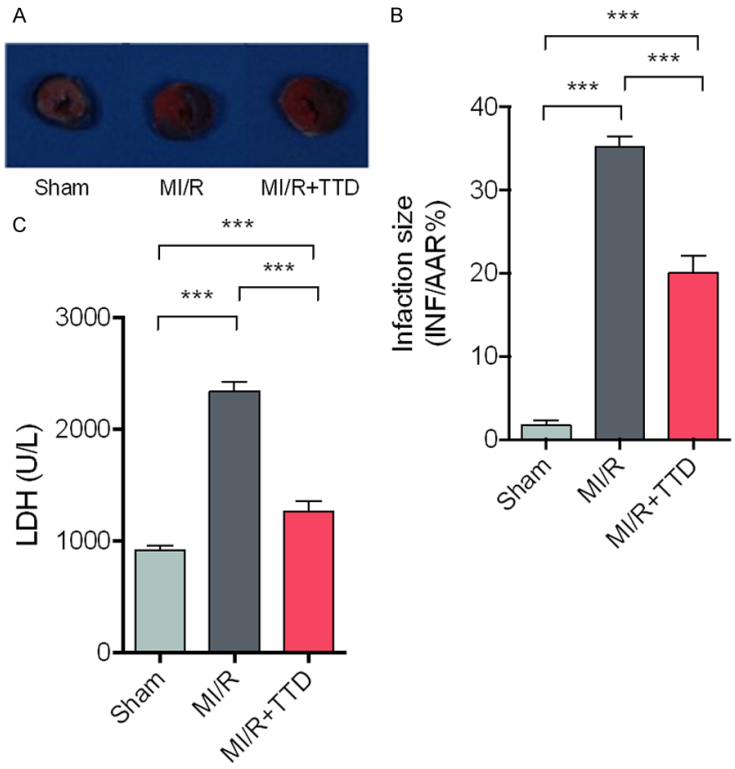

Figure 2. Tetrandrine reduced infarct size and necrosis post MI/R. A. Myocardial infarct size in rats subjected to 30 minutes I, followed by 3 hours R. B. Myocardial infarct size (INF) expressed as percentage of area at risk (AAR). C. Plasma creatine kinase (CK) and lactate dehydrogenase (LDH) levels. n=10/group. ***P < 0.001.

were determined by Protein Assay Kit (Invitrogen, USA). Samples were loaded onto 12% SDS polyacrylamide gel electrophoresis. Proteins were transferred to nylon membranes by electrophoretic transfer system (Bio-Rad, USA). Membranes were blocked in 5% skim milk for 1 hour at room temperature. Incubation with primary antibody commenced overnight at 4°C, followed by secondary antibody conjugated to horseradish peroxidase for 2 hours. Immunoblot was visualized with ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare Bio-sciences).

Statistical analysis

Results are presented as means ± SEM. The significant differences were determined using one-way ANOVA when 3 groups were compared. Values of $P < 0.05$ were regarded as statistically significant. All statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

Results

Pretreatment with tetrandrine significantly augmented rat cardiac function

There were no significant hemodynamic differences existed between groups at baseline conditions, and no significant differences in heart rate (HR) and mean arterial pressure (MAP) between any groups during MI/R. Pretreatment with TTD enhanced LV dP/dt max after 3 hours reperfusion compared to MI/R group (**Figure 1**). Additionally, TTD remarkably decreased LVEDP and LVSP post-I/R compared to MI/R group (P < 0.01). Hemodynamic data support TTD improved rat cardiac systolic and diastolic function after MI/R.

Tetrandrine reduced the infarct size and blood LDH levels after MI/R

To assess the myocardial injury post I/R, we measured the myocardial infarct size and plasma LDH. Representative AAR and INF images are shown in **Figure 2A**, **2B**. No myocardial infarction was observed in hearts of sham operation group. 30 minutes MI followed by 3 hours reperfusion leads to significant infarction in MI/R group rats compared to sham-group (P < 0.01). TTD treatment significantly decreased infarct size (P < 0.01). No significant difference were found in AAR between all groups. Cardiomyocyte necrosis is characterized by cellular content release. Plasma LDH levels increased in the MI/R-group (**Figure 2C**). TTD treatment decreased LDH level (P < 0.01) in the MI/R group. These data support TTD decreased in vivo myocardial necrosis post-MI/R.

Tetrandrine ameliorated in vitro cell death post SI/R

Cells were treated with varying concentrations of TTD (0.1-20 mM) to determine the effects of TTD alone on NRCs. TTD alone at these concentrations for 24 hours was not cytotoxic by MTT

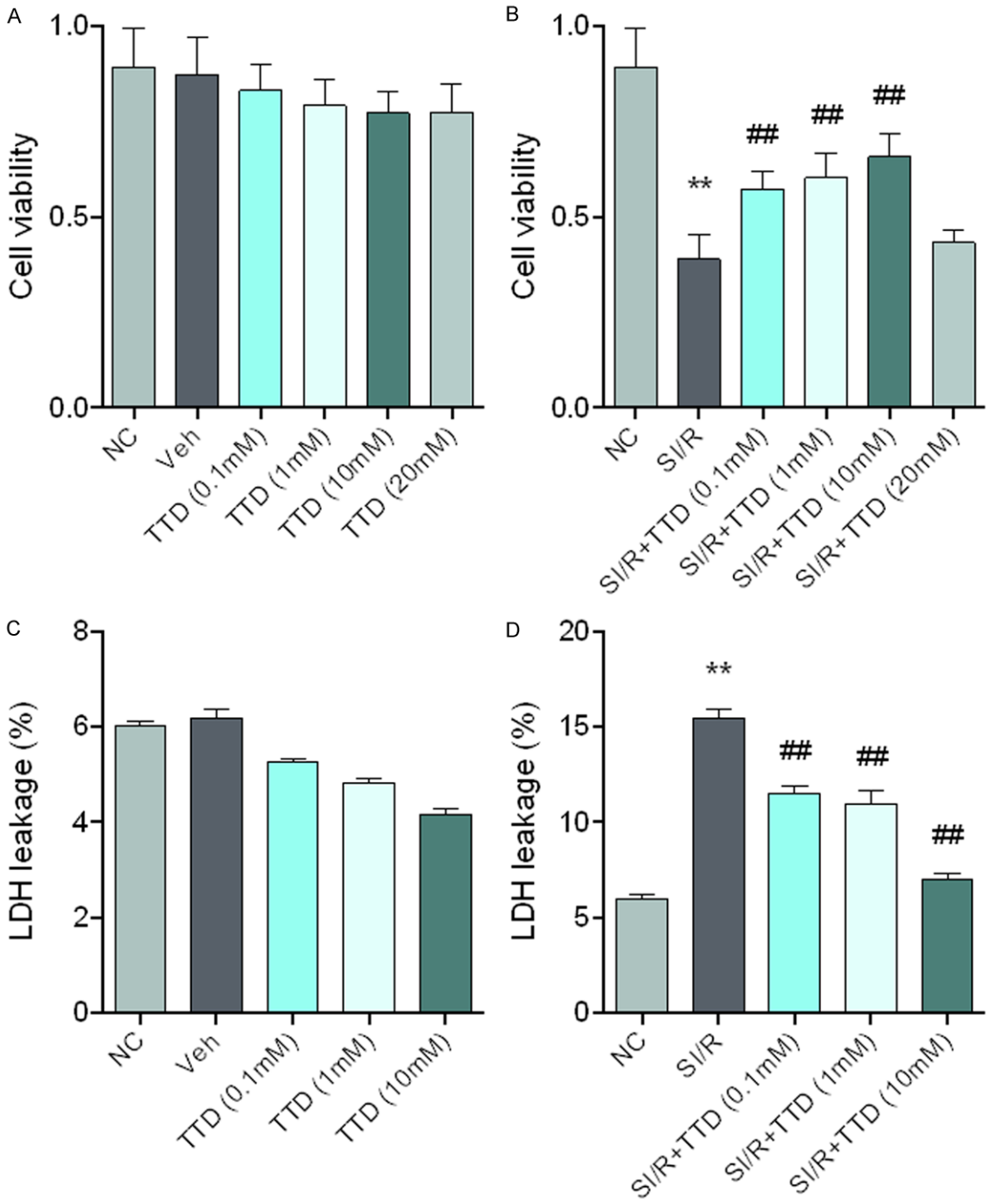

Figure 3. Tetrandrine ameliorated SI/R-induced in vitro cell injury. A. TTD treatment alone (0.1-20 mM) for 24 hours did not alter NRC viability, suggesting no TTD-induced toxicity at concentrations up to 10 mM (n=10; *P < 0.05 vs. Control). B. Cellular viability as determined by MTT assay after SI/R (3 hours hypoxia followed by 2 hours reoxygenation). C. Cellular death post TTD treatment alone for 24 hours as determined by LDH leakage into medium (n=10; *P < 0.05 vs. Control). D. LDH assay in cells administered TTD (0.1, 1, 10 mM) 30 minutes prior to SI/R.

and LDH leakage assay (**Figure 3A**, **3C**). Concentration response curves determining cellular viability are shown in **Figure 3B**. Peak cellular viability was observed at TTD dose 10 mM. Cellular viability and LDH leakage are indices of NRCs injury. After being subject to SI/R, cellular

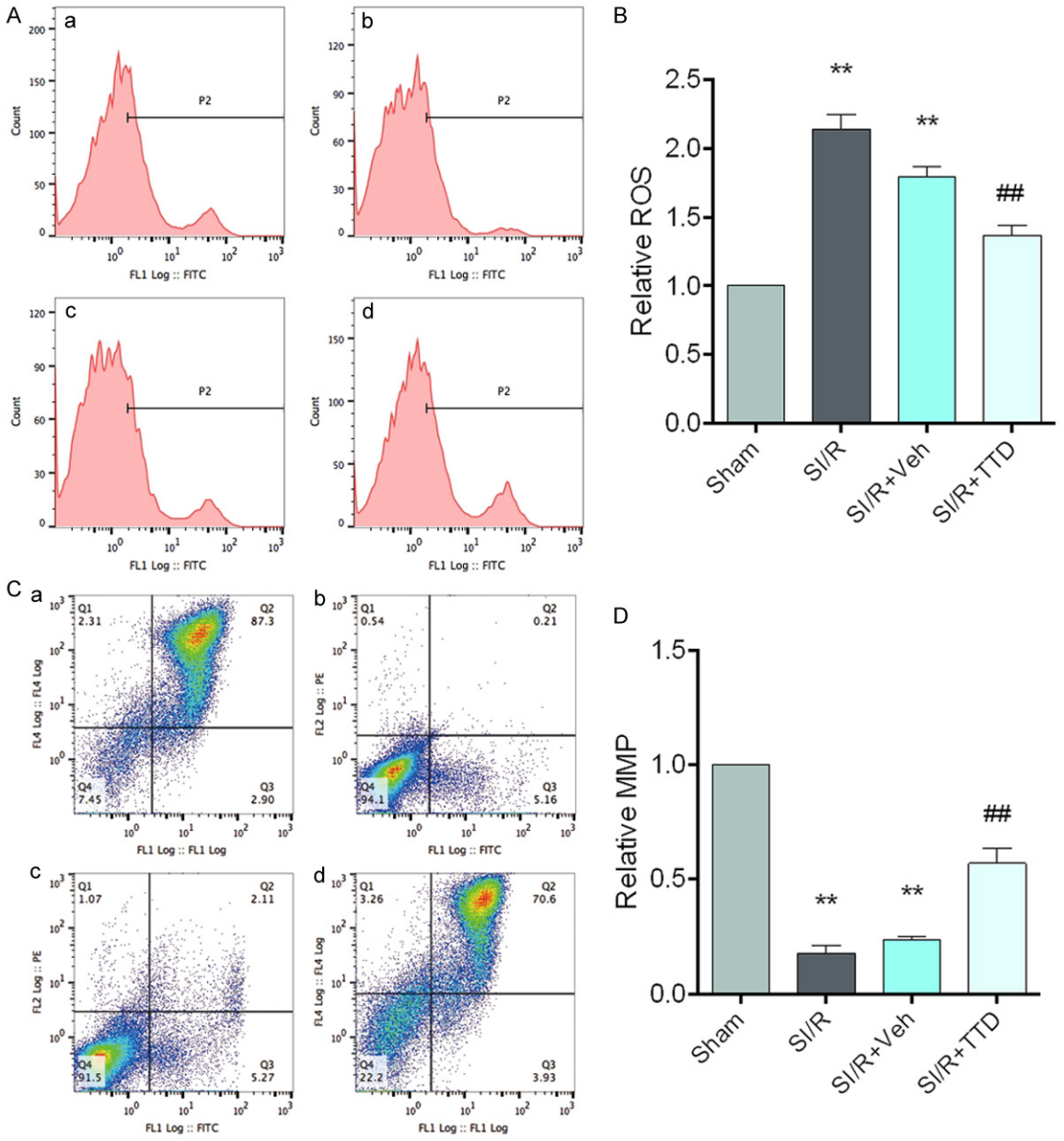

Figure 4. Tetrandrine reduces intracellular ROS generation and increases mitochondrial membrane potential (MMP) in NRCs subjected to SI/R. A. Fluorescent intensity was determined at excitation wavelength 488 nm and emission wavelength 525 nm via flow cytometry. B. Values presented are mean ± SEM. **P < 0.01 vs. Control, #P < 0.05 vs. SI/R. These experiments were performed in triplicate with similar results. a: Control group; b: Vehicle group; c: SI/R group; d: TTD+SI/R group. n=10/group. C. MMP was measured with fluorescent dye JC-1. 10 mM TTD was administered 30 minutes prior to SI/R. Fluorescent intensity of JC-1 was determined at excitation wavelength 488 nm and emission wavelength 530 nm via flow cytometry. D. Values presented are mean ± SEM. **P < 0.01 vs. Control, ##P < 0.01 vs. SI/R. These experiments were performed in triplicate with similar results. a: Control group; b: Vehicle group; c: SI/R group; d: TTD+SI/R group. n=10/group.

viability in the vehicle group was significantly reduced compared to control, and LDH leakage were increased compared to control (P < 0.01). TTD remarkably reduced SI/R induced cell death (P < 0.01, **Figure 3B**, **3D**). Together, these results indicate TTD significantly preserved cellular viability post-SI/R injury in a dose-dependent manner (at concentrations up to 10 mM).

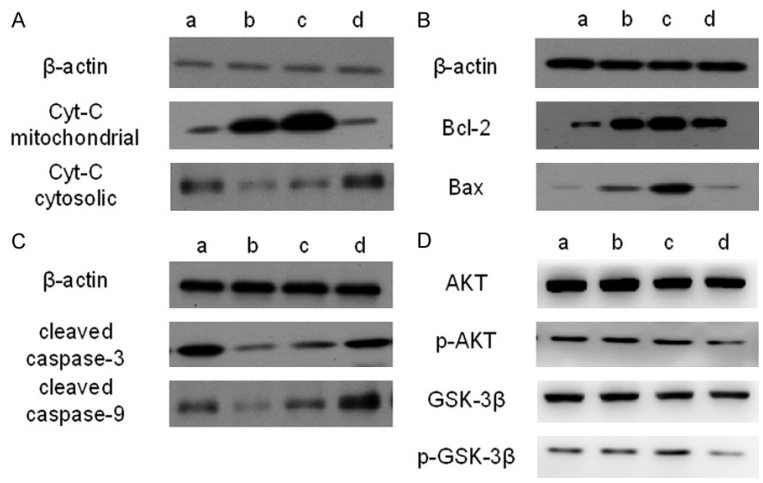

Figure 5. Tetrandrine inhibits mitochondrial-mediated apoptosis and increases phosphorylation of Akt and GSK-3b in NRCs subjected to SI/R. A. Western blot for cytochrome c release. SI/R increased cytosolic translocation of mitochondrial cytochrome c. a: Control group; b: Vehicle group; c: SI/R group; d: TTD+SI/R group. B. Representative western blot for Bcl-2 and Bax expression after various experimental treatments. a: Control group; b: Vehicle group; c: SI/R group; d: TTD+SI/R group. C. Representative western blot for SI/R-induced casepase-3 and caspase-9 activation. a: Control group; b: Vehicle group; c: SI/R group; d: TTD+SI/R group. D. Densitometric analysis demonstrates GSRd increased the ratio of P-Akt/Akt and P-GSK-3b/GSK-3b, which was significantly blocked by Akt-inhibitor LY294002. a: Control group; b: SI/R group; c: TTD+SI/R group; d: TTD+SI/R+LY294002 group.

Tetrandrine inhibited SI/R-induced ROS generation increases mitochondrial membrane potential, and decreases cytochrome c release in NRCs subjected to SI/R

SI/R induced rapidly and significantly increased DCF fluorescence ($P < 0.01$, **Figure 4A**). However, pretreatment of NRCs prior to SI/R significantly decreased DCF fluorescence ($P < 0.05$, **Figure 4B**), suggesting TTD significantly reduced ROS generation during SI/R in NRCs.

Mitochondrial membrane potential (MMP) is an important early determinant of the mitochondrial apoptotic pathway. We investigated the effects of TTD upon MMP and cytochrome c release. MMP detection was performed utilizing JC-1 dye to assess mitochondrial membrane depolarization. NRCs subjected to SI/R exhibited substantially decreased mitochondrial depolarization compared to control ($P < 0.01$, **Figure 4C**). Pretreatment with 10 mM TTD significantly stabilized the MMP ($P < 0.01$, **Figure 4D**). Mitochondrial depolarization releases several apoptogenic proteins, most notably cytochrome c into the cytosol. Western blot analysis demonstrated SI/R increased mitochondrial cytochrome c release into cytosol, and 10 mM TTD decreased cytochrome c release ($P < 0.05$, **Figure 5A**). Together, these results suggest TTD may attenuate apoptosis by potentially involving the mitochondrial apoptotic pathway.

Tetrandrine inhibited activation of caspase-9 and caspase-3, increased the phosphorylated Akt and GSK-3b, and increased the Bcl-2/Bax ratio

Caspases regulate cellular apoptosis. Cytochrome c release activates caspase-9, which activates caspase-3. SI/R significantly increased expression of both cleaved caspase-9 and caspase-3, which was attenuated by 10 mM TTD pretreatment (**Figure 5B**).

To further investigate the molecular mechanism underlying TTD-mediated cardioprotection, we determined P-Akt/Akt and P-GSK-3b/GSK-3b in NRCs post SI/R by western blot. There was no significant difference in Akt and GSK-3b expression between treatment groups at baseline (**Figure 5C**). Consistent with previous reports, SI/R alone increased phosphorylation of Akt and GSK-3b. Pretreatment with 10 mM TTD significantly increased phosphorylation of Akt and GSK-3b ($P < 0.01$).

Whether TTD protects against SI/R induced apoptosis in NRCs by modulating the Bcl-2 family proteins were determined. SI/R treatment decreased Bcl-2 expression, and increased Bax expression, decreasing the Bcl-2/Bax ratio (**Figure 5D**). Pretreating NRCs with 10 mM TTD prior to SI/R promoted Bcl-2 expression and inhibited Bax expression, increasing the Bcl-2/Bax ratio (**Figure 5D**).

Discussion

There are much remarkable observations were found in this study. Primarily, we demonstrate

that pretreatment with TTD attenuated in vivo MI/R injury in a rat model, and it can reduce in vitro SI/R injury in cultured NRCs. Then we provide the evidence that TTD reduces intracellular ROS generation in cardiomyocytes, and inhibits myocardial apoptosis induced by SI/R via the mitochondrial-dependent apoptotic pathway. Finally, we demonstrate Akt/GSK-3b signaling pathway activation significantly contributes to the anti-apoptotic effect of TTD.

Pathological conditions such as ischemia and reperfusion tilt the balance in favor of ROS overproduction which increases oxidative stress is a major apoptotic stimulus. In current studies, MI/R injury caused infarction and cardiac dysfunction. SI/R injury in cultured NRCs induced cell death significantly. TTD limited infarct size together withe augmented cardiac function in the employed rat MI/R model.

Cardiomyocyte apoptosis is one of the major pathogenic mechanisms underlying MI/R injury [10]. Cumulative evidence suggests that ROS, implicated in reperfusion toxicity, can trigger cardiomyocyte apoptosis via the mitochondrial apoptosis pathway [11]. During the early phase of myocardial reperfusion, ROS were released strongly oxidizes cardiomyocytes been damaged by ischemia. Cardiomyocytes are rich in mitochondria, a major endogenous source and susceptible target of ROS damage [12]. Mitochondrial-mediated apoptosis plays an important role in MI/R injury pathogenesis [5]. Under general conditions, cytochrome c is located in mitochondria. During intracellular ROS overproduction, collapse of the mitochondrial membrane potential (MMP) results in mitochondrial permeability transition pore (mPTP) opening, and rapidly releasing cytochrome c into the cytoplasm. Once released, cytochrome c binds the C-terminal domain of the apoptotic protease activating factor-1 (Apaf-1), inducing a conformation change. The activated Apaf-1/cytochrome c complex promotes caspase activation [13]. Caspases transduce and execute apoptotic signaling [14]. Caspase-3 (of the terminal common apoptotic pathway) is also activated by caspase-9, which is activated by the mitochondria-mediated apoptotic pathway. In the current study, we demonstrate TTD pretreatment mitigated SI/R-induced intracellular ROS, MMP, and mitochondrial release of cytochrome c into the cytosol, suggesting involvement of the mitochondrial pathway in TTD-mediated cardioprotection.

The Bcl-2 protein family are important mitochondrial regulators during cardiomyocyte apoptosis [7]. Bcl-2 regulates mPTP opening in opposition to Bax, blocking cytochrome c release, inhibiting caspase activity, and decreasing cell apoptosis [15]. As a result, altering the Bcl-2/Bax ratio affects apoptotic balance. Result of western blot revealed SI/R significantly decreased the Bcl-2/Bax ratio, an effect reversed by TTD administration, suggesting TTD-mediated cardioprotection against SI/R injury may occur partially via modulating Bcl-2/Bax expression.

Overall, our results demonstrate that TTD exerts cardioprotection against myocardial MI/R injury by both reducing intracellular ROS and inhibiting mitochondria mediated apoptosis. Activation of Akt/GSK-3b signaling is involved in the cardioprotective effect of TTD. The traditional herbal medicine TTD may have therapeutic potential attenuating myocardial ischemia/reperfusion injury.

Disclosure of conflict of interest

None.

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