

# Pre-treatment with a combination of Shenmai and Danshen injection protects cardiomyocytes against hypoxia/reoxygenation- and H<sub>2</sub>O<sub>2</sub>-induced injury by inhibiting mitochondrial permeability transition pore opening

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**Abstract.** Increasing evidence has indicated that opening of the mitochondrial permeability transition pore (mPTP) has a vital role in myocardial ischemia/reperfusion (I/R) injury. Shenmai injection (SMI) plus Danshen injection (DSI) combination, termed Yiqi Yangyin Huoxue (YYH) therapy is used in the clinic to treat cardiovascular diseases, including myocardial I/R injury. Previous studies by our group have demonstrated the protective effect of pretreatment with YYH against myocardial I/R injury in isolated rat hearts. The present study aimed to examine the protective effect of YYH against hypoxia/reoxygenation (H/R)- and H<sub>2</sub>O<sub>2</sub>-induced cardiomyocyte injury, and to determine whether this effect is produced by inhibition of mPTP opening. Primary cardiomyocytes isolated from neonatal rats were cultured and randomly grouped into a control group,

injury group and pretreatment group, with six duplicated wells in each group during each assay. Cardiomyocytes in the injury group were subjected to H/R to simulate I/R or exposed to H<sub>2</sub>O<sub>2</sub> for 2 h to induce oxidative injury. Cellular injury was assessed via release of creatine kinase (CK) and lactate dehydrogenase (LDH), and cell viability was measured by an MTT assay. The mitochondrial membrane potential ( $\Delta\Psi_m$ ) and cytosolic reactive oxygen species (ROS) were detected using the fluorescent probes rhodamine123 (Rh123) and chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H<sub>2</sub>DCFDA), respectively. Intracellular Ca<sup>2+</sup>, mitochondrial Ca<sup>2+</sup> and mPTP opening were measured using fluo-4 acetoxymethyl (Fluo-4/AM), rhodamine-2 acetoxymethyl (Rhod-2/AM) and calcein acetoxymethyl (Calcein/AM) probes, respectively. The results indicated that pretreatment with YYH enhanced cell viability, increased  $\Delta\Psi_m$ , reduced CK and LDH release, and decreased intracellular ROS and Ca<sup>2+</sup>, thus reducing cardiomyocyte injury induced by H/R or H<sub>2</sub>O<sub>2</sub>. LY294002, a specific phosphoinositide 3-kinase (PI3K) inhibitor, and PD98059, a specific inhibitor of the extracellular signal-regulated kinase 1/2 (Erk1/2) pathway, eliminated the protective effects of the combination therapy on cell viability and the change in the  $\Delta\Psi_m$  in cardiomyocytes. In conclusion, pre-treatment with YYH has cardioprotective effects against H/R injury and oxidative stress via activation of the PI3K/Akt and Erk1/2 signaling pathways, which reduces mPTP opening, overproduction of ROS and calcium overload.

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**Abbreviations:** BrdU, bromodeoxyuridine; CHD, coronary heart disease; CK, creatine kinase; DSI, Danshen injection; H/R, hypoxia/reoxygenation; IPC, ischemic pre-conditioning; I/R, ischemia/reperfusion; LDH, lactate dehydrogenase; mPTP, mitochondrial permeability transition pore; PCI, percutaneous transluminal coronary intervention; Rh123, Rhodamine123; ROS, reactive oxygen species; SMI, Shenmai injection; TCM, Traditional Chinese Medicine; YYH, YiqiYangyinHuoxue;  $\Delta\Psi_m$ , mitochondrial membrane potential

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**Key words:** Shenmai injection, Danshen injection, neonatal rat cardiomyocytes, hypoxia/reoxygenation injury, H<sub>2</sub>O<sub>2</sub> injury, mitochondrial permeability transition pore opening

## Introduction

Ischemic heart disease is a major cause of mortality worldwide, and the World Health Organization predicts that it will be the leading cause of mortality by 2030 (1). For patients that present with acute myocardial infarction, the most effective therapeutic strategy to preserve their myocardial tissue is timely reperfusion; however, the process of reperfusion may prompt further injury, which is a major feature of morbidity and mortality following infarction and has a direct correlation with the occurrence of coronary heart disease (CHD) (2). At present, there is

no effective treatment that protects the heart against reperfusion injury. Cyclosporine-A acts by inhibiting the opening of the mitochondrial permeability transition pore (mPTP) and phase II trials are exploring the use of cyclosporine-A immediately prior to percutaneous transluminal coronary intervention (PCI) (3); however, the severe adverse reactions associated with cyclosporine-A limit its clinical use.  $\beta$ -blocker agents have been in use for numerous years. Metoprolol was reported to reduce the infarct area in patients with anterior ST-elevation myocardial infarction undergoing PCI (4); however, it was also previously reported that metoprolol increases mortality in patients with a systolic blood pressure of <120 mmHg (5). Therefore, novel therapies to reduce myocardial ischemia/reperfusion (I/R) injury are required to improve clinical outcomes for patients with CHD.

Although the molecular mechanisms mediating reperfusion injury remain to be fully elucidated, the underlying pathophysiology of myocardial I/R injury may involve reactive oxygen species (ROS) generation, cytosolic and mitochondrial  $\text{Ca}^{2+}$  overload, cell apoptosis and inflammatory responses (6). Numerous studies have demonstrated the importance of mitochondrial dysfunction due to opening of the mPTP in I/R injury. Opening of the mPTP results in the non-selective permeability of the inner mitochondrial membrane to small molecules, resulting in the collapse of the mitochondrial membrane potential ( $\Delta\Psi_m$ ) and uncoupling of oxidative phosphorylation. Finally, cell death occurs due to ATP depletion (7). Therefore, inhibition of mPTP opening may be an important cardioprotective strategy. Increasing evidence has indicated that  $\text{Ca}^{2+}$  overload in the cytosol and mitochondria, and mitochondrial oxidative stress are the key inducers of mPTP opening, with ROS generation from the electron transport chain appearing within the first few minutes after myocardial reperfusion (8). Akt and extracellular signal-regulated kinase 1/2 (Erk1/2) are components of the reperfusion injury salvage kinase (RISK) pathway, which is thought to be the major signaling pathway involved in cardioprotection following myocardial reperfusion. The pathway is activated by ischemic pre-conditioning (IPC) and post-conditioning, and may be targeted by various pharmacological agents. An association between the activation of the phosphoinositide 3-kinase (PI3K)/Akt and Erk1/2 pathways, and the inhibition of mPTP opening has been previously suggested (9). Activation of the RISK pathway stimulates mPTP, enhances cardiomyocyte survival and reduces I/R injury (10).

Traditional Chinese Medicine (TCM) has received increasing attention regarding applications of multi-target therapies. Radix Ginseng Rubra, Radix Ophiopogonis and *Salvia miltiorrhiza* Bunge are well-known Chinese herbal medicines frequently used together to enhance their therapeutic efficacy. Shenmai injection (SMI) is composed of water-soluble extracts from Radix Ginseng Rubra and Radix Ophiopogonis. Danshen injection (DSI) is composed of aqueous extracts of *S. miltiorrhiza* Bunge. Ginsenosides, including protopanaxatriol-type ginsenosides (Re, Rf, Rg1), protopanaxadiol-type ginsenosides (Rb2, Rb1, Rd, Rc) and oleanolic acid-type ginsenosides (Ro), are the major active components of SMI (11). Previous studies by our and other groups have isolated and identified >15 phenolic acids in the water-soluble constituents of *S. miltiorrhiza*, including salvanic acid, protocatechuic acid, protocatechuic aldehyde and caffeic acid, as well as salvianolic acid A and B (12,13).

SMI, DSI and their combination, termed Yiqi Yangyin Huoxue (YYH), are clinically used to treat cardiovascular diseases, including CHD, myocardial infarction, congestive heart failure and myocardial I/R injury. Ginsenosides are the primary bioactive components of SMI, and have been confirmed to have various effects, including blocking  $\text{Ca}^{2+}$  channels, scavenging oxygen free radicals (14,15), and attenuating I/R injury in cardiovascular and cerebrovascular diseases. Furthermore, SMI has also been demonstrated to inhibit apoptosis and  $\text{Ca}^{2+}$  influx in neurocytes subjected to hypoxia-reoxygenation (H/R) (15,16). *In vitro* and *in vivo* studies suggest that DSI may be vasoactive, able to scavenge ROS, promote circulation and inhibit platelet aggregation (17). Clinical studies have reported that the combination of SMI and DSI therapy improved myocardial reperfusion injury following IPC in patients with acute myocardial infarction by reducing oxidative stress (18). A previous study by our group has demonstrated the protective effect of pretreatment with YYH, against myocardial I/R injury via the PI3K/Akt and Erk1/2 signaling pathways in isolated rat hearts (Fig. 1) (19). However, the mechanisms underlying the cardioprotective effect of YYH have remained elusive. The present study aimed to investigate the underlying mechanisms by which YYH attenuates H/R- and hydrogen peroxide ( $\text{H}_2\text{O}_2$ )-induced cardiomyocyte injury, focusing on the inhibition of mPTP opening via the PI3K/Akt and Erk1/2 signaling pathways.

## Materials and methods

**Reagents.** SMI and DSI were donated by Chiatai-Qing-Chun-Bao Pharmaceutical Co., Ltd. (Hangzhou, China). Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H<sub>2</sub>DCFDA), fluo-4 acetoxymethyl (Fluo-4/AM) and calcein acetoxymethyl (Calcein/AM) were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Collagenase II, bromodeoxyuridine (BrdU), rhodamine123 (Rh123), MTT,  $\text{H}_2\text{O}_2$  and PD98059 were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Rhodamine-2 acetoxymethyl (Rhod-2/AM) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). LY294002 was purchased from Apollo Scientific Ltd. (Stockport, UK). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium were obtained from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). Creatine kinase [(CK); cat. no. A0032] and lactate dehydrogenase [(LDH); cat. no. C0016] assay kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Trypsin (1:25) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

**Isolation and culture of neonatal rat cardiomyocytes.** A total of 30 neonatal Sprague Dawley rats (age, 1-3 days; body weight, 10-15 g) were obtained from Beijing HFK Bioscience Co. Ltd. (Beijing, China). Without further housing, these neonatal rats were anaesthetized in a container with metofane-saturated gauze and ventricular cardiomyocytes were isolated (20,21). In brief, after administration of metofane, the newborn rats exhibited no sign of consciousness, indicated by absence of reaction to soft poking and disappearance of skin pinch reaction, which indicated full anesthesia. Furthermore, vital signs and absence of any indications of toxicity were confirmed. The neonatal rats

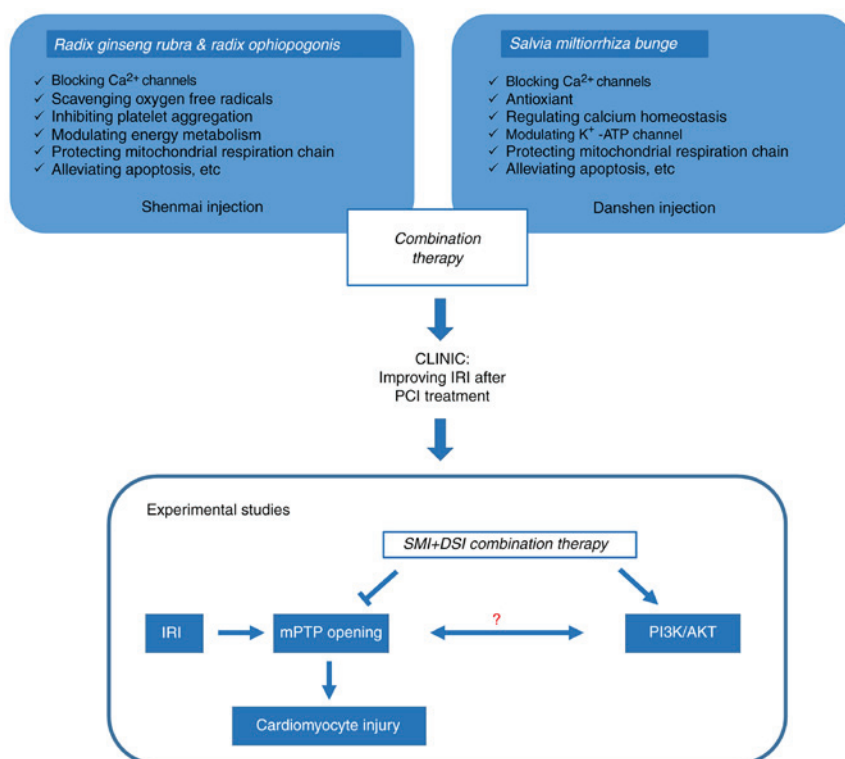


Figure 1. Schematic summarizing the thematic background and questions to be addressed in the present study. The possible implication of PI3K signaling in inhibition of mPTP opening as a mechanism of the protective effects of YYH against cardiomyocytes H/R injury was investigated. SMI, Shenmai injection; DSI, Danshen injection; mPTP, mitochondrial permeability transition pore; PI3K, phosphoinositide 3-kinase; IRI, ischemia/reperfusion injury; PCI, percutaneous transluminal coronary intervention.

were disinfected with 75% ethanol and the hearts were rapidly harvested. The ventricular myocardium was minced into 1 mm<sup>3</sup> pieces using scissors in PBS. Samples were digested in PBS containing 0.0625% (w/v) trypsin and 0.1% (w/v) collagenase II with gentle agitation at 37°C for 5 min. Digestion was repeated for 5-8 cycles in total, and the digestion was stopped by addition of medium containing 10% (v/v) FBS. Subsequently, the cell suspension was filtered through a 200-mesh sieve and centrifuged at 560 x g at 4°C for 10 min. The harvested cell pellet was re-suspended in basic medium containing 10% (v/v) FBS and incubated at 37°C for 90 min to allow for fibroblast adhesion. Non-adherent cells were collected and seeded in a culture flask at 3x10<sup>5</sup> cells/mm<sup>2</sup>. Cells were incubated with 95% air and 5% CO<sub>2</sub> for 1 h. To enhance the purity of the cardiomyocytes, BrdU (0.1 mM) was added to the culture medium for the first 3 days according to previously described methods (22). Cardiomyocytes were then used for subsequent experiments.

**H/R injury in cardiac myocytes.** H/R was simulated as previously described (23). In brief, the cardiomyocyte medium for hypoxia was deprived of glucose and serum. Then the cells were deposited into a hypoxia chamber (Stemcell Technologies, Inc., Vancouver, BC, Canada) containing with 95% (v/v) N<sub>2</sub> and 5% (v/v) CO<sub>2</sub> at 37°C for 20 h of hypoxia. The medium was replaced with high-glucose medium and the cells were transferred to the regular incubator and maintained for 4 h for reoxygenation.

**H<sub>2</sub>O<sub>2</sub>-induced oxidative stress injury.** In the clinic, adult patients receive an i.v. infusion of 30 ml Shenmai injection and 30 ml Danshen injection (18,24), which are diluted with

250 ml saline solution containing 5% glucose. As the maximum concentration, 10 µl/ml [SMI/DSI/culture medium, 5:5:990 (v/v/v)] is equivalently used in the clinic. In the present study, cells were pre-treated with a combination of SMI and DSI (2.5, 5 and 10 µl/ml) for 10 h. Oxidative stress was induced in cultured cardiac myocytes by adding 100 µM H<sub>2</sub>O<sub>2</sub> for 120 min, or 90 min for the mitochondrial Ca<sup>2+</sup> experiment. Cells were pre-treated with specific probes and with Hank's solution in the absence or presence of 100 µM H<sub>2</sub>O<sub>2</sub>, and fluorescence was monitored.

**Cell viability, CK and LDH activity assays.** Following reoxygenation, the medium was removed and cells were incubated with a solution of 1.2 mM MTT for 4 h at 37°C. Subsequently, 150 µl dimethylsulfoxide was added to each well following removal of the medium. Mitochondrial dehydrogenase activity, which reflects cell viability, was measured at 490 nm. Cell viability was expressed as a percentage of the control. CK and LDH activity in the medium was measured using the CK or LDH assay kits according to the manufacturer's protocol, respectively.

**Assessment of  $\Delta\Psi_m$ .** Fluorescence quenching of Rh123 was used to assess  $\Delta\Psi_m$  as previously described (25). Cardiomyocytes were cultured with 5 µM Rh123 at 37°C for 30 min and then washed three times with PBS. Fluorescence was measured using a Multimode plate reader (PerkinElmer, Inc., Waltham, MA, USA) at excitation/emission (ex/em) wavelengths of 488/535 nm. Values are expressed as a percentage of the control. Images of the cells were captured under a fluorescence microscope (Olympus IX73; Olympus Corp., Tokyo, Japan).



**Measurement of intracellular ROS.** Intracellular ROS were detected using the fluorescent dye CM-H<sub>2</sub>DCFDA. In brief, following the specific treatments, samples were washed with PBS and incubated with 5  $\mu$ M CM-H<sub>2</sub>DCFDA for 20 min at 37°C, and then washed again twice with PBS. Fluorescence intensity was measured at ex/em wavelengths of 488/525 nm using a Multimode plate reader (PerkinElmer, Inc.).

**Determination of cytosolic and mitochondrial Ca<sup>2+</sup>.** To monitor cytosolic Ca<sup>2+</sup>, cells were loaded with 4  $\mu$ M Fluo-4/AM at 37°C for 30 min and then washed three times with dye-free buffer. Following further incubation with 90  $\mu$ l Hank's solution at 37°C for 20 min, cells were exposed to H<sub>2</sub>O<sub>2</sub> (10  $\mu$ l H<sub>2</sub>O<sub>2</sub> added to 90  $\mu$ l Hank's solution) at 37°C for 2 h, and the fluorescence was measured at ex/em wavelengths of 494/516 nm using a Multimode plate reader (PerkinElmer, Inc.).

The Ca<sup>2+</sup>-sensitive dye Rhod-2AM was used to monitor mitochondrial Ca<sup>2+</sup> (26,27). Cells were washed with Hank's solution, followed by incubation with 4  $\mu$ M dihydro Rhod-2/AM containing 0.05% (v/v) Pluronic F-127 at 37°C for 45 min. Rhod-2 fluorescence was measured at ex/em wavelengths of 552/581 nm as a baseline value. Subsequently, cells were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> (diluted with Hank's solution) at 37°C for 90 min and the fluorescence was measured at a series of time-points. Results are expressed as a percentage of the baseline fluorescence intensity.

**Monitoring of mPTP opening.** mPTP opening was monitored by co-loading cells with Calcein/AM and CoCl<sub>2</sub> as previously described (28,29). In brief, cardiomyocytes were incubated with 2  $\mu$ M Calcein/AM and 1 mM CoCl<sub>2</sub> at room temperature for 35 min, and then washed with 1 mM CoCl<sub>2</sub> for 25 min. Calcein fluorescence was measured at ex/em wavelengths of 488/515 nm as the baseline value. Subsequently, cells were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> (diluted with Hank's solution) at 37°C for 120 min and the fluorescence was measured at a series of time-points. The abrupt loss of fluorescence was regarded as an indicator of mPTP opening. Results are expressed as a percentage of the baseline fluorescence intensity.

**Statistical analysis.** Statistical analyses were performed using the SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). All values are expressed as the mean  $\pm$  standard deviation (number of replicate wells, n=6). Differences between two groups were assessed using Student's t-test and one-way analysis of variance followed by the least-significant differences method was employed for comparison between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Cardioprotective effects of YYH in cardiomyocytes subjected to H/R.** YYH improves the viability of cardiomyocytes subjected to H/R injury. H/R resulted in an 82.46 $\pm$ 3.52% reduction in cardiomyocyte viability compared with that in the control group. YYH pretreatment at increasing concentrations (2.5, 5 and 10  $\mu$ l/ml) enhanced the cell viability following H/R injury (93.40 $\pm$ 3.81, 92.66 $\pm$ 3.66 and 113.83 $\pm$ 7.57% of the control, respectively; Fig. 2A).

YYH prevents H/R-induced CK and LDH release in cardiomyocytes. The CK and LDH levels were higher in the H/R group (19.3 $\pm$ 1.5 and 278.3 $\pm$ 30.0 U/l, respectively) than those in the control group (4.6 $\pm$ 2.8 and 25.3 $\pm$ 5.5 U/l, respectively; P<0.01). YYH pretreatment (5 and 10  $\mu$ l/ml) significantly reduced the levels of CK and LDH in a concentration-dependent manner (Fig. 2B and C).

YYH preserves  $\Delta\Psi$ m in cardiomyocytes subjected to H/R. H/R increased  $\Delta\Psi$ m depolarization to 76.61 $\pm$ 3.33% of that in the control group (P<0.01). YYH pretreatment (2.5, 5 and 10  $\mu$ l/ml) increased mitochondrial depolarization to 89.4 $\pm$ 5.4, 91.9 $\pm$ 8.1 and 97.2 $\pm$ 4.0% of the control, respectively (P<0.01; Fig. 2D).

YYH reduces H/R-induced ROS generation. H/R resulted in increased generation of intracellular ROS compared with the control group (190.82 $\pm$ 7.24%; P<0.01). Pretreatment with YYH (2.5, 5 and 10  $\mu$ l/ml) significantly reduced intracellular ROS generation compared with the H/R treatment group (148.35 $\pm$ 2.82, 134.88 $\pm$ 1.58 and 130.74 $\pm$ 10.01% of the control, respectively) after 4 h of reperfusion following hypoxia (P<0.01; Fig. 2E).

**Cardioprotective effect of YYH determined in cardiomyocytes challenged with H<sub>2</sub>O<sub>2</sub>.**

YYH reduces H<sub>2</sub>O<sub>2</sub>-induced LDH release in cardiomyocytes. LDH activity was detected at 2 h after treatment with H<sub>2</sub>O<sub>2</sub>. Exposure to H<sub>2</sub>O<sub>2</sub> induced an increase in LDH release from 117.5 $\pm$ 21.9 to 709.0 $\pm$ 37.0 U/l, which was significantly suppressed by YYH preincubation (10  $\mu$ l/ml) for 10 h (490.0 $\pm$ 31.4 U/l; P<0.05; Fig. 3A).

YYH attenuates H<sub>2</sub>O<sub>2</sub>-induced ROS generation. Aberration in DCF fluorescence reflects the change in ROS levels. H<sub>2</sub>O<sub>2</sub> induced a marked increase in ROS levels compared with those in the control group (P<0.01). Treatment with YYH (2.5, 5 and 10  $\mu$ l/ml) resulted in a significant decrease in ROS levels compared with those in the H<sub>2</sub>O<sub>2</sub> group (P<0.01; Fig. 3B). ROS levels in middle and high dose of YYH (5 and 10  $\mu$ l/ml) were closed to those in the control group, indicating a better inhibitory effect on ROS overproduction.

YYH reduces cytosolic and mitochondrial Ca<sup>2+</sup> overload in H<sub>2</sub>O<sub>2</sub>-challenged cardiomyocytes. The cytosolic Ca<sup>2+</sup> level was monitored using Fluo-4AM. The Ca<sup>2+</sup> level in the H<sub>2</sub>O<sub>2</sub> group was significantly increased compared with that in the control group (P<0.01), which was reversed by the pretreatment with YYH (P<0.01; Fig. 3C). Among these, the high dose of YYH significantly decreased cytosolic Ca<sup>2+</sup> levels, beyond that of the control group (P<0.01).

Mitochondrial Ca<sup>2+</sup> overload induced by H<sub>2</sub>O<sub>2</sub> was evaluated using time-lapse fluorescence microscopy, monitoring changes in Rhod-2 fluorescence. There was an obvious increase of mitochondrial fluorescence appeared at 30 min after exposure to H<sub>2</sub>O<sub>2</sub>. The high fluorescence was maintained for the remaining time period after 60 min. Pretreatment with YYH did not alter the baseline level of mitochondrial Ca<sup>2+</sup>. Of note, after 30 min of exposure to H<sub>2</sub>O<sub>2</sub>, the associated increases in the mitochondrial Ca<sup>2+</sup> levels were reduced in the groups pretreated with YYH (2.5, 5 and 10  $\mu$ l/ml; Fig. 3D). Rhod-2 fluorescence at 90 min after exposure to H<sub>2</sub>O<sub>2</sub> is presented in the bar graph.

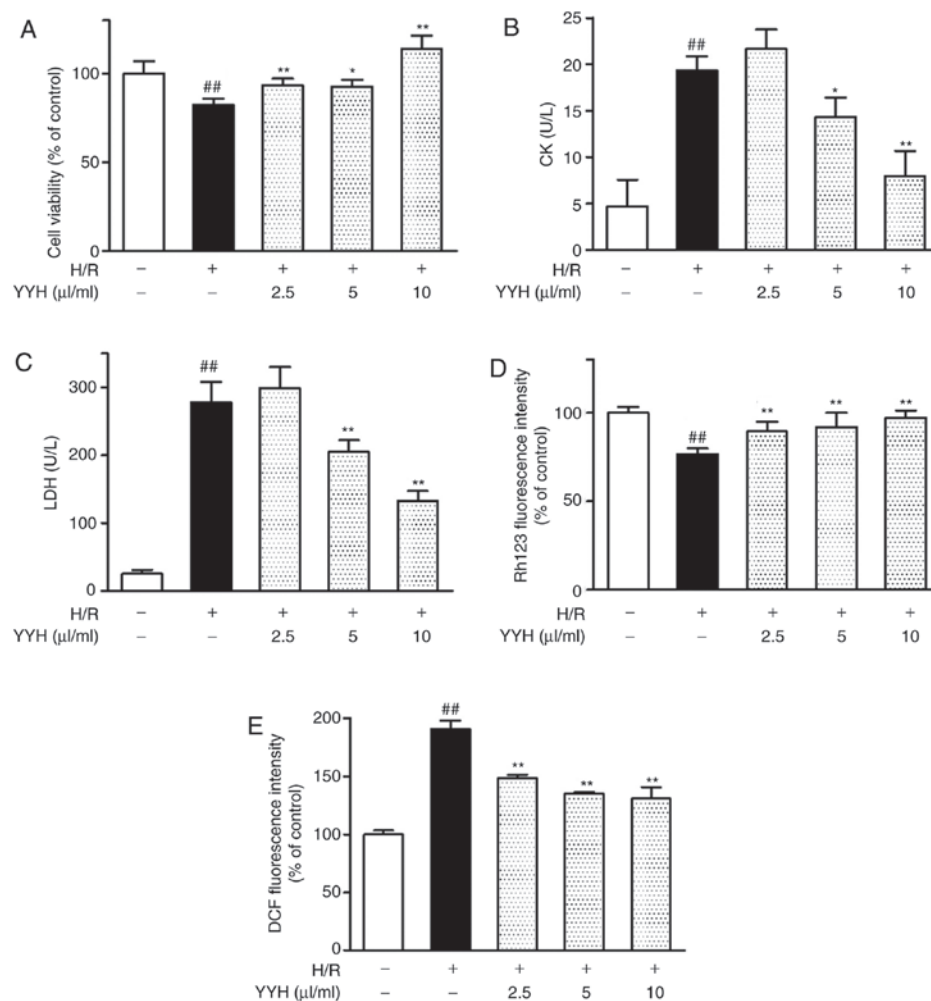


Figure 2. Protective effects of YYH (2.5, 5 and 10 µl/ml) on H/R-induced injury of neonatal rat cardiomyocytes. (A) Effect of YYH on cell viability was determined by an MTT assay. (B) Effect of YYH on the level of extracellular CK was measured using a CK assay kit. (C) Effect of YYH on the level of extracellular LDH was measured using an LDH assay kit. (D) Evaluation of mitochondrial membrane potential of cardiomyocytes using Rh123 fluorescence. (E) Intracellular reactive oxygen species levels were measured using the DCF probe. Values are expressed as the mean ± standard deviation (n=6). <sup>##</sup>P<0.01 vs. control; <sup>\*</sup>P<0.05 vs. H/R, <sup>\*\*</sup>P<0.01 vs. H/R. H/R, hypoxia/reoxygenation; YYH, YiqiYangyinHuoxue, referring to the combination of Shenmai injection and Danshen injection; CK, creatine kinase; LDH, lactate dehydrogenase; Rh123, Rhodamine123; DCF, dichlorodihydrofluorescein.

Compared with the control group, H<sub>2</sub>O<sub>2</sub> exposure significantly increased fluorescence intensity (P<0.01), indicating increased mitochondrial Ca<sup>2+</sup> levels. Pretreatment with YYH (2.5, 5 and 10 µl/ml) significantly inhibited H<sub>2</sub>O<sub>2</sub>-induced mitochondrial Ca<sup>2+</sup> overload (P<0.01). However, levels of mitochondrial Ca<sup>2+</sup> in the pretreatment groups remain higher than those in the control group. With the increasing concentration of YYH, the fluctuating fluorescence intensity indicated that the inhibitory effect of YYH (10 µl/ml) on mitochondrial Ca<sup>2+</sup> overloading may be reach the level of saturation.

**YYH inhibits mPTP opening induced by H<sub>2</sub>O<sub>2</sub>.** mPTP opening in intact cells was analyzed by monitoring the fluorescence of mitochondrial-entrapped calcein. A rapid decrease in calcein fluorescence was detected at ~35 min after exposure to H<sub>2</sub>O<sub>2</sub>, indicating mPTP opening. YYH pretreatment (2.5, 5 and 10 µl/ml) suppressed the sudden drop of the fluorescence value and the 10 µl/ml dose exerted the greatest effect (Fig. 3E). Differences in calcein fluorescence at 120 min after exposure to H<sub>2</sub>O<sub>2</sub> are presented in the bar graph. Compared with the control group, H<sub>2</sub>O<sub>2</sub> exposure decreased the fluorescence

intensity from 65.8±7.8 to 21.0±4.3% of the baseline value (P<0.01), indicating increased mPTP opening. By contrast, YYH pretreatment (2.5, 5 and 10 µl/ml) inhibited mPTP opening, as indicated by the increase in fluorescence from 21.0±4.3 to 44.1±3.9, 46.6±4.7 and 52.9±3.2% of the baseline value, respectively (P<0.01). Therefore, YYH inhibited H<sub>2</sub>O<sub>2</sub>-induced mPTP opening.

**Inhibition of PI3K/Akt and ERK1/2 pathways attenuates YYH cardioprotection from H/R injury.** To further explore whether the cardioprotective effect of YYH is associated with the activation of PI3K/Akt and Erk1/2 signaling, a PI3K-specific inhibitor, LY294002, and an ERK1/2-specific inhibitor, PD98059, were used to investigate cell viability and ΔΨ<sub>m</sub>. Compared with that in the H/R group, pre-treatment with YYH (10 µl/ml) resulted in a marked increase in cell viability (P<0.01; Figs. 4A and 5A). However, these effects were partially attenuated by LY294002 (P<0.01) or PD98059 (P<0.01) compared with combination group LY294002 reduced cell viability compared with that in the H/R group, indicating that the protective effect of the PI3K signaling pathway was inhibited by LY294002.

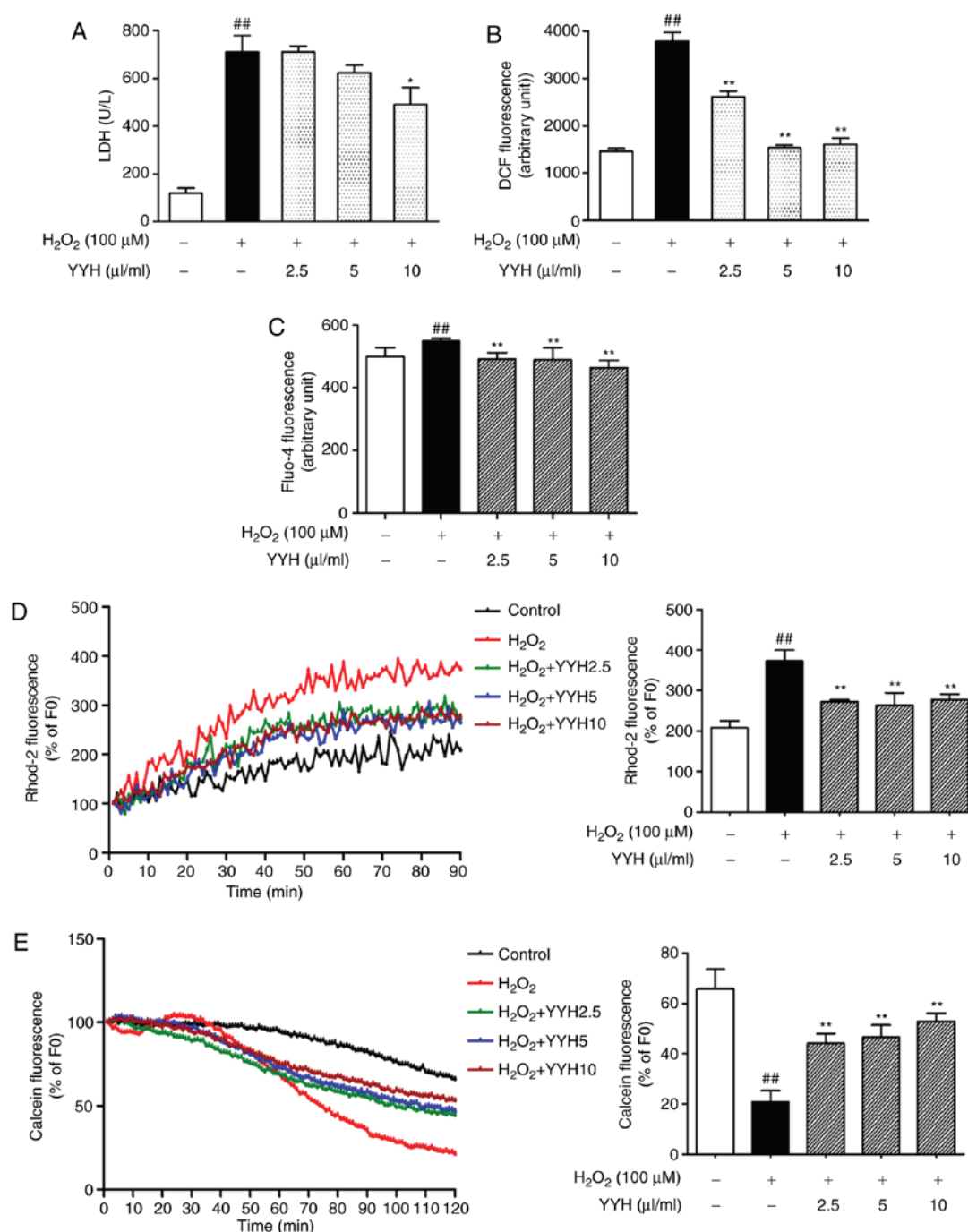


Figure 3. Effects of YYH (2.5, 5 and 10  $\mu$ l/ml) on H<sub>2</sub>O<sub>2</sub>-induced injury of cardiomyocytes isolated from neonatal rats. (A) Extracellular LDH was measured using LDH assay kit. (B) Intracellular reactive oxygen species levels were measured using the chloromethyl H<sub>2</sub>DCF diacetate probe. (C) Effect of YYH on cytosolic Ca<sup>2+</sup> was measured using Fluo-4 acetoxymethyl ester. (D) Time-dependent changes in mitochondrial Ca<sup>2+</sup> levels were measured using Rhod-2 (left). Bar graph of Rhod-2 fluorescence intensity at 90 min following exposure to H<sub>2</sub>O<sub>2</sub> (right). (E) Time-dependent changes in mitochondrial permeability transition pore opening indicated by calcein (left). Bar graph of calcein fluorescence intensity at 120 min following exposure to H<sub>2</sub>O<sub>2</sub> (right). Values are expressed as the mean  $\pm$  standard deviation (n=6). <sup>##</sup>P<0.01 vs. Control; <sup>\*</sup>P<0.05 vs. H<sub>2</sub>O<sub>2</sub>; <sup>\*\*</sup>P<0.01 vs. H<sub>2</sub>O<sub>2</sub>. YYH, YiqiYangyinHuoxue, referring to the combination of Shenmai injection and Danshen injection; LDH, lactate dehydrogenase; DCF, dichlorodihydrofluorescein; Rhod, rhodamine; F0, baseline value.

Compared with that in the H/R group, pretreatment with YYH (10  $\mu$ l/ml) resulted in a marked increase in the  $\Delta\Psi_m$  (P<0.01; Fig. 4B and C, Fig. 5B). However, these effects were partially abolished by LY294002 (P<0.01) or PD98059 (P<0.01). LY294002 or PD98059 alone exerted no effect on cell viability and  $\Delta\Psi_m$ . The results indicate that the PI3K/Akt pathway and the ERK1/2 pathway may be involved in the protective effect of YYH.

## Discussion

Blockage of cardiac blood flow deprives the heart of its oxygen supply, resulting in myocardial injury. Timely restoration of the blood flow effectively attenuates ischemic injury; however, subsequent reperfusion induces secondary damage to the ischemic myocardium, known as reperfusion injury (30). The mechanisms underlying reperfusion injury are complex and

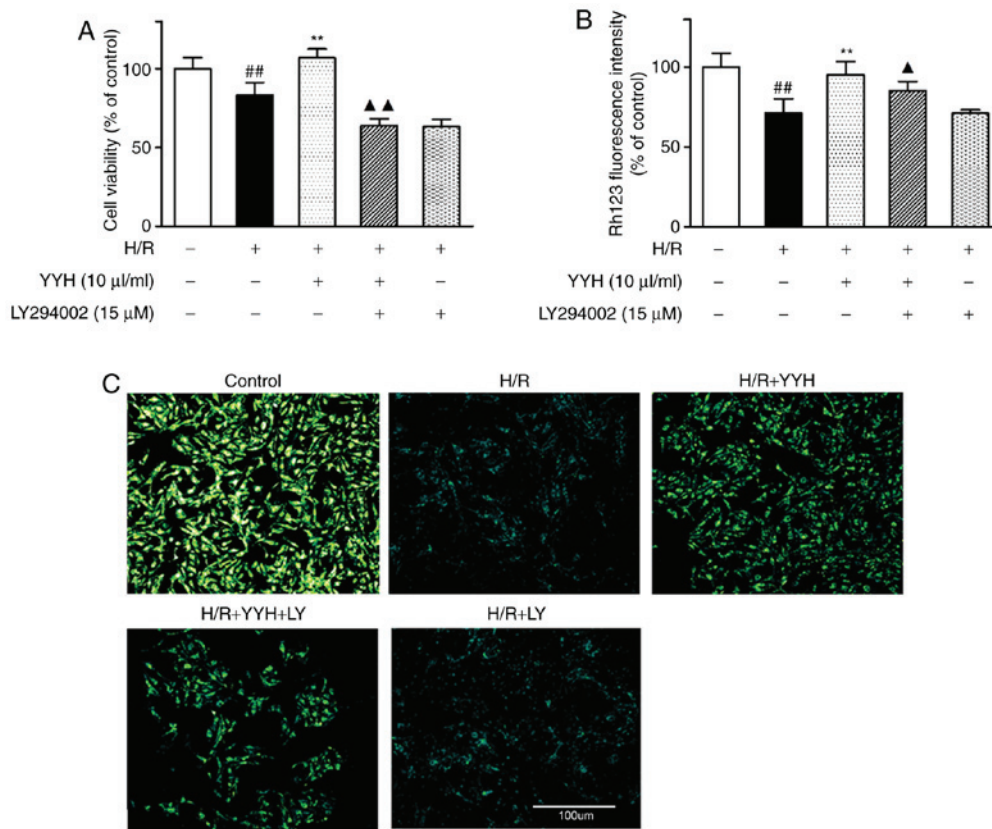


Figure 4. LY294002 antagonizes the protective effects of YYH (10 μl/ml) on cardiomyocytes that were subjected to H/R. (A) Cell viability was measured via an MTT assay and cells were incubated with a solution of 1.2 mM MTT for 4 h at 37°C. (B) Mitochondrial membrane potential was assessed via Rh123 fluorescence. Quantified Rh123 fluorescence intensity is provided in a bar graph. (C) Fluorescence microscopy images of cells in the different groups subjected to Rh123 staining (scale bar, 100 μm). Values are expressed as the mean ± standard deviation (n=6). <sup>##</sup>P<0.01 vs. Control; <sup>\*\*</sup>P<0.01 vs. H/R; <sup>▲</sup>P<0.05, <sup>▲▲</sup>P<0.01 vs. YYH group. H/R, hypoxia/reoxygenation; YYH, YiqiYangyinHuoxue, referring to the combination of Shenmai injection and Danshen injection; Rh123, Rhodamine123; LY, LY294002.

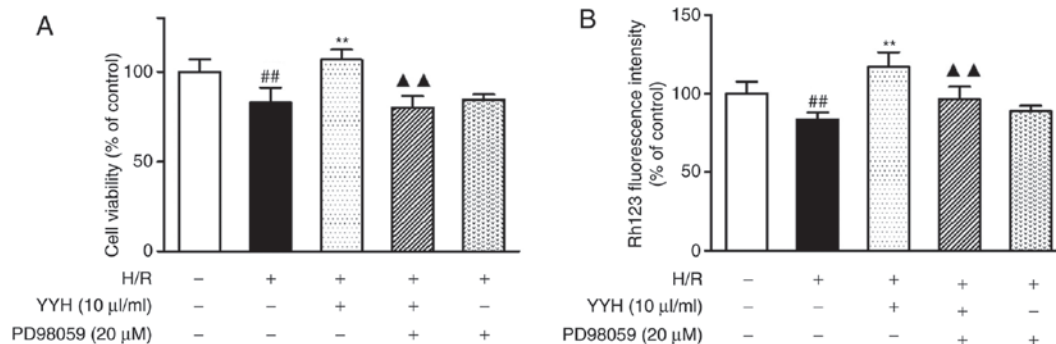


Figure 5. Protective effect of YYH (10 μl/ml) on cardiomyocytes subjected to H/R is partially inhibited by PD98059. (A) Cell viability was measured via an MTT assay. (B) Mitochondrial membrane potential. Values are presented as the mean ± standard deviation (n=6). <sup>##</sup>P<0.01 vs. Control; <sup>\*\*</sup>P<0.01 vs. H/R; <sup>▲▲</sup>P<0.01 vs. combination group. H/R, hypoxia/reoxygenation; YYH, YiqiYangyinHuoxue, referring to the combination of Shenmai injection and Danshen injection; Rh123, Rhodamine123.

multifactorial, with ROS generation, Ca<sup>2+</sup> overload, opening of the mPTP, endothelial dysfunction and pronounced inflammatory responses all implicated in causing the damage (31). Radix Ginseng Rubra, Radix Ophiopogonis and *S. miltiorrhiza* Bunge, which are included in YYH, have been investigated to determine their potential combined pharmaceutical properties, including anti-inflammatory, anti-oxidant, microcirculation promotion and cardioprotective abilities (32,33). In the clinic, it has been reported that YYH improved myocardial reperfusion

injury following PCI in patients with acute myocardial infarction (18).

In the present study, cardiomyocytes were subjected to H/R- and H<sub>2</sub>O<sub>2</sub>-induced injury. YYH was administered at three concentrations (2.5, 5 and 10 μl/ml) to investigate its cardioprotective actions. The results of the present study indicated that H/R reduced cell viability and ΔΨ<sub>m</sub>, suggesting disruption of mitochondrial integrity and function. Of note, pre-treatment with YYH inhibited these decreases. The protective effects



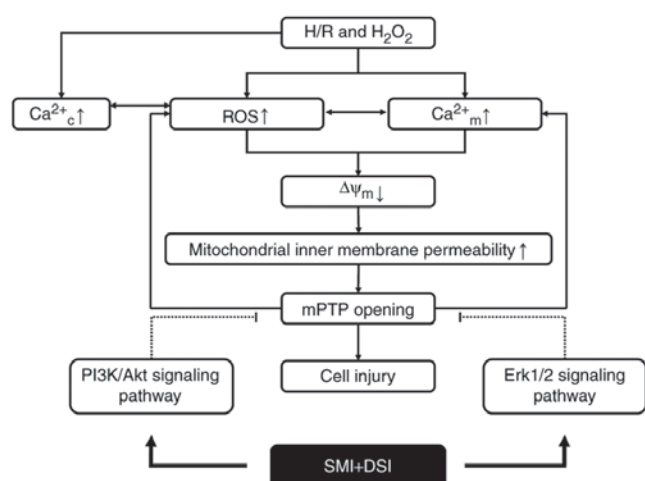


Figure 6. Summary scheme of the mechanism underlying inhibition of SMI plus DSI combination on H/R and H<sub>2</sub>O<sub>2</sub>-induced mPTP opening. H/R and H<sub>2</sub>O<sub>2</sub> treatment induces accumulation of ROS, Ca<sup>2+</sup><sub>c</sub> overload and an increase of mitochondrial inner membrane permeability. SMI plus DSI combination therapy activates the PI3K/Akt and Erk1/2 signaling pathways, therefore inhibiting mPTP opening and cardiomyocyte injury. H/R, hypoxia/reoxygenation; Ca<sup>2+</sup><sub>c</sub>, cytosolic calcium; Ca<sup>2+</sup><sub>m</sub>, mitochondrial calcium; ROS, reactive oxygen species; ΔΨ<sub>m</sub>, mitochondrial membrane potential; mPTP, mitochondrial permeability transition pore; PI3K, phosphoinositide 3-kinase; Erk1/2, extracellular signal-regulated kinase; SMI, Shenmai injection; DSI, Danshen injection.

of YYH were abolished by LY294002, a specific inhibitor of PI3K, and PD98059, a specific inhibitor of the Erk1/2 pathway, suggesting that the PI3K/Akt and Erk1/2 signaling pathways are involved in the cardioprotective effects of YYH. A previous study by our group demonstrated that ginsenoside Rb1, a principal active component of SMI, directly inhibited mPTP opening on mitochondria isolated from rat hearts *in vitro* (34). Its effect on mPTP opening is mediated via phosphorylation of Akt and glycogen synthase kinase-3β, as demonstrated in a Langendorff-perfused rat heart model and in experiments using a H/R induced cardiomyocyte injury model that was subjected to H/R (34). Increasing evidence suggests that the PI3K/Akt and Erk1/2 pathways are involved in signaling cascades in myocardial IPC and have important roles in cardioprotection following myocardial I/R injury (35,36). Activation of the PI3K/Akt and Erk1/2 signaling stimulates mPTPs downstream, enhances cardiomyocyte survival and reduces morbidity and mortality following I/R injury (37).

mPTP is a non-selective conductance pore located in the inner mitochondrial membrane. mPTP opening contributes to the transition from reversible to irreversible myocardial I/R injury (38) by inducing mitochondrial swelling and outer membrane rupture, subsequently facilitating activation of caspases and release of pro-apoptotic proteins, and ultimately contributing to the induction of apoptosis. This process is initiated shortly after ischemia and amplified by reperfusion. ROS and Ca<sup>2+</sup> are also elevated during myocardial I/R, following activation of mPTP opening, particularly during reperfusion. I/R injury induces mitochondria to produce high level of ROS, including superoxide anion (O<sub>2</sub><sup>-</sup>), hydroxyl radical (OH<sup>•</sup>) and hydrogen peroxide H<sub>2</sub>O<sub>2</sub> (39). Excessive amounts of ROS cause damage to mitochondria, inducing mPTP opening and mitochondrial depolarization (40). Furthermore,

an increased concentration of cytosolic Ca<sup>2+</sup> activates a variety of cell death-associated processes following I/R. Ca<sup>2+</sup> is then transported into the mitochondria of cardiomyocytes via mitochondrial Ca<sup>2+</sup> uniporters. Once mitochondrial Ca<sup>2+</sup> overloading occurs, the mPTP response is triggered. Therefore, numerous cardioprotective processes primarily function via direct inhibition of mPTP opening or upstream factors (23,41).

The results of the present study revealed that YYH protects cardiomyocytes by blocking H/R- and H<sub>2</sub>O<sub>2</sub>-induced CK and LDH release, inhibiting ROS production, reducing oxidative stress-induced cytosolic and mitochondrial Ca<sup>2+</sup> overload, and subsequently suppressing mPTP opening. Inhibition of mPTP opening may be a key event in mediating myocardial protection against I/R injury. This beneficial effect is associated with activation of the PI3K/Akt and Erk1/2 signaling pathways (19). However, it has not been established whether the mechanisms that mediate the effects of YYH *in vitro* are also involved in the therapeutic effects *in vivo*, and further studies are required to support this. The mitochondrial protection mechanism of YYH therapy also requires validation using animal models. Further research is also required to explore mechanisms of potential mPTP-targeting strategies and other mechanisms that may be involved in the effects of SMI and DSI combination using *in vitro* and *in vivo* analyses.

In summary, the present study demonstrated that YYH protects cardiomyocytes against H/R- and H<sub>2</sub>O<sub>2</sub>-induced injury through activation of the PI3K/Akt and Erk1/2 signaling pathway and inhibition of mPTP opening resulting from ROS generation and calcium overload (Fig. 6). The *in vitro* molecular mechanisms of action of YYH therapy and their protective effects against myocardial I/R *in vivo* require further exploration.

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## Availability of data and materials

The datasets generated and/or analyzed during the present study are included in this published paper.

## Authors' contributions

LL, ZS and YW performed the experiments and wrote the paper. ZD revised the paper. DY, JL and HW performed the experiments. YL designed the experiments. All of the authors read and approved the final manuscript.

## Ethics approval and consent to participate

This study was performed in strict accordance with the recommendations in the Guidance Suggestions for the Care and Use of Laboratory Animals issued by the Ministry of Science and



Technology of China. The protocols were approved by the Laboratory Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine (Tianjin, China; permit no. TCM-LAEC20160035).

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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