Original Article

Protective effects of Shen Yuan Dan on myocardial ischemia-reperfusion injury via the regulation of mitochondrial quality control

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Background: Myocardial cell death resulting from ischemia-reperfusion (I/R) injury has been a predominant contributor to morbidity and mortality globally. The mitochondria-centered mechanism plays an important role in the formation of I/R injury. This study intended to discuss the protective mechanism of Shen Yuan Dan (SYD) on cardiomyocytes hypoxia-reoxygenation (H/R) injury via the regulation of mitochondrial quality control (MQC). Additionally, this study clarified the mechanism by which SYD suppressed mitophagy activity through the suppression of the PTEN-induced kinase 1 (PINK1)/Parkin pathway.

Methods: To induce cellular injury, H9c2 cardiomyocytes were exposed to H/R stimulation. Following the pretreatment with SYD, cardiomyocytes were subjected to H/R stimulation. Mitochondrial membrane potential (MMP), adenosine triphosphate (ATP), superoxide dismutase (SOD), and methane dicarboxylic aldehyde (MDA) were detected to evaluate the degree of cardiomyocyte mitochondrial damage. Laser confocal microscopy was applied to observe the mitochondrial quality, and the messenger (mRNA) levels of mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), optic atrophy protein 1 (Opa1), dynamin-related protein 1 (Drp1), fission 1 (Fis1), and peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) in cardiomyocytes were assessed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Western blotting was employed for the estimation of light chain 3 (LC3)-I, LC3-II, PINK1, and Parkin in cardiomyocytes.

Results: It was discovered that SYD pretreatment elevated MMP in H/R injury cardiomyocytes, enhanced ATP content, activated SOD activity, and reduced MDA level. SYD treatment increased the mRNA levels of Mfn1, Mfn2, Opa1 and PGC-1α decreased the mRNA levels of Drp1 and Fis1, and reduced the protein levels of LC3, PINK1, and Parkin.

Conclusions: SYD plays a protective role in H/R injury to cardiomyocytes by regulating mitochondrial quality. Meanwhile, SYD may inhibit mitophagy activity through inhibiting the PINK1/Parkin pathway. This study provides insights into the underlying mechanism of SYD in alleviating myocardial I/R injury.

Keywords: Myocardial ischemia-reperfusion injury; Shen Yuan Dan (SYD); mitochondrial quality control; PINK1/Parkin pathway

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Introduction

Cardiovascular diseases are major contributors to death worldwide. According to published data, the incidence, mortality, and disability rates of acute myocardial infarction (MI) are still increasing (1). Cardiovascular diseases leverage a considerable burden upon society and individuals and thus have become a major public health problem (2). When myocardial ischemia occurs, restoring myocardial blood supply as soon as possible is the most important means of treatment. However, regardless of whether treatment consists of emergency drug thrombolytic therapy, interventional therapy, or cardiac bypass surgery, when the ischemic myocardial tissue is restored to blood perfusion, if the myocardial injury caused by ischemia is not completely recovered, even more serious damage may occur (3). This paradoxically kills (as opposed to rescuing) previously abundant ischemic myocytes in a process referred to as lethal myocardial ischemia-reperfusion injury (MIRI) (4-6). Fortunately, many studies have been performed to explore the drugs to improve MIRI. For example, polyphyllin I was evidenced to protect against MIRI via the suppression of inflammatory response and oxidative stress (7). The protective role of curcumin in MIRI was testified by Mokhtari-Zaer and co-workers (8).

Recent researches have proposed several molecular mechanisms that may account for the pathological changes in MIRI, including rapid reactive oxygen species (ROS) release, energy depletion, dysfunctional mitochondria, and programmed cell death trigger (9-12). Among these, mitochondria have been considered to be the critical trigger of MIRI. It has been reported that cardiomyocytes highly depend on mitochondria, which determine more than 90% of the energy supply of cardiomyocytes (13). In the course of ischemia-reperfusion (I/R), the large-scale damage of mitochondrial quality in cardiomyocytes can lead to a sharp increase in ROS level and the triggering of the mitochondrial apoptosis pathway, which are key factors in inducing MIRI (14,15). At the same time, other pathologic conditions, such as oxidative stress, immune responses, and endoplasmic reticulum stress can be triggered, integrated, or enhanced via dysfunctional mitochondria (16). The dysfunctional mitochondria can result in I/R-mediated endothelial cell damage; therefore, clearing out injured mitochondria and generating new and healthy mitochondria is pivotal for the amelioration of I/R damage (17,18).

Mitochondrial quality control (MQC) is an important mechanism to ensure mitochondrial homeostasis and keep cardiomyocytes functionally viable (19,20). MQC system is involved in a range of processes such as mitochondrial dynamics/mitophagy and mitochondria-mediated cell death (21-23), the function of which is mainly reflected in regulating the quantity and quality of mitochondria. Well-organized MQC promotes the regeneration of mitochondria, improves the biochemical processes and signaling transduction of mitochondria, and modulates the related cell death of mitochondria (24-26). Interestingly, strategies to promote mitochondrial recovery are modulated in part through the enhancement of MQC, thereby improving mitochondrial turnover as well as protecting against mitochondria-induced cell death (10,27-29). It was also reported that the regulation of mitochondrial function by COX6B1 could relieve cardiomyocytes hypoxia-reoxygenation (H/R) injury (30). Existing study considered mitochondrial dysfunction as a hallmark behind the development of cardiac malfunction induced by MIRI (31). Therefore, initiating MQC and improving dysfunctional mitochondria are pivotal approaches to preventing and treating MIRI.

Shen Yuan Dan (SYD) consists of eight crude Chinese medicinal agents named Salvia miltiorrhiza Bge, Astragalus membranaceus Bge, root of Pilose Asiabell, Radix Scrophulariae, Hirudo nipponica (Whitman), Lumbricus, Eupolyphaga sinensis (Walker), and Rhizoma Corydalis (7). As a traditional Chinese medicine (TCM) compound preparation, SYD has been shown to be effective in the clinical treatment of ischemic cardiomyopathy (32). A previous study has demonstrated that SYD can increase superoxide dismutase (SOD) activity and decrease methane dicarboxylic aldehyde (MDA) level in MIRI rats, while exerting myocardial protection through antioxidative stress (33);

Highlight box

Key findings

- Shen Yuan Dan (SYD) might be a promising drug for the treatment of myocardial ischemia/reperfusion injury (MIRI).

What is known and what is new?

- SYD plays a protective role in H/R-injured cardiomyocytes by regulating mitochondrial quality.
- SYD inhibits mitophagy activity through inhibiting the PINK1/Parkin pathway.

What is the implication, and what should change now?

- The application of SYD might be effective for the amelioration of MIRI.

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meanwhile, SYD was also shown to effectively maintain Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase at relatively high levels and ameliorate the energy metabolism of ischemic myocardial tissue (33). Nevertheless, the mechanism underlying the effect of SYD on the myocardial energy metabolism is obscure. In view of this, the present study investigated the efficacy of SYD pretreatment on dysfunctional mitochondria in myocardial cells injured by hypoxia-reoxygenation (H/R) and determined whether SYD could improve energy metabolism and protect against MIRI by regulating MQC. We present the following article in accordance with the MDAR reporting checklist (available at https://cdt.amegroups.com/article/view/10.21037/cdt-23-86/rc).

Methods

Reagents and instruments

TRNzol (#DP424) was acquired from Tiangen Biotech Co., Ltd. (Beijing, China); PrimeScript RT reagent Kit with gDNA Eraser (#RR047B), SYBR Premix Ex Taq II (Tli RNaseH Plus), and ROX plus (#RR82LR) were acquired from Takara Bio (Tokyo, Japan); an methyl thiazolyl tetrazolium (MTT) assay kit, bicinchoninic acid (BCA) protein quantification kit, and a NanoDrop 2000 spectrophotometer were acquired from Thermo Fisher Scientific (Waltham, MA, USA); a RIPA Total Protein Extraction Kit was acquired from Sigma-Aldrich (St. Louis, MI, USA); a mitochondria extract kit, enzyme-linked immunosorbent assay (ELISA) kits, and mitochondrial membrane potential JC-1 kit were acquired from Solarbio Science & Technology (Beijing, China); an adenosine triphosphate (ATP) kit, superoxide dismutase (SOD) kit, and methane dicarboxylic aldehyde (MDA) kit were acquired from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); a FACSCalibur II Flow Cytometer was acquired from BD Biosciences (Franklin Lakes, NJ, USA); an XDS-2B inverted fluorescence microscope was acquired from Chongqing Optical & Electrical Instrument Co., Ltd. (Chongqing, China); and an ABI 7500 real-time PCR instrument was acquired from Applied Biosystems (Thermo Fisher Scientific); the autophagy inhibitor 3-methyladenine (3MA) was purchased from Selleck Chemicals (Houston, TX, USA).

Drugs and concentration selection

The preparation of SYD lyophilized powder was completed by the Institute of Traditional Chinese Medicine, Chinese Academy of Traditional Chinese Medicine, under the following parameters: cold trap temperature, −45 ℃; and vacuum, −0.1. Furthermore, 4 mL of Dulbecco's modified Eagle medium (DMEM) was applied to dissolve 100 mg of SYD freeze-dried powder, which was followed by filtration and sterilization. Subsequently, the storage of the collected power was conducted at 4 ℃. The dilution of powder to favorable concentration was implemented prior to experimentation (34). MTT was employed to assess the nontoxic concentration of the drug, and the results indicated that 100 µg/mL was the optimal nontoxic concentration of SYD.

Cardiomyocyte resuscitation culture and H/R model establishment

H9c2 cardiomyocytes provided by Shanghai Beinuo Biotechnology Co., Ltd. (Shanghai, China) were incubated in DMEM with high glucose containing 10% fetal bovine serum (FBS) at 37 ℃ and 5% CO_2. To establish H/R model *in vitro*, H9c2 cells were subjected to hypoxic conditions equilibrated with 0.1% O_2, 5% CO_2 and 95% N_2 at 37 ℃ for 6 h. After that, H9c2 cells were reoxygenated under normoxic conditions supplemented with 95% air and 5% CO_2 for 12 h at 37 ℃. Following the implementation of H/R, further experiments were conducted.

Methyl thiazolyl tetrazolium (MTT) assay

The toxicity of SYD with varying concentrations (0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200 µg/mL) on H9c2 cells was detected using MTT assay kit according to the manufacturer's instructions. Briefly, the H9c2 cells were injected into 96-well plates at the density of 2×10^3/well and then incubated for 24 h. Subsequently, MTT solution was added into each well and the cells were incubated for another 4 h. After that, MTT solution was discarded and the cells were exposed to dimethyl sulfoxide. Finally, the absorbance at 570 nm was measured with a spectrophotometer.

Experimental grouping and cell processing

H9c2 cells were incubated in DMEM supplemented with 10% FBS at 37 ℃ and 5% CO_2. All cells were divided into four groups: (I) control group (without any treatment), (II) H/R model group [hypoxia (6 h, 0.1% O_2, 5% CO_2 and 95% N_2) and reoxygenation (12 h, 95% air and 5% CO_2)],

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Table 1 Sequences of primers

Genes	Primer	Primer sequence (5' to 3')
Mfn1	Mfn1-F	CTTCCCTTGTACATCGATTCCT
	Mfn1-R	GGGTTAGAAGGAGCAGTAGGAG
Mfn2	Mfn2-F	ATTTCTCCCTTGGATGGACTA
	Mfn2-R	AGAGAGGCCAGGCCAGTAA
Opa1	Opa1-F	AGTGATGAGATTACCACAGTCC
	Opa1-R	CCTCTCCGACAAAGGTTACAGT
Drp1	Drp1-F	TCTTCTAAAGTTCCAAGTGCTTTG
	Drp1-R	GAAGTTTTCAGCATTCCTCTCC
Fis1	Fis1-F	AGGAAATTTCAGTCTGAGCAGG
	Fis1-R	GCCAGGTAGAAGACATAATCCC
PGC-1a	PGC-1a-F	CCAAACCAACAACTTTATCTCTTC
	PGC-1a-R	CTGCAGTTCCAGAGAGTTCCAC

Mfn1, mitofusin 1; Mfn2, mitofusin 2; Opa1, optic atrophy protein 1; Drp1, dynamin-related protein 1; Fis1, fission 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α.

(III) H/R model + 3MA group: (H9c2 cells were pre-treated with 2 mM 3MA for 6 h and then exposed to H/R induction, (IV) H/R model + SYD group (H9c2 cells were pre-treated with 100 µg /mL SYD for 6 h and then exposed to H/R induction).

Evaluation of change in mitochondrial membrane potential of cardiomyocytes with JC-1 staining

After the rinse with PBS, 100 µL sample cells were collected for staining. Subsequently, 1 µL of JC-1 reagent was placed into each tube and then reacted for 30 min at room temperature. After suspension, 400 µL of PBS was added, and flow cytometry was employed to count the number of JC-1 cells, with the JC1-1 fluorescence channel being FL1 and JC1-2 fluorescence channel being FL2. Data were analyzed by CellQuest software (BD Biosciences): the membrane potential was considered to be the numerical ratio of JC1-1 + JC1-2+ to JC1-1 + JC1-2−.

ATP measurement

The concentration of ATP in H9c2 cells was detected using ATP assay kits according to manufacturer's instructions. In brief, H9c2 cells were mixed with cell lysis buffer for 10 min and then centrifugated at 12,000 g at 4 ℃ for 5 min. After the incubation with 100 µL kit solution at room temperature for 5 min, the ATP level in cell supernatant was detected.

The measurement of SOD activity and MDA content

All operations were implemented according to the manufacturer's instructions. Following ultrasonic treatment, cell lysate was centrifugated and subsequently, the supernatant was collected to measure the levels of SOD and MDA with SOD assay kits and MDA assay kits.

Detection mitochondrial quality and dynamic changes via laser confocal microscopy

Following treatment, cells were rinsed with PBS or normal saline twice (200 µL each time) and mixed with 1:100 diluted Mito-Tracker Green dye (MTH; 150 µL/dish) away from light for 1 h. After the subjection to 4',6-diamidino-2-phenylindole (DAPI) working solution (150 µL/dish), the cells were cultivated away from light for 15 min. Finally, confocal microscopy-green-blue fluorescence was applied to capture the fluorescence of PBS-rinsed cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Following the extraction with TRNzol, the RNA was synthesized into complement DNA (cDNA) by QuantiTect Reverse Transcription kit (Qiagen GmbH, Hilden, Germany) according to established protocols. PCR reaction was implemented using SYBR Green PCR Master Mix (Takara Bio) on a 7500 Fast Real-time PCR system. $2^{-\Delta\Delta CT}$ was employed for the determination of relative gene expression (35). The primer sequences are listed in *Table 1*.

Western blot analysis

The quantification of total proteins, which were isolated with RIPA lysis buffer, was conducted by means of the BCA method. Following separation with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 50 µg of protein lysates was transferred to a polyvinylidene fluoride (PVDF) membrane. The overnight cultivation of membranes, which were blocked by 5% skimmed milk, was operated at 4 ℃ with primary antibodies targeting LC3-I/

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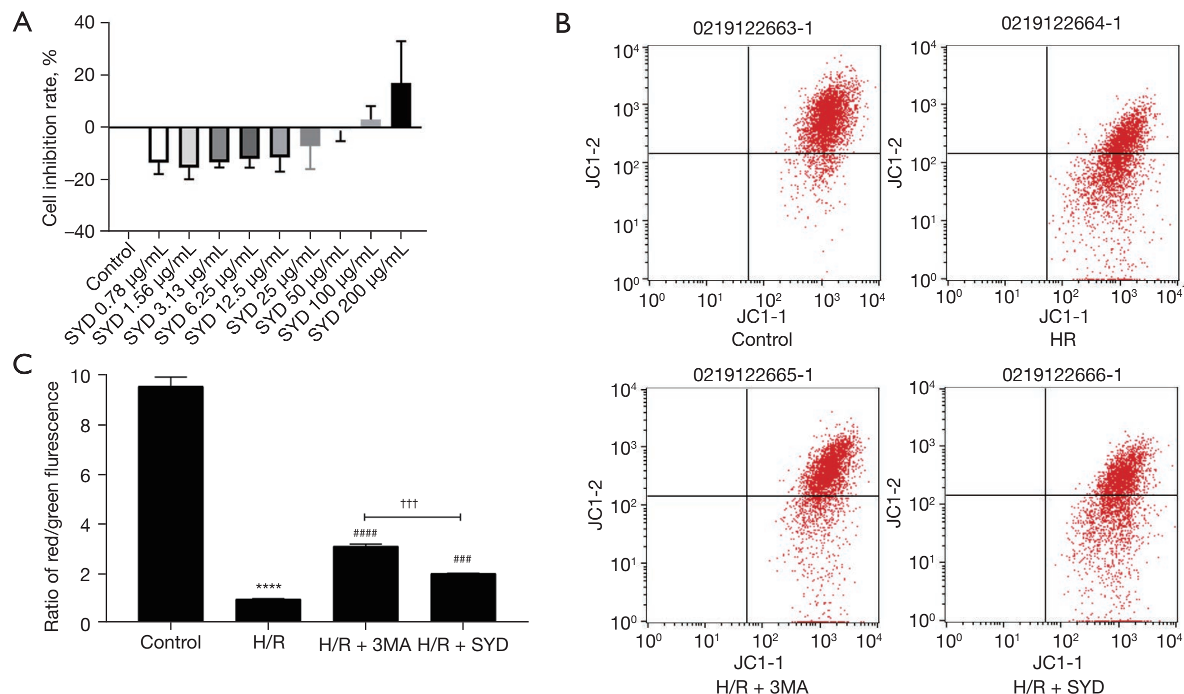

Figure 1 Effects of SYD on the MMP of cardiomyocytes (n=3). (A) The toxicity of SYD on cardiomyocytes. (B,C) The effects of SYD on the MMP of cardiomyocytes. ****, P<0.0001 *vs.* control group; ####, P<0.0001, ###, P<0.001 *vs.* H/R group; †††, P<0.001 *vs.* H/R + 3MA group. SYD, Shen Yuan Dan; MMP, mitochondrial membrane potential; H/R, hypoxia/reoxygenation; 3MA, 3-methyladenine.

LC3-II (ab128025; 1:1,000; Abcam), PINK1 (ab186303; 1:1,000; Abcam), Parkin (ab77924; 1:2,000; Abcam) or GAPDH (ab8245; 1:1,000; Abcam) and followed by exposure to rabbit anti-mouse IgG H&L (ab6728; 1:2,000; Abcam) or goat anti-rabbit IgG H&L antibodies (ab6721; 1:2,000; Abcam) at room temperature for 1 h. Finally, enhanced chemiluminescence (ECL) was employed for the visualization of protein blots, and the images were captured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

SPSS software version 27.0 (IBM Corp., Armonk, NY, USA) was applied for the analysis of data derived from at least 3 independent experiments and expressed as mean ± standard error of mean (SEM). To determine the differences between 2 groups, Student *t*-test was applied; to determine the differences across multiple groups, 1-way analysis of variance (ANOVA) Tukey's *post-hoc* test was applied. The experimental data were considered to be statistically significant at a P value less than 0.05.

Results

Determination of SYD concentration and the effects of SYD on mitochondrial membrane potential of cardiomyocytes injured by H/R

As shown in *Figure 1A*, 100 μg/mL was the optimal nontoxic concentration of SYD. In view of this, we chose 100 μg/mL SYD for following experiments. In relation with the Control group, the mitochondrial membrane potential (MMP) in H/R group was markedly reduced, indicating that H/R caused significant damage to the mitochondria of cardiomyocytes. SYD pretreatment evidently alleviated the MMP of cardiomyocytes after H/R stimulation, suggesting that SYD administration could alleviate cardiomyocyte damage induced by H/R stimulation while the effect was not as good as that in the H/R + 3MA group when compared with the H/R + SYD group (*Figure 1B,1C*).

Effects of SYD on ATP content in H/R-injured cardiomyocytes

As shown in *Figure 2A*, ATP content in the H/R group was

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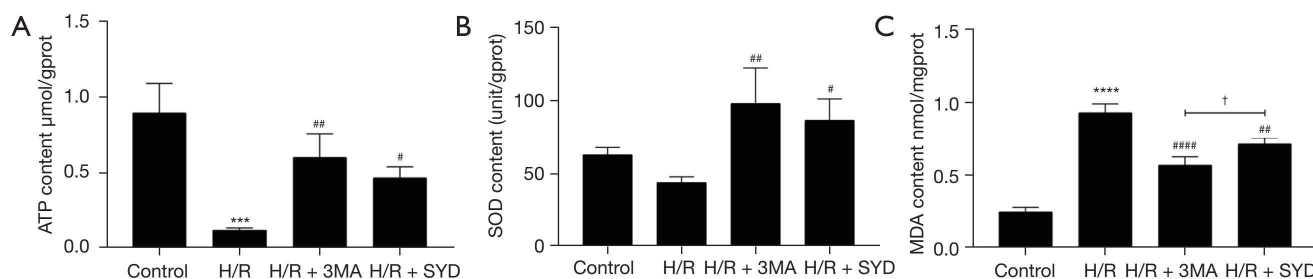

Figure 2 Effects of SYD on ATP, SOD and MDA of H/R cardiomyocytes (n=3). (A) The effects of SYD on the content of ATP. (B) The effects of SYD on the content of SOD. (C) The effects of SYD on the content of MDA. ****, P<0.0001, ***, P<0.001 *vs.* control group; $^{####}$, P<0.0001, $^{##}$, P<0.01, $^{#}$, P<0.05 *vs.* H/R group; †, P<0.05 *vs.* H/R + 3MA group. ATP, adenosine triphosphate; H/R, hypoxia/reoxygenation; 3MA, 3-methyladenine; SYD, Shen Yuan Dan; SOD, superoxide dismutase; MDA, methane dicarboxylic aldehyde.

markedly reduced relative to the Control group. Compared with that in the H/R group, the content of ATP in the H/R-induced cardiomyocytes was dramatically increased in the H/R + SYD group and H/R + 3MA group. Nevertheless, SYD pretreatment group caused lower expression level of ATP compared to the H/R + 3MA group.

Effects of SYD on SOD and MDA in H/R-injured cardiomyocytes

As shown in *Figure 2B*, SOD activity was reduced by H/R stimulation, indicating that H/R could impair the antioxidant capacity of cardiomyocytes. Compared with the H/R group, both the H/R + SYD and H/R + 3MA groups showed improved SOD activity in cardiomyocytes after H/R. The high activity of SOD in the H/R + SYD group suggested that SYD could improve the antioxidant effect of H/R damage to cardiomyocytes.

Compared with the Control group, MDA content was conspicuously elevated in H/R group, indicating that H/R could significantly aggravate oxidative damage of cardiomyocytes. However, in comparison with that in the H/R group, MDA level in both the H/R + SYD and H/R + 3MA groups was diminished, indicating that SYD and 3MA could alleviate H/R-induced oxidative damage of cardiomyocytes, with the protective effect in the H/R + 3MA group being greater (*Figure 2C*).

Effects of SYD on the mitochondrial quality and mitochondrial dynamics in H/R-injured cardiomyocytes

In order to investigate the impact of SYD on mitochondrial quality and the dynamic changes in cardiomyocytes resulting from H/R stimulation, a mitochondria-specific fluorescent mito-tracker green (MTG) probe was applied for the co-staining of cardiomyocytes. As seen in *Figure 3A,3B*, the degree of mitochondrial networking was remarkably diminished in H/R group compared with the Control group, suggesting that mitochondrial division and fusion might be affected, resulting in a decreased number of mitochondria. This further indicated that H/R might lead to mitochondrial dysfunction in the cardiomyocytes and precipitate mitochondrial biosynthesis. Nevertheless, SYD strengthened the degree of mitochondrial networking in H/R-injured cardiomyocytes, and the distribution and number of mitochondria were greatly increased, suggesting that SYD regulated the number and network morphology of mitochondria by regulating the dynamic changes of mitochondria. In comparison with that in the H/R + 3MA group, the degree of mitochondrial improvement was slightly lower in H/R + SYD group.

Effects of SYD on mRNA expression levels of key regulatory factors of mitochondrial fission, fusion, and mitochondrial biogenesis in H/R-injured cardiomyocytes

The role of SYD in mitochondrial fission and fusion in H/R-injured cardiomyocytes was examined. Results obtained from RT-qPCR demonstrated that the H/R induction reduced the mRNA levels of mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), optic atrophy protein 1 (Opa1), and increased the mRNA levels of dynamin-related protein 1 (Drp1), and fission 1 (Fis1) relative with the Control group (*Figure 4A,4B*). Following the treatment with SYD, the mRNA levels of Mfn1, Mfn2 and Opa1 were increased while the expressions of Drp1 and Fis1 were reduced by contrast with those in H/R group.

Additionally, results obtained from RT-qPCR also

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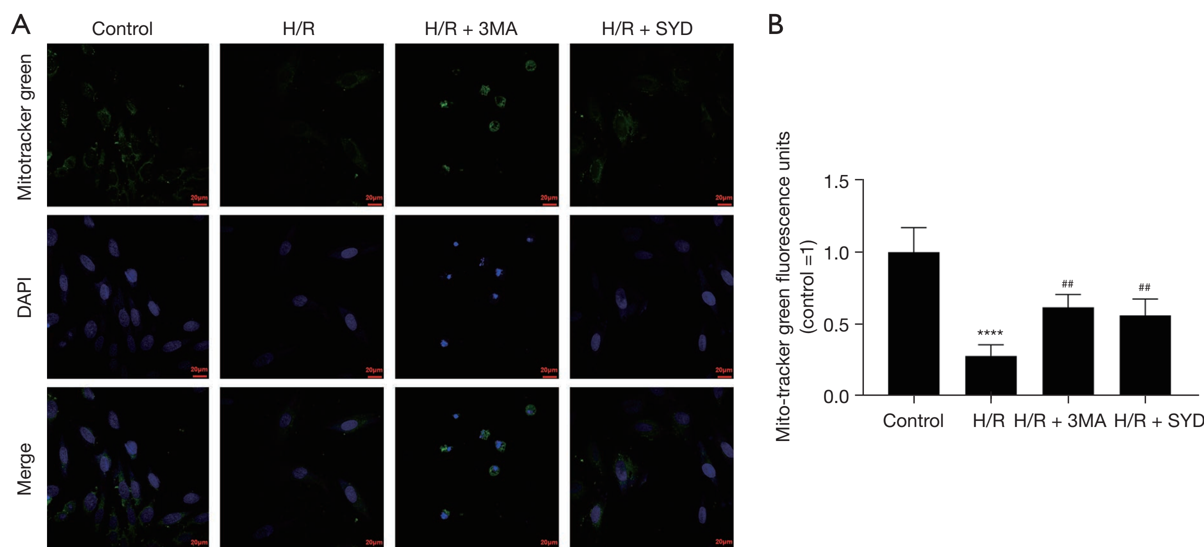

Figure 3 Effects of SYD on mitochondrial quality and mitochondrial dynamics in H/R-injured cardiomyocytes (n=3). (A) Representative pictures of mitochondrial probed with MTG through laser-scanning confocal microscopy. DAPI was used for staining (magnification, ×600). (B) Impacts of SYD on mitochondrial dynamics in cardiomyocytes injured by hypoxia/reoxygenation. ****, P<0.0001 *vs.* control group; ##, P<0.01 *vs.* H/R group. H/R, hypoxia/reoxygenation; 3MA, 3-methyladenine; SYD, Shen Yuan Dan; DAPI, 4',6-diamidino-2-phenylindole; MTG, mito-tracker green.

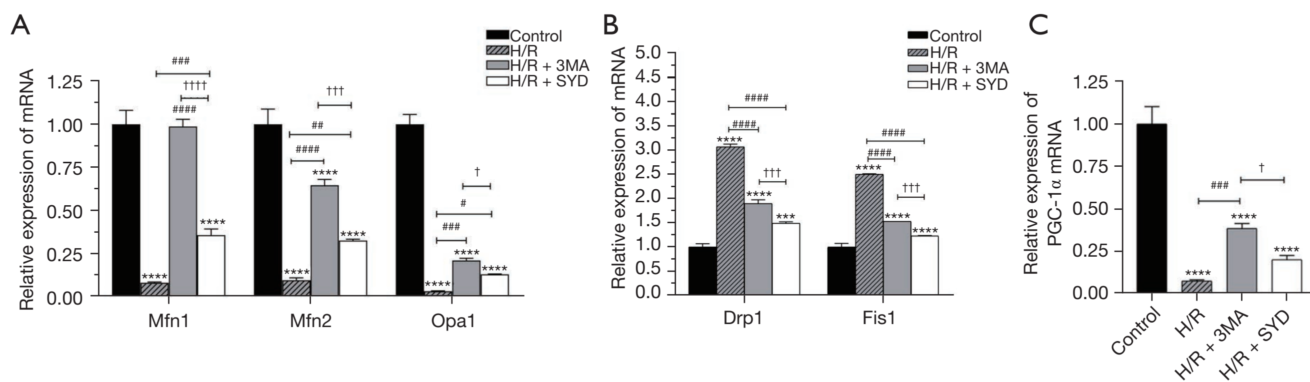

Figure 4 The mRNA expression of Mfn1, Mfn2, Opa1, Drp1, Fis1, and PGC-1α in cardiomyocytes by qRT-PCR (n=3). (A) The effects of SYD on the mRNA expression of Mfn1, Mfn2 and Opa1. (B) The effects of SYD on the mRNA expression of Drp1 and Fis1. (C) The effects of SYD on the expression of PGC-1α. ***, P<0.001, ****, P<0.0001 *vs.* control group; ####, P<0.0001, ###, P<0.001, ##, P<0.01, #, P<0.05 *vs.* H/R group; ††††, P<0.0001, †††, P<0.001, †, P<0.05 *vs.* H/R + 3MA group. Mfn1, mitofusin 1; Mfn2, mitofusin 2; Opa1, optic atrophy protein 1; Drp1, dynamin-related protein 1; Fis1, fission 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; H/R, hypoxia/reoxygenation; 3MA, 3-methyladenine; SYD, Shen Yuan Dan.

revealed that the level of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) mRNA in H/R + SYD group was dramatically increased compared with the H/R group, suggesting that SYD promoted the transcription of PGC-1α in H/R-induced cardiomyocytes (*Figure 4C*).

Effects of SYD on the expression levels of LC3-II, PINK1, and Parkin in H/R-injured cardiomyocytes

As seen in *Figure 5*, by contrast with the Control group, LC3-II content was markedly increased in H/R group, implying that mitophagy was induced after H/R. Relative with the H/R group, the content of LC3-II in H/R + 3MA

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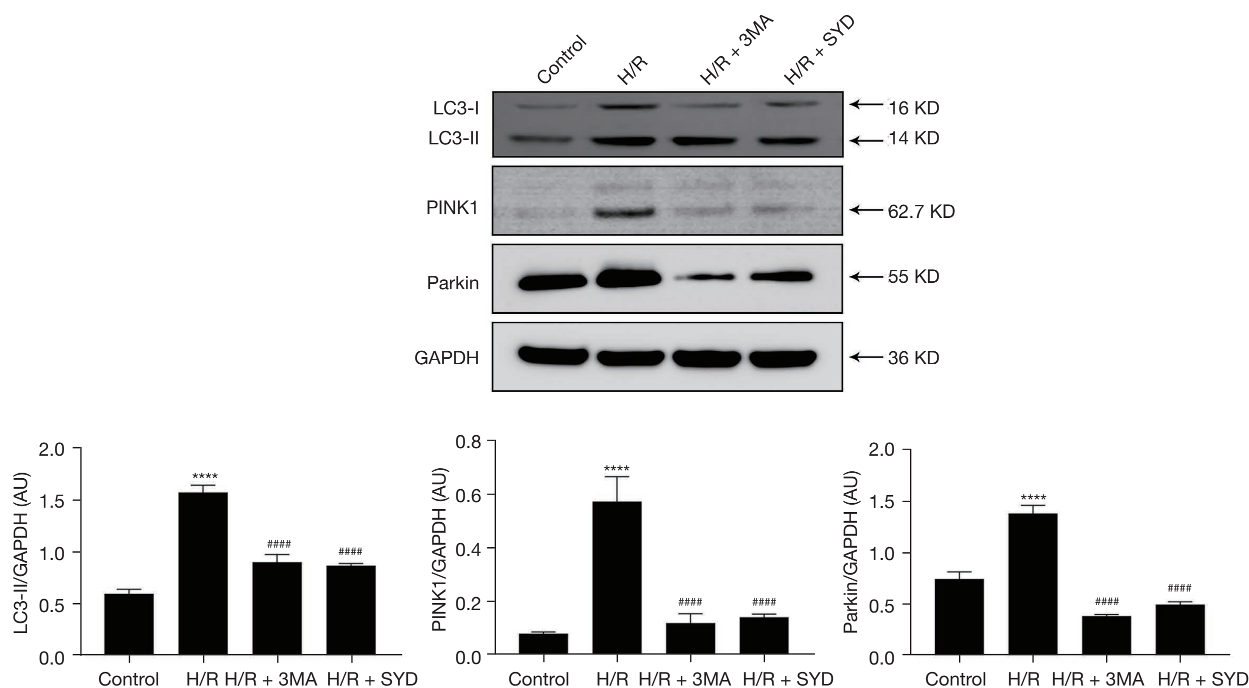

Figure 5 The expression of Parkin, PINK1, and LC3-II in cardiomyocytes according to Western blot (n=3). The effects of SYD on the protein expression of Parkin, PINK1, and LC3-II. ****, P<0.0001 *vs.* control group; ####, P<0.0001 *vs.* H/R group. H/R, hypoxia/reoxygenation; 3MA, 3-methyladenine; SYD, Shen Yuan Dan; AU, arbitrary unit.

or H/R + SYD group was markedly decreased, suggesting that 3MA or SYD could suppress the mitophagy activity of H/R-damaged cardiomyocytes.

To clarify the role of PINK1/Parkin, a key regulatory pathway of mitophagy, in H/R-injured cardiomyocytes, PINK1/Parkin expression in cardiomyocytes was evaluated. As seen in *Figure 5*, the levels of PINK1 and Parkin proteins in cardiomyocytes were increased considerably after H/R stimulation, indicating that H/R could induce elevated mitophagy activity. Nevertheless, the levels of PINK1 and Parkin in H/R + SYD group were markedly reduced in comparison with the H/R group, indicating that SYD could inhibit the overexpression of PINK1 and Parkin in H/R-injured cardiomyocytes, thereby suppressing the occurrence of mitophagy.

Discussion

In our study, it was discovered that SYD pretreatment increased levels of MMP; raised cellular ATP content; activated mitochondrial antioxidant SOD activity; reduced MDA level; elevated the mRNA levels of Mfn1, Mfn2, Opa1 and PGC-1α; decreased the mRNA levels of Drp1 and Fis1; and reduced the protein levels of LC3 in H/R-injured cardiomyocytes. Therefore, our results demonstrated that SYD can reduce cardiomyocyte H/R injury by regulating MQC. Additionally, this study confirmed that SYD could downregulate the expressions of PINK1 and Parkin proteins in H/R-injured cardiomyocytes, thus confirming that SYD inhibited mitophagy activity by downregulating the PINK1/Parkin pathway, thereby exerting a protective effect against MIRI.

As a clinical condition that has a close relation with MI, MIRI contributes to ventricular arrhythmias, contractile dysfunction, and mortality (36). MI results from the occlusion or blockage of one or more coronary vessels supplying a region of the heart (37). As the coronary blood flow recanalizes or the collateral branches open, reperfusion of the ischemic myocardium and the immediate reintroduction of oxygen and nutrients become vital for the rescue of the reversibly injured myocardium and the limitation of infarct progression. Nevertheless, for subpopulations of reversibly injured cardiomyocytes, recovery of blood flow facilitates the death of necrotic and apoptotic cells (6,38,39). Myocardial cell death resulting from I/R is a prominent contributor to morbidity and

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mortality worldwide. Despite the fact that great efforts have been made, the mechanisms of lethal I/R injury, which are complicated and multifactorial, still remain obscure (40-44). Research results show that the mitochondria-centered mechanism plays an important role in the formation of I/R injury (45,46). It was also evidenced that mitochondrial dysfunction has currently been considered as a promising therapeutic target (47).

As an adaptive response, MQC adjusts the morphology and function of mitochondria in the course of MIRI (48). Following exposure to stress, mitochondrial fission is activated, and with the cooperation of mitophagy, part of the damaged mitochondria can be removed from the mitochondrial network, with small mitochondrial fragments being integrated into healthy long mitochondria. In this way, the resistance of the whole mitochondria is enhanced. Therefore, MQC is predominantly modulated by mitochondrial dynamics and mitophagy (49), while mitochondrial biogenesis is accompanied by mitophagy; these different mechanisms maintain mitochondrial quality in cells. They coordinate with each other to retain healthy mitochondria to the maximum extent while removing irreversible mitochondrial damage, and finally complete the dual regulation of mitochondrial quantity and quality. Nevertheless, it has been found that while autophagy confers protective effects to cells in the ischemic stage, in the reperfusion stage, excessive autophagy will aggravate cell damage, which may result in cell death in severe cases (50,51). The occurrence of MIRI is often accompanied by the pathological overexpression of mitochondrial fission and mitophagy, which can exacerbate the damage to heart muscle cells and even lead to cell death. Therefore, via the modulation of MQC, the trigger of mitophagy that promotes specificity, targeting, and balance may be the key factor for cardiac protection (52-54). In this study, MQC was used as the entry point to investigate the protective impacts of SYD on H/R-injured cardiomyocytes, which is expected to clarify the mechanism of SYD's anti-MIRI action.

In TCM, the typical feature of MIRI is qi deficiency and clinical blood stasis (55). SYD was prepared in the Beijing Hospital of Traditional Chinese Medicine Capital Medical University and was registered in the Beijing Medical Products Administration (approval document No. 99 Jingwei Zi [056] F-203; National Invention Patent No. ZL201410363400.9). SYD has been broadly applied in clinical for the treatment of coronary heart disease by invigorating qi and removing blood stasis. A previous case study reported that SYD exerted myocardial protection through antioxidative stress and clearly relieved energy metabolism of the ischemic myocardial tissue (33). Liu and co-workers have evidenced that SYD could increase SOD expression in MIRI rats (7). In one study, SYD was shown to promote autophagy via the inactivation of PI3K/Akt/mTORC1 signaling pathway, thus attenuating atherosclerosis and foam cell formation (34). Additionally, SYD reduced myocardial cell injury and apoptosis in rats with acute myocardial infarction (AMI) via modulating mitochondrial apoptosis signaling pathway (56). Our study showed that SYD pretreatment could increase MMP in H/R-injured cardiomyocytes, elevate ATP content, trigger SOD activity, and reduce the MDA level in H/R-injured cardiomyocytes. This suggests that SYD can reduce oxidative stress injury and ameliorate mitochondrial function, thereby imparting a protective impact on H/R-injured cardiomyocytes. In addition, we investigated the molecular mechanism of SYD's regulation of mitochondrial quality and anti-MIRI by observing the regulation of SYD pretreatment on mitochondrial division and fusion, mitochondrial biogenesis, and mitophagy in H/R-injured cardiomyocytes, the results from which may lay pharmacological basis for SYD's prevention and treatment of MIRI. The *Figure 6* is about the description of MQC system.

The mitochondrial fusion process is primarily regulated by 3 dynamin-related guanosine triphosphatases (GTPases) (57). Mitochondrial Fis1 protein and Drp1 are critical regulators in mitochondrial fission (58). PGC-1α is the most pivotal regulatory factor in the PPAR γ coactivator (PGC) (59,60). This study showed that SYD could regulate the mRNA levels of mitochondrial fission and fusion joint regulatory factors in H/R-injured cardiomyocytes, inhibit mitochondrial fission and promote fusion, imped the fission of the damaged mitochondria and mitochondrial network, repress substance exchange between mitochondria, and regulated mitochondrial quality. SYD also elevated PGC-1α expression in H/R-injured cardiomyocytes, thereby promoting mitochondrial biogenesis and modulating mitochondrial quality.

The classic understanding for the mechanism of mitophagy includes the mitochondrial serine/threonine PINK1/Parkin (61-63). A study has shown that the PINK1/Parkin pathway may induce excessive mitophagy in myocardial I/R, thereby promoting cell death (64), which plays an important role in maintaining the normal function of cardiomyocytes and myocardial protection in MIRI by

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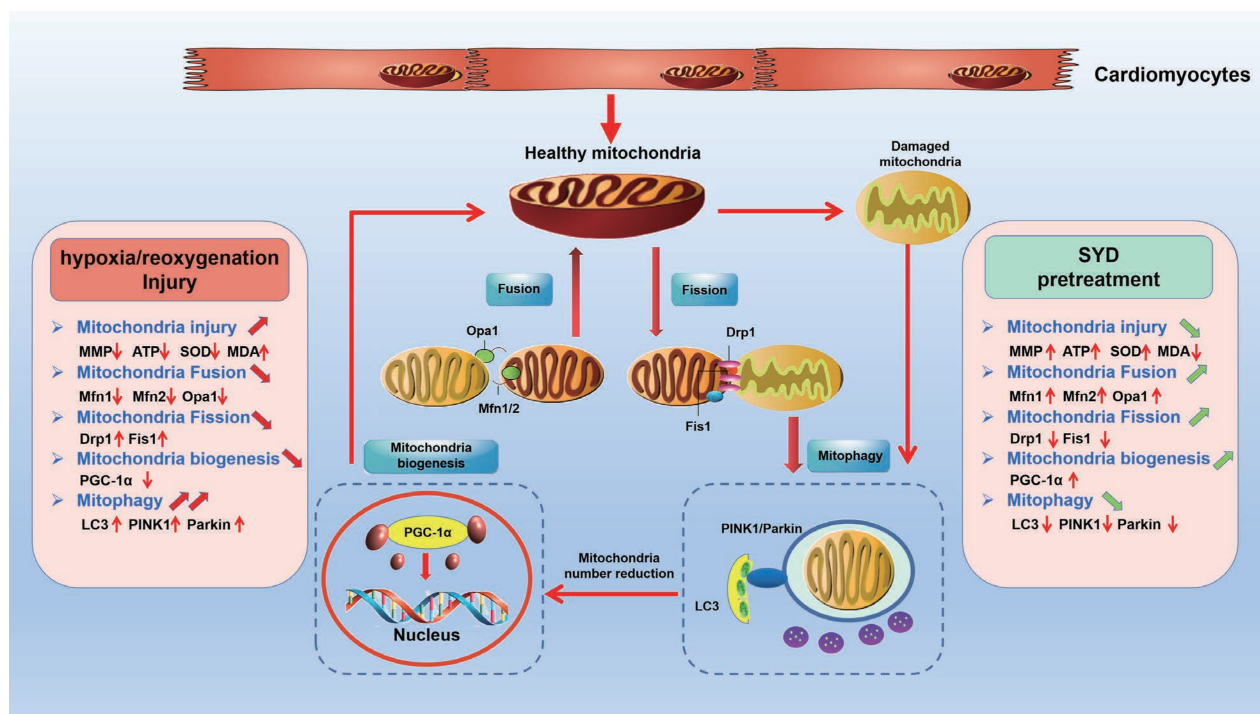

Figure 6 Mitochondrial quality control system. Under H/R injury: the contents of MMP, ATP and SOD activity in cardiomyocytes decreased, while the content of MDA increased, mitochondrial damage was aggravated. The mRNA levels of Mfn1, Mfn2 and Opa1 were reduced while the mRNA expressions of Drp1 and Fis1 were up-regulated. The activation of Pink1/Parkin pathway induced excessive mitophagy, which aggravated mitochondrial damage. After SYD pretreatment: the contents of MMP, ATP and SOD activity in H/R injury cardiomyocytes were increased, while the content of MDA was decreased, and mitochondrial damage was alleviated. The mRNA levels of Mfn1, Mfn2 and Opa1 were increased, the mRNA expressions of Drp1 and Fis1 were reduced and mitochondrial biosynthesis was increased. The excessive mitophagy was inhibited through inhibiting the Pink1/Parkin pathway, and mitochondrial damage was alleviated. H/R, hypoxia/reoxygenation; MMP, mitochondrial membrane potential; ATP, adenosine triphosphate; SOD, superoxide dismutase; MDA, methane dicarboxylic aldehyde; SYD, Shen Yuan Dan; Mfn1, mitofusin 1; Mfn2, mitofusin 2; Opa1, optic atrophy protein 1; Drp1, dynamin-related protein 1; Fis1, fission 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; LC3, light chain 3.

mediating mitophagy activity (3). A growing number of studies have revealed that the regulation of PINK1/Parkin pathway is critical for the alleviation of MIRI. For example, Yang and co-workers have found that the suppression of PINK1/Parkin pathway by VDAC1 downregulation could attenuate MIRI (65). Additionally, the regulation of PINK1/Parkin pathway by the inhibition of myosin IIA-actin interaction-induced actomyosin contractility could impede MIRI (66). In this study, we found that SYD pretreatment can reduce the expression of LC-II in H/R-injured cardiomyocytes, reduce mitophagy activity, and ameliorate the injury to cardiomyocytes. Moreover, SYD pretreatment downregulated the expressions of PINK1 and Parkin proteins in H/R-injured cardiomyocytes. Thus, it is speculated that SYD may suppress excessive mitophagy activity by regulating PINK1/Parkin pathway to alleviate the myocardial injury effect of MIRI.

Conclusions

The results of our study demonstrate that SYD pretreatment significantly increases mitochondrial antioxidant capacity, alleviates the mitochondrial damage caused by H/R, regulates mitochondrial fission and fusion, upregulates mitochondrial biogenesis, and downregulates excessive mitophagy; therefore, it exerts a protective effect against H/R injury to cardiomyocytes by regulating mitochondrial quality. Meanwhile, SYD may inhibit mitophagy activity by inhibiting the PINK1/Parkin pathway, thereby protecting cardiomyocytes from I/R

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injury. This study offers novel insights into the underlying mechanism of the SYD alleviation of myocardial I/R injury and may provide a theoretical basis for the clinical strategies involving SYD treatment in MIRI.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://cdt.amegroups.com/article/view/10.21037/cdt-23-86/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://cdt.amegroups.com/article/view/10.21037/cdt-23-86/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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