Chinese Journal of Integrative Medicine
Available online at link.springer.com/journal/11655
Journal homepage: www.cjim.cn/zxyjhen/zxyjhen/ch/index.aspx
E-mail: cjim_en@cjim.cn

Original Article

Shexiang Tongxin Dropping Pill (麝香通心滴丸) Protects against $Na_2S_2O_4$-Induced Hypoxia-Reoxygenation Injury in H9c2 Cells*

LIN Shan[1,2,3], LIN Jiu-mao[1,2], ZHANG Ling[1,2], CHEN Da-xin[1,2,3], XIAO Fei[1,2], CHEN Hong-wei[1,2,3], CHEN You-qin[4], ZHU Yu-ling[5], CHU Jian-feng[1,2,3], and PENG Jun[1,2,3]

ABSTRACT **Objectives**: To investigate the protective effects of Shexiang Tongxin Dropping Pill (麝香通心滴丸, STP) on $Na_2S_2O_4$-induced hypoxia-reoxygenation injury in cardiomyoblast H9c2 cells. **Methods**: The cell viability and levels of mRNA and protein expression in H9c2 cells were determined following $Na_2S_2O_4$-induced hypoxia using Hoechst staining, annexin V/propidium iodide (PI) flow cytometry, real-time polymerase chain reaction and Western blot analysis. **Results**: STP pretreatment significantly increased the viability and inhibited aberrant morphological changes in H9c2 cardiomyoblast cells induced by $Na_2S_2O_4$ treatment (P<0.05). In addition, STP pretreatment attenuated $Na_2S_2O_4$-induced hypoxic damage, down-regulated the expression of pro-apoptotic Bax, and up-regulated the expression of anti-apoptotic Bcl-2 in H9c2 cells (P<0.05). **Conclusions**: STP was strongly cardioprotective in hypoxia-reoxygenation injury by preventing hypoxic damage and inhibiting cellular apoptosis. These results further support the use of STP as an effective drug for the treatment of ischemic heart disease.

KEYWORDS myocardial ischemia, apoptosis, Shexiang Tongxin Dropping Pill, Chinese medicine, $Na_2S_2O_4$-induced hypoxia-reoxygenation injury

Myocardial ischemia occurs when coronary blood flow to the heart is impaired due to atherosclerosis, thrombosis or vascular spasm. The imbalance between oxygen demand and supply as a result of myocardial ischemia can result in damage and necrosis of cardiac tissues. Cardiovascular disease, in particular ischemic heart disease (IHD), is one of the leading causes of morbidity and mortality worldwide.[1,2] There is mounting evidence which suggests that cardiomyocyte apoptosis occurs during the first few hours of myocardial ischemia, which can lead to widespread myocardial cell damage, cardiac dysfunction and ultimately heart failure. Current treatments for IHD often involve the use of antianginal drugs to relieve or prevent acute ischemic episodes via increasing myocardial oxygen supply and/or decreasing myocardial oxygen demand.[3] Cardiomyocyte hypoxia is therefore one of the most important aspects of ischemia in the treatment of IHD. However, despite recent technological advances and research into myocardial ischemia, there have been little or no fundamental breakthroughs in drug treatment.[4] Thus, the ability to protect cardiomyocytes from hypoxic injury is a crucial therapeutic strategy for the treatment of IHD.

Shexiang Tongxin Dropping Pill (麝香通心滴丸, STP) is a prescription medicine approved by the Chinese Food and Drug Administation (No. Z20080018), which consists of *Moschus*, *Radix rhizoma ginseng*, *Calculus bovis*, *Fel ursi*, *Venenum bufonis*, *Borneolum syntheticum* and *Salvia miltiorrhiza*. We had previously analyzed and identified the major constituents of STP using high performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (HPLC-Q-TOF-MS/MS), which included triterpene saponins, bufadienolides, bile acids and phenylally compounds, and quantified the identified

*Supported by the Foundation of Fujian University of Traditional Chinese Medicine (No. X2013026), the Developmental Fund of Chen Ke-ji Integrative Medicine (No. CKJ2013016) and the Education Department of Fujian Province (No. JA14163)

1. Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou (350122), China; 2. Fujian Key Laboratory of Integrative Medicine on Geriatric, Fujian University of Traditional Chinese Medicine, Fuzhou (350122), China; 3. Rainbow Babies Children's Hospital, Case Western Reserve University School of Medicine, Cleveland, OH (44106), USA; 4. Inner Mongolia Conba Pharmaceutical Co., Ltd., Shanghai (201318), China; 5. CHEN Ke-ji Academic Thought Heritage Studio, Fuzhou (350122), China
Correspondence to: Prof. PENG Jun, Tel: 86-591-22861303, E-mail: pjunlab@hotmail.com

DOI: https://doi.org/10.1007/s11655-018-2976-4

Chin J Integr Med 2019 Jun;25(6):439-445

constituents using UPLC-QqQ-MS/MS.[5] STP has been widely used for the clinical treatment of cardiovascular diseases, particularly coronary heart disease in China and Southeast Asia.[6-9] STP has been shown to protect endothelial cells from atherosclerotic lesions by decreasing the levels of endothelin-1 (ET-1), C-reactive protein (CRP) and tumor necrosis factor-α (TNF-α), while increasing nitrogen oxide content in the blood.[10] STP has also been shown to attenuate atherosclerotic lesions in ApoE deficient mice, suggesting its multi-target effects during atherosclerosis, including lipid regulation and protecting against fibrosis, inflammation and oxidative stress.[11] However, the precise mechanisms of STP in protecting cardiomyocytes against hypoxic injury remain largely unknown.

In our present study, we examined the cardioprotective effects of STP on $Na_2S_2O_4$-induced hypoxia in cardiomyoblast H9c2 cells, and elucidated its underlying mechanisms of action in protecting against ischemic-hypoxic injury. This study provided a theoretical basis for the enhanced use of STP in the treatment and prevention of IHD.

METHODS

Materials

STP was provided by Inner Mongolia Conba Pharmaceutical Co., Ltd. (lot No. 20140522337, Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-ethylene diaminete traacetic acid (EDTA) and Trizol reagent were purchased from Invitrogen (Carlsbad, USA). SuperScript Ⅱ reverse transcriptase was obtained from Promega (Madison, USA). Bcl-2 and Bax antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, USA). Unless indicated otherwise, all chemicals were obtained from Sigma Chemicals (St. Louis, USA). Bax and Bcl-2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Annexin V-FITC apoptosis detection kit and Hoechst 33258 were obtained from KeyGen Biotech. Co. Ltd. (Nanjing, China). All primers used for real-time polymerase chain reaction (RT-PCR) were synthesized by Sangon Biotechnology (Shanghai, China).

STP Extraction

The STP powder was first dissolved in double-distilled water with 0.5% dimethyl sulphoxide (DMSO), and the resulting solution was centrifuged, then sterilized by filtering through a 0.22 μm syringe filter and stored at –20 ℃ until further use.

Cell Culture and Treatments

H9c2 cells (ATCC, USA) were cultured in high-glucose DMEM (4.5 g/L glucose) supplemented with 10% (v/v) fetal bovine serum, penicillin G (100 U/mL), and streptomycin (100 mg/mL in a 37 ℃ humidified incubator with 5% CO_2. Cells were seeded in 96-well plates at a density of 2×10^4 cells/well and treated with STP for 24 h to first determine the optimal safe concentrations for use in subsequent bioassays.

The effect of STP on $Na_2S_2O_4$-induced cell injury model was explored based on four experimental groups: control group, model group, model + low-dose STP group (25 μg/mL), model + high-dose STP group (50 μg/mL). H9c2 cells were seeded in 96-well plates at a density of 1×10^5 cells/well or in 6-well plates at a density of 5×10^5 cells/well, and pretreated with STP (25 or 50 μg/mL) at 37 ℃ for 12 h. The cell culture medium was then replaced with 1 mmol/L $Na_2S_2O_4$ for 4 h to induce cell hypoxia. Following induction of hypoxia, cells were reoxygenated for 6 h.[12] H9c2 cells cultured in 96-well plates were subsequently used for microculture tetrazolium (MTT) and Hoechst staining assays, and those cultured in 6-well plates underwent flow cytometry analysis and determination of mRNA and protein expression.

Cytotoxicity Assay

In order to test the cytotoxicity of STP and $Na_2S_2O_4$-induced hypoxia on H9c2 cardiomyoblast cells, the survival rate of H9c2 cells were measured using MTT assays (Solarbio, USA). Following treatment of STP or $Na_2S_2O_4$, MTT solution (2.5 mg/mL) was added to each well and incubated for 4 h at 37 ℃. The formazan precipitate was resuspended in DMSO and the absorbance (OD) was measured at 490 nm using a microplate reader. Cell viability (%) was calculated based on the absorbance of treatment sample group/control group × 100.

Hoechst 33258 Staining Analysis

To observe nuclear changes that occurred during apoptosis, H9c2 cells were treated with the chromatin specific dye Hoechst 33258. After $Na_2S_2O_4$ treatment, cells were fixed with 4% paraformaldehyde solution for 10 min at room temperature prior to

Hoechst staining assay (Beyotime, China), according to the manufacturer's instructions. Images were taken using a fluorescence microscope.

Flow Cytometry Analysis

For quantification of cell apoptosis, we performed annexin V-fluorescein isothiocyanate (V-FITC) staining assay (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Viable cells were indicated as both annexin V and propidium iodide (PI) negative, whereas cells in early apoptosis were annexin V positive and PI negative (LR), and cells in late apoptosis or non-viable cells were both annexin V and PI positive (UR). Following staining, the cell characteristics were analyzed using a flow cytometer (BD Biosciences, USA).

RT-PCR

Total RNA of H9c2 cells was extracted using Trizol reagent according to the manufacturer's instruction. Oligo(dT)-primed RNA (1 μg) was reverse transcribed to cDNA using SuperScript Ⅱ reverse transcriptase according to the manufacturer's instructions. Real-time PCR analysis was performed to determine the level of mRNA expression of Bcl-2 and Bax using 7500 fast real time PCR system and SYBR Green RT-PCR reagents kit (ABI, USA). Data were represented as the fold change compared to the control. GAPDH was used to normalize all sample data.

Western Blot Analysis

Cells were lysed using mammalian cell lysis buffer containing protease and phosphatase inhibitor cocktails. Protein concentration was measured using Bradford assay (Bio-Rad). Equal amounts of protein lysates were loaded onto and separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membrane (Millipore, MA, USA). Membranes were blocked in 5% non-fat dry milk for 1 h, and probed with primary antibodies at 4 ℃ overnight. Signals were amplified with HRP-conjugated secondary antibodies (1:1000 dilution) and observed under enhanced chemiluminescence Western Blot Detection System (Bio-rad, USA). Band intensity was measured and densitometry analysis was performed using Scion-Image software for Windows.

Statistical Analysis

All data were representative of at least three separate experiments. Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). A one-way analysis of variance (ANOVA) with Dunnett's test was used to compare between groups. Data were analyzed using SPSS 18.0 software. $P<0.05$ was considered to be statistically significant.

RESULTS

Effect of STP on H9c2 Cell Viability

At concentrations of 100 μg/mL or lower, STP treatment for 24 h had no noticeable cytotoxic effect on H9c2 cells, whereas concentrations above 200 μg/mL resulted in a significant decrease in cell viability (Figure 1A). $Na_2S_2O_4$-induced hypoxia significantly reduced cardiomyocyte viability (64.30% ± 3.59%), which was rescued following STP pretreatment (Figure 1B). STP significantly attenuated $Na_2S_2O_4$-induced hypoxia in a dose-dependent manner, and restored cell viability to 83.06% ± 8.77% and 91.57% ± 5.17% at concentrations of 25 and 50 μg/mL, respectively. Therefore, STP at concentrations of 25 and 50 μg/mL were used for the subsequent experiments.

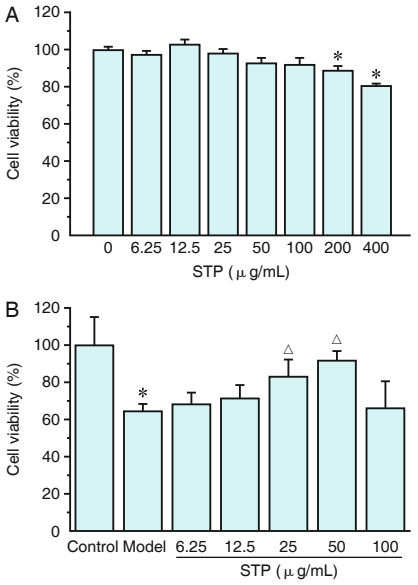

Figure 1. Effect of STP on H9c2 Cell Viability ($\bar{x} \pm s$)

Notes: (A) H9c2 cells were treated with various concentrations of STP for 24 h. (B) H9c2 cells were pretreated with STP prior to $Na_2S_2O_4$-induced hypoxia. Cell viability was determined using MTT assay. Data were normalized to the viability of control cells. $^*P<0.05$ *vs*. control cells, $^\triangle P<0.05$ *vs*. $Na_2S_2O_4$ treated model cells

Effect of STP on $Na_2S_2O_4$-Induced Apoptosis

$Na_2S_2O_4$ treatment markedly induced apoptosis in H9c2 cells observed under bright-field and fluorescence microscope, which was rescued by pretreatment with 25 and 50 μg/mL STP (Figure 2).

Chin J Integr Med 2019 Jun;25(6):439-445

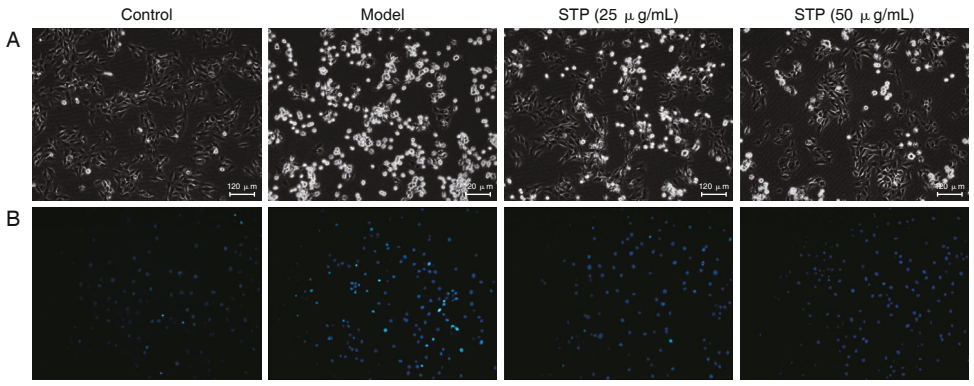

Figure 2. STP Pretreatment Attenuates $Na_2S_2O_4$-Induced Apoptosis in H9c2 Cells

Notes: Morphology of $Na_2S_2O_4$-induced hypoxia in H9c2 cells pretreated with STP observed under a bright-field microscope (A) and by Hoechst staining observed under a fluorescence microscope (B). Original magnifications: 200×.

The percentage of cells undergoing apoptosis (both early and late apoptotic cells) was 3.59%±0.36% following $Na_2S_2O_4$-induced hypoxia (Figure 3). However, following STP pretreatment, the percentage of cells undergoing apoptosis decreased significantly to 1.38%±0.34% and 1.85%±0.37% at concentrations of 25 and 50 μg/mL, respectively (P<0.05).

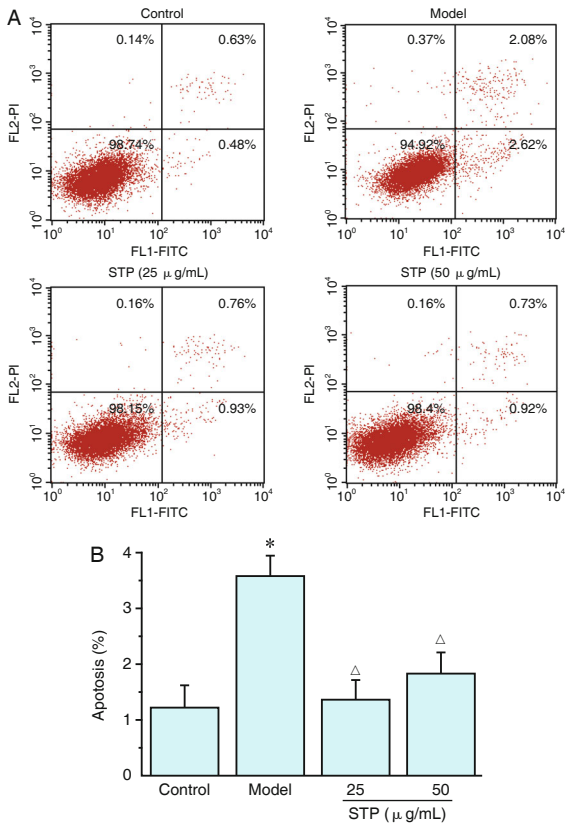

Figure 3. Effect of STP on $Na_2S_2O_4$-Induced Apoptosis in H9c2 Cells

Notes: (A) Representative flow cytometry graphs following $Na_2S_2O_4$-induced apoptosis in H9c2 cells. Images are representative of three independent experiments. (B) Quantification of cell apoptosis analysis by flow cytometer. $^*P$<0.05 *vs*. control cells, $^\triangle P$<0.05 *vs*. model cells

STP Increases Anti-apoptotic Bcl-2 and Decreases Pro-apoptotic Bax Expression Following $Na_2S_2O_4$-Induced Hypoxia

Following $Na_2S_2O_4$ treatment, the level of Bcl-2 was significantly reduced, whereas the level of Bax was significantly increased compared with control group (P<0.05, Figure 4). In contrast, cells pretreated with STP (both 25 and 50 μg/mL) had significantly increased levels of Bcl-2 and decreased levels of Bax compared with the model group (P<0.05).

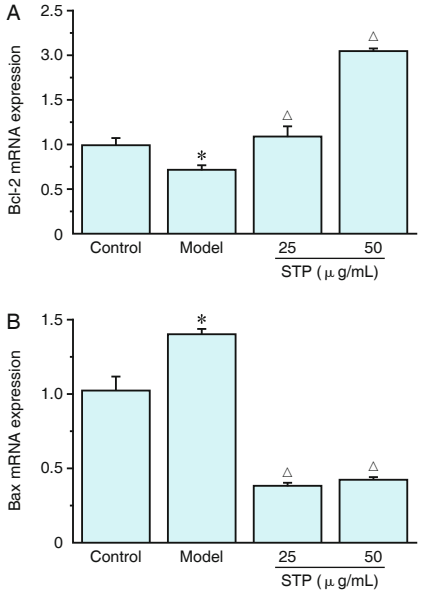

Figure 4. Effect of STP on mRNA Expression Levels of Bcl-2 and Bax Following $Na_2S_2O_4$-Induced Hypoxia in H9c2 Cells

Notes: (A) Level of Bcl-2 mRNA expression. (B) Level of Bax mRNA expression. $^*P$<0.05 *vs*. control cells, $^\triangle P$<0.05 *vs*. model cells

Effect of STP on Bcl-2 and Bax Protein Expression

The level of anti-apoptotic Bcl-2 was significantly reduced using Western Blot analysis, whereas

the level of pro-apoptotic Bax was significantly increased following $Na_2S_2O_4$ treatment (P<0.05), and furthermore, STP pretreatment could attenuate these changes in Bcl-2 and Bax expression (Figure 5).

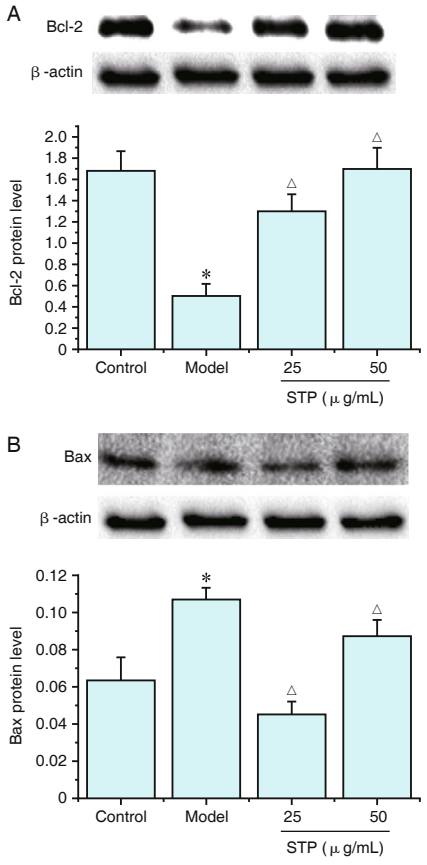

Figure 5. Effect of STP on Protein Expression Levels of Bcl-2 and Bax Following $Na_2S_2O_4$-Induced Hypoxia in H9c2 Cells

Notes: Protein expression levels of Bcl-2 (A) and Bax (B). $^*P$<0.05 *vs*. control cells, $^\triangle P$<0.05 *vs*. model cells

DISCUSSION

The cardioprotective effect of STP is clinically well known,[5-7] although its underlying mechanisms remain unclear. Our present study aims to define the mechanism of action of STP following $Na_2S_2O_4$-induced hypoxia in H9c2 cardiomyoblasts. We determined the protective effect of STP on $Na_2S_2O_4$-injured H9c2 cells based on increased cell viability, decreased aberrations in cellular morphology and decreased markers of cellular apoptosis following STP pretreatment. In addition, the protective effect of STP against $Na_2S_2O_4$-induced apoptotic cell death was associated with the down-regulation of pro-apoptotic Bax expression and up-regulation of anti-apoptotic Bcl-2 expression. Notably, pretreatment with STP markedly protected H9c2 cells from $Na_2S_2O_4$-induced hypoxic injury.

Hypoxia is a common pathological feature of cardiovascular diseases which mediates cardiac remodeling and adverse cardiomyocyte function. Similar to ischemic-reperfusion injury, cardiomyocyte reoxygenation often does not reverse hypoxia-induced changes but rather worsens cardiomyocyte damage. Numerous studies have indicated that apoptosis plays a crucial role in cardiomyocyte death during hypoxia-reoxygenation.[13,14] $Na_2S_2O_4$ is a chemical that is commonly used to mimic the hypoxic response in various cells.[15] In preliminary study, we investigated the cell hypoxic injury induced by 0.1% O_2 oxygen-deprived environments, $Na_2S_2O_4$ and $CoCl_2$. The results showed that the $Na_2S_2O_4$-induced hypoxia model we established was successful compared with the other two models. Therefore, the use of $Na_2S_2O_4$ treatment to model hypoxia-induced myocardial injury in H9c2 cells provides a simple and convenient method to elucidate the molecular mechanisms of hypoxia-associated cell death.[12,16]

STP had no cytotoxic effect on H9c2 cells below 200 μg/mL based on MTT assay, while STP pretreatment at a concentration between 25 and 50 μg/mL effectively prevented $Na_2S_2O_4$-induced cell damage and apoptosis in a dose-dependent manner. These results suggest that STP has a strong protective effect against $Na_2S_2O_4$-induced apoptosis. In preliminary study, we investigated the effects of pre-, co- and post-treatment with STP on $Na_2S_2O_4$-induced hypoxia model. Then the results showed that pretreatment of STP can increase hypoxic cell viability.

In addition, STP pretreatment prevented the deterioration in cell morphology and necrosis of H9c2 cells following $Na_2S_2O_4$ treatment, which is likely due to the anti-apoptosis properties of STP. Hoechst staining also revealed that STP pretreatment resulted in decreased H9c2 cardiomyoblast apoptosis. As we know, myocardial apoptosis often occurs during the early stage, which can lead to extensive loss of cardiomyocytes, contribute to the pathogenesis of hypoxic injury and ultimately result in the deterioration of cardiac function. These results suggest that STP is cardioprotective by alleviating myocardial ischemic injury and preventing myocardial apoptosis.

There has been considerable evidence that

indicate myocardial apoptosis plays a crucial role in the pathogenesis of various cardiovascular diseases, such as myocardial hypertrophy, myocardial ischemia and heart failure.[17-19] Therefore, prevention of hypoxia-induced cardiomyocyte apoptosis might be a critical step in the management of cardiomyocyte hypoxia-reoxygenation injury, myocardium remodeling after myocardial infarction and heart failure. Preventing cardiomyocyte death is therefore a key strategy in proventing from cardiovascular diseases.[20,21]

In order to better understand the mechanism of mitochondrial-mediated apoptosis, we analyzed the expression of Bcl-2 family of proteins, which have important functions in the regulation of myocardial apoptosis. Notably, the Bcl-2 family of proteins includes anti-apoptotic members such as Bcl-2 and pro-apoptotic members such as Bax, and these proteins act on the outer mitochondrial membrane to initiate or inhibit cellular apoptosis.[22,23] Typically, the balance between the pro-apoptotic and anti-apoptotic proteins determines the fate of the cells, to either undergo apoptosis or survive under pathophysiological stress following injury.[24,25] Our study showed that STP pretreatment up-regulated the expression of Bcl-2 and down-regulated the expression of Bax following $Na_2S_2O_4$-induced hypoxia, thereby demonstrating the anti-apoptotic and cardioprotective properties of STP following hypoxic-ischemic injury. However, the exact mechanism by which STP regulate the expression of apoptosis-associated genes is still unclear. Additional studies are required in order to better understand the mechanisms of STP in protecting against myocardial ischemic and hypoxic injury.

In conclusion, our study demonstrated that STP protected against $Na_2S_2O_4$-induced hypoxic injury by preventing apoptosis in cardiomyoblast H9c2 cells. Furthermore, the cardioprotective effect of STP on myocardial hypoxic-ischemic injury was mediated by rescuing the abnormal upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2. Thus, our study implicates STP as an effective and promising drug for the clinical treatment and prevention of IHD. In the future, further studies are required to investigate the signaling pathways involved in STP-induced protection against $Na_2S_2O_4$-induced hypoxic injury. The mechanisms of STP on the mitochondrial-mediated apoptosis pathway will be further studied.

Conflict Interest

The authors report no conflicts of interest.

Author Contributions

Lin S was in charge of data analysis, and drafted this paper. Peng J granted proposal for funding, led study design, reviewed and revised the manuscript. Zhang L, Chen DX, Xiao F and Chen HW were responsible for data collection in each experiment. Lin JM, Chu JF, Chen YQ and Zhu YL organized the whole research group and guide the study experiments. All authors have read and approved the final manuscript.

Acknowledgements

The project was completed in Academy of Integrative Medicine and Fujian Key Laboratory of Integrative Medicine on Geriatric of Fujian University of Traditional Chinese Medicine. We thank Inner Mongolia Conba Pharmaceutical Co., Ltd. for providing STP and support.

REFERENCES

1. Fan J, Li GQ, Liu J, Wang W, Wang M, Qi Y, et al. Impact of cardiovascular disease deaths on life expectancy in Chinese population. Biomed Environm Sci 2014;27:162-168.
2. Sivaraman V, Yellon DM. Pharmacologic therapy that simulates conditioning for cardiac ischemic/reperfusion injury. J Cardiovasc Pharmacol Ther 2014;19:83-96.
3. Al-Nimer MSM. Evaluation of anti-ischemic therapy in coronary artery disease: a review. In: Chaikovsky, ed. Coronary Artery Diseases; Europ: In Tech: 2012;151-196.
4. Wu LX, Gu XF, Zhu YC, Zhu YZ. Protective effects of novel single compound, hirsutine on hypoxic neonatal rat cardiomyocytes. Eur J Pharmacol 2011;650:290-297.
5. Chen DX, Lin S, Xu W, Huang MQ, Chu JF, Xiao F, et al. Qualitative and quantitative analysis of the major constituents in Shexiang Tongxin Dropping Pill by HPLC-Q-TOF-MS/MS and UPLC-QqQ-MS/MS. Molecules 2015;20:18597-18619.
6. Ning HH. The analysis of Shexiang Tongxin Dropping Pill on treating coronary heart disease angina pectoris curative effect. Chin J Geriatric Care (Chin) 2012;10(3):46.
7. Zhang J, Xu XM, Wang WM. Shexiang Tongxin Dropping Pill combined with aspirin for treatment of unstable angina. Chin J Integr Med Cardio/Cerebrovasc Dis (Chin) 2011;9:917-918.
8. Hua XP, Zhan Y, Li ZX. Effect of Shexiang Tongxin Dropping Pill on cardiac function in patients with chronic heart failure. Chin J Integr Med Cardio/Cerebrovasc Dis (Chin) 2011;9:143-145.
9. Sui W. Clinical observation of Shexiang Tongxin Dropping Pill in treating senile unstable agina pectoris. Chin Comm Doc (Chin) 2011;9:177-178.
10. Zhang HQ, Xu DL, Yan, L, Yuan LY, Jia JG, Sun AJ, et al.

The protective function of Shexiang on endothelial injury induced by angiotensin Ⅱ osmotic pump in the rat. Chin J Clin Med 2009;16:669-672.
11. Xiong MQ, Jia CL, Cui JG, Wang PW, Du XY, Yang QB, et al. Shexiang Tongxin Dropping Pill attenuates atherosclerotic lesions in ApoE deficient mouse model. J Ethnopharmacol 2015;159:84-92.
12. Shi MF, He WJ, Liu YL, Li XR, Yang SL, Xu QM. Protective effect of total phenylethanoid glycosides from *Monochasma savatieri* Franch on myocardial ischemia injury. Phytomed Int J Phytother Phytopharmacol 2013;20:1251-1255.
13. Kang PM, Haunstetter A, Aoki H, Usheca A, Izumo S. Morphological and molecular characterization of adult cardiomyocyte apoptosis during hypoxia and reoxygenation. Circul Res 2000;87:118-125.
14. Chen DL, Zhe J, Zhang JJ, Jiang LL, Chen K, He XH, et al. HO-1 protects against hypoxia/reoxygenation-induced mitochondrial dysfunction in H9c2 cardiomyocytes. PLos One 2016;11:e0153587.
15. Zhang XQ, Eyzaguirre C. Effects of hypoxia induced by $Na_2S_2O_4$ on intracellular calcium and resting potential of mouse glomus cells. Brain Res 1999;818:118-26.
16. Lin L, Pan YX, Yuan WJ. Effects of endothelin-1 antagonist BQ610 on hypoxia-induced injury and [Ca^{2+}] (1) changes in cultured neonatal rat cardiomyocytes. Drug Develop Res 2003;58:74-78.
17. Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. J Mol Cell Cardiol 2001;33:907-921.
18. Guo Y, Zhang L, Li F, Hu CP, Zhang Z. Restoration of sirt1 function by pterostilbene attenuates hypoxia-reoxygenation injury in cardiomyocytes. Eur J Pharmacol 2016;776:26-33.
19. Shen M, Wu RX, Zhao L, Li J, Guo HT, Fan R, et al. Resveratrol attenuates ischemia/reperfusion injury in neonatal cardiomyocytes and its underlying mechanism. PLos One 2012;7:e51223.
20. Caroppi P, Sinibaldi F, Fiorucci L, Santucci R. Apoptosis and human diseases: mitochondrion damage and lethal role of released cytochrome C as proapoptotic protein. Curr Med Chem 2009;16:4058-4065.
21. Mohsen F, Mansour E, Hosein M, Seid HS, Leila G, Hannaneh W, et al. Pretreatment with hyperoxia reduces *in vivo* infarct size and cell death by apoptosis with an early and delayed phase of protection. Eur J Cardio-Thoracic Sur 2011;39:233-240.
22. Xu JY, Zhou M, Ouyang J, Wang J, Zhang QG, Xu Y, et al. Gambogic acid induces mitochondria-dependent apoptosis by modulation of Bcl-2 and Bax in mantle cell lymphoma JeKo-1 cells. Chin J Cancer Res 2013;25:183-191.
23. Wang YL, Wang CY, Zhang BJ, Zhang ZZ. Shenfu Injection suppresses apoptosis by regulation of Bcl-2 and Caspase-3 during hypoxia/reoxygenation in neonatal rat cardiomyocytes *in vitro*. Mol Biol Rep 2009;36:365-370.
24. Liao YH, Xia N, Zhou SF, Tang TT, Yan XX, Lv BJ, et al. Interleukin-17A contributes to myocardial ischemia/reperfusion injury by regulating cardiomyocyte apoptosis and neutrophil infiltration. J Am Coll Cardiol 2012;59:420-429.
25. Ji LL, Fu F, Zhang LH, Liu WC, Cai XQ, Zhang L, et al. Insulin attenuates myocardial ischemia/reperfusion injury via reducing oxidative/nitrative stress. Am J Physiol Endocrinol Metab 2010;298:E871-E880.

(Accepted July 14, 2017; First Online April 4, 2018)
Edited by YUAN Lin