International Immunopharmacology 71 (2019) 361–371

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Trans sodium crocetinate alleviates ischemia/reperfusion-induced myocardial oxidative stress and apoptosis *via* the SIRT3/FOXO3a/SOD2 signaling pathway☆

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ARTICLE INFO

Keywords:
Trans sodium crocetinate
Ischemia/reperfusion
Myocardial injury

ABSTRACT

Trans sodium crocetinate (TSC) has been reported to exert a protective effect against cerebral ischemia/reperfusion (I/R) injury. However, whether TSC protects against myocardial ischemia/reperfusion (MI/R) injury remains unknown. Herein, we found that TSC treatment reduced myocardial infract size and elevated serum LDH and CK activities of MI/R rats. TSC administration attenuated oxidative stress in MI/R rats and H9C2 cells exposed to oxygen glucose deprivation/reperfusion (OGD/R). TSC administration relieved I/R-induced myocardial apoptosis *in vivo* and *in vitro*, as evidenced by reduced number of TUNEL positive cells, accompanying with marked decreases in caspase-3 activity and Bax protein level and an increase in Bcl-2 protein level. TSC treatment markedly increased SIRT3 activity and SIRT3 and SOD2 protein levels, and could also diminished the phosphorylation of FOXO3a protein. Additionally, TSC treatment attenuated the acetylation of FOXO3a and SOD2 protein. But, these effects were obviously blocked by SIRT3 knockdown. Besides, SIRT3 knockdown blocked the cardioprotective effect of TSC on OGD/R-induced oxidative stress, apoptosis and mitochondrial dysfunction *in vitro*. In summary, TSC alleviates I/R-induced myocardial oxidative stress and apoptosis *via* the SIRT3/FOXO3a/SOD2 signaling pathway. Our study suggests that TSC may become a novel drug for the treatment of MI/R injury.

1. Introduction

Myocardial infarction (MI), one of the most common cardiovascular diseases, is hallmarked by coronary obstruction and interruption of blood flow [1,2]. Myocardial ischemia/reperfusion (MI/R) injury initiates various deleterious cascades such as inflammation, oxidative stress, and apoptosis [3,4]. Restoration of blood flow to the heart without delay is considered as the most effective therapy for myocardial infarction [5]. In most situations, reperfusion therapy produces positive results, but it may also cause additional myocardial damage, known as MI/R injury [6].

Trans sodium crocetinate (TSC) is a derivative compound of the carotenoid crocetin that can enhance the diffusivity of small molecules in aqueous solutions by modifying the structure of water [7]. TSC is capable of increasing the movement of oxygen from red blood cells into hypoxic tissues [8] Moreover, TSC treatment promoted cerebral tissue oxygenation under conditions of vascular occlusion, whereas, attenuated cerebral tissue hyperoxygenation during vascular perfusion [9]. Additionally, TSC as a radiosensitizer increased the sensitivity of glioblastoma cancer cells to radiation therapy and enhanced survival [10–12]. However, whether TSC could protect heart from MI/R injury remains unclear.

Reactive oxygen species (ROS) are important oxygen derivatives and function as signaling molecules to participate in various physiology processes, such as gene expression [13]. Overproduction of ROS provokes a state of oxidative stress. Oxidative stress results from an imbalance between accumulation of ROS and biological antioxidant systems, and is regarded as an important causative factor of MI/R injury

☆ Declarations of interest: None.

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https://doi.org/10.1016/j.intimp.2019.03.056
Received 21 December 2018; Received in revised form 26 March 2019; Accepted 27 March 2019
Available online 02 April 2019

[14]. Myocardial ischemic causes the depletion of ATP, the accumulation of H^+ and Ca^{2+} and the opening of the mitochondrial permeability transition pore, leading to the superfluous generation of ROS and thereby activating the apoptosis cascade [15]. Excessive ROS generation induces oxidative damage to proteins, lipids and DNA, resulting in cell death [16].

Sirtuin-3 (SIRT3), located in the mitochondrial matrix, belongs to the sirtuin family and is highly expressed in cardiac myocytes [17]. Of note, SIRT3 has been postulated to be implicated in the pathological mechanism of MI/R injury [18]. FOXO3a, also known as FKHRL1, is a forkhead transcription factor and belongs to the FOXO family. FOXO3a is capable of regulating the expression of genes involved in cellular development, longevity, autophagy, apoptosis and stress resistance [19]. Superoxide dismutase (SOD), also known as manganese-dependent superoxide dismutase, belongs to the iron/manganese superoxide dismutase family and is a superoxide scavenger enzyme that plays a vital role in maintenance of cellular redox balance [20].

In this study, we explored the cardioprotective effect of TSC in MI/R rats and H9C2 cells exposed to oxygen glucose deprivation/reperfusion (OGD/R) injury. Further, the exact molecular mechanism underlying the anti-oxidative and anti-apoptosis effects of TSC was investigated *in vitro*.

2. Materials and methods

2.1. Animal

Male Wistar rats (weighing 180–230 g) were supplied by Vital River Lab Animal Technology Co., Ltd. (Beijing, China). Rats were penned individually, kept under a specific pathogen-free condition and had free access to standard pellet diet. Rats were randomized into sham + saline group ($n = 8$), MI/R + saline group ($n = 8$), MI/R + TSC (25 μg/kg; $n = 8$) group, MI/R + TSC (50 μg/kg; $n = 8$) group and MI/R + TSC (100 μg/kg; $n = 8$) group. TSC (ChemFaces, Wuhan, China) or vehicle (saline) was administered to rats with myocardial ischemia *via* the femoral vein at 10 min prior to reperfusion. All experimental procedures were approved by the Ethics Committee of The First People's Hospital of Shangqiu.

2.2. Rat model of MI/R injury

To establish an *in vivo* model of MI/R injury, rats were fasted overnight and anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg; Sigma, St. Louis, USA). During the operation, rectal temperature was maintained at 37.0 ± 0.5 °C. The heart was exposed with a left thoracotomy, and a 4–0 silk suture slipknot was placed at the distal 1/3 of the left anterior descending coronary artery (LAD) origin. After 30 min of LAD occlusion, the slipknot was released to allow reperfusion for 24 h. Rats in the sham + saline group underwent surgical procedures without coronary occlusion.

2.3. Cells culture and transfection

H9C2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM) contained 10% fetal bovine serum (Solarbio, Beijing, China) and 1% penicillin/streptomycin (Solarbio).

For downregulation of SIRT3, sh-SIRT3_1, sh-SIRT3_2 or negative control (sh-NC) were synthesized by GeneChem (Shanghai, China) and transfected into H9C2 cells using Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) as instructed by the manufacturer.

To mimic MI/R conditions *in vitro*, H9C2 cells were incubated in glucose free, serum-free DMEM medium for 4 h in a humidified incubator with 5% CO_2/95% N_2 at 37 °C. Afterwards, cells were incubated in DMEM medium for 24 h in normal cell incubator at 37 °C. After 4 h of OGD insult, 0.5 μM TSC was added to the cells and then incubated under normal conditions for 24 h.

2.4. Determination of myocardial infarct size

The myocardial infarct size was determined using 2,3,5-triphenyltetrazolium chloride triazole (TTC; Solarbio) staining. At 24 h after MI/R surgery, rats were sacrificed and the hearts were excised, frozen and cut into slices. The slices were incubated with 2% TTC in PBS for 15 min at 37 °C in the dark, followed by fixation with 10% formalin solution for 12 h. The infarct area (white) and non-infract area (red) were analyzed using Image J software (NIH, Rockville, MD, USA).

2.5. Detection of cardiac marker enzymes

At 24 h after MI/R surgery, blood samples were collected and stored at −80 °C. Myocardium damage was assessed by determining serum lactate dehydrogenase (LDH) and creatine kinase (CK) activities. Serum LDH and CK activities were determined using the LDH assay kit (Jiancheng, Nanjing, China) and the CK assay kit (Jiancheng), according to the manufacturer's instructions.

2.6. Detection of ROS, GSH, SOD and MDA levels

The levels of ROS, glutathione (GSH), SOD and methane dicarboxylic aldehyde (MDA) *in vitro* and *in vivo* were determined using the ROS assay kit (Jiancheng), total glutathione assay kit (Beyotime, Shanghai, China), total superoxide dismutase assay kit with wst-8 (Beyotime) and MDA assay kit (Jiancheng), respectively.

2.7. Terminal deoxynucleotidyl transferase dUTP nick end labeling

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Roche, Penzberg, Germany) assay was performed to evaluate cell apoptosis. Tissue slices or adherent cells were fixed in formalin solution for 30 min, followed by permeation with 0.3% Triton X-100 for 5 min. After washing with PBS, tissue slices or cell smears were incubated with TUNEL reaction mixture for 1 h at 37 °C in darkness, followed by staining with DAPI or hematoxylin.

2.8. Detection of caspase-3 activity

The activity of caspase-3 was measured using a Caspase-3 assay kit (Beyotime). Tissues or cells were homogenized or lysed in ice-cold lysis buffer and then incubated with Ac-DEVD-*p*NA (2 mM) for 1 h at 37 °C. The activity of caspase-3 was measured at 405 nm using a microplate spectrophotometer (SpectraMax M5, Molecular Devices, and Sunnyvale, CA, USA).

2.9. Detection of SIRT3 activity

The activity of SIRT3 *in vitro* and *in vivo* was determined using the CycLex SIRT3 deacetylase fluorometric assay kit (MBL, Tokyo, Japan) in keeping with the manufacturer's specifications. The activity of SIRT3 was evaluated spectrophotometrically using a SpectraMax M5 (Molecular Devices).

2.10. Western blot analysis

Proteins were extracted from tissues and cells using RIPA buffer in the presence of proteinase cocktail. Protein extracts were subjected to 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted into polyvinylidene fluoride membranes. After blocking with 5% non-fat milk, membranes were incubated with primary antibodies against SIRT3 (Cell Signaling Technology, Beverly, MA, USA), p-forkhead box O3 (p-FOXO3a; Cell Signaling Technology), superoxide dismutase 2 (SOD2; Abcam, Cambridge, UK), Bcl-2-associated X protein

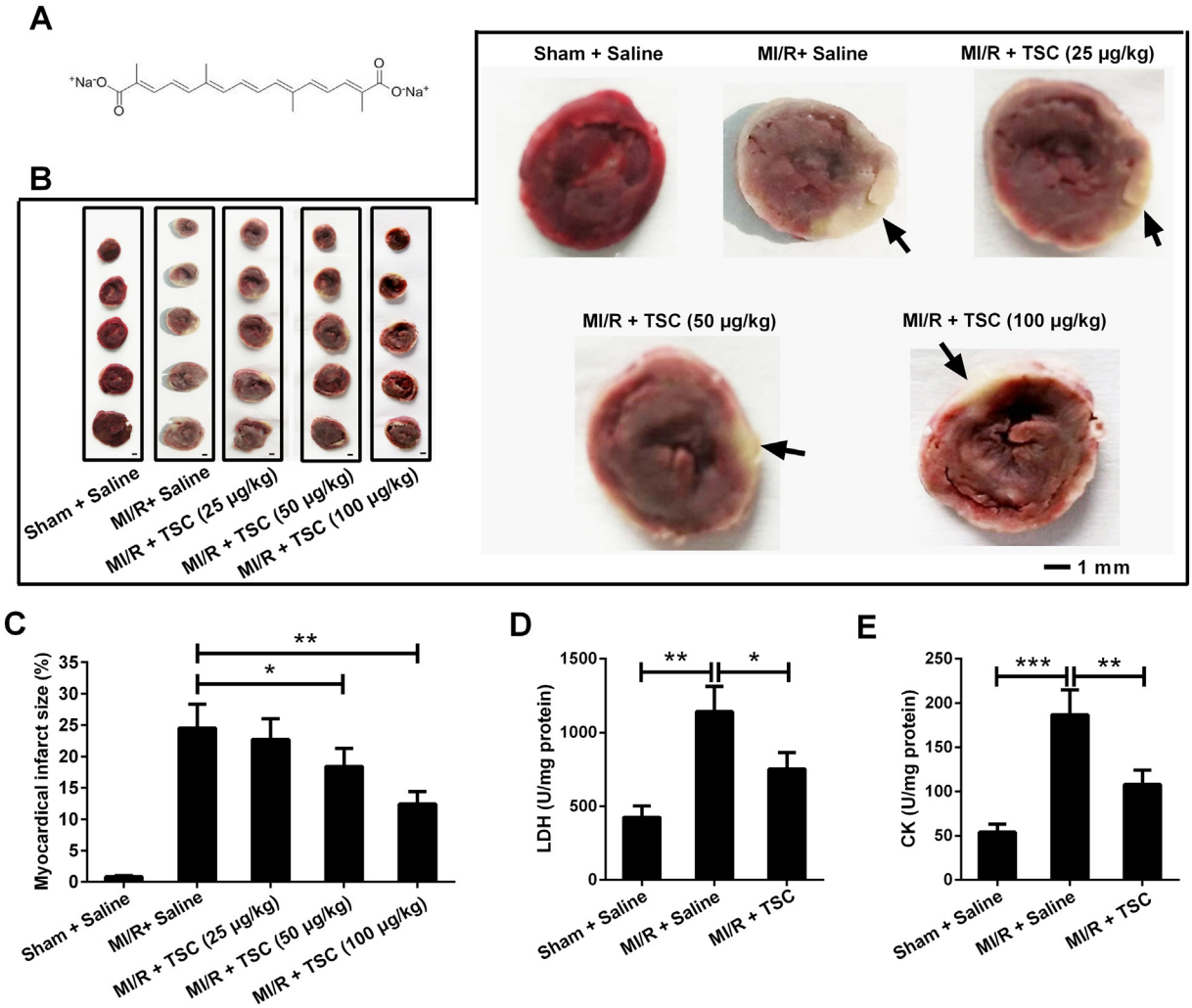


Fig. 1. TSC alleviates MI/R injury. (A) The chemical structure of TSC was illustrated. (B) TSC (25, 50, 100 μg/kg) was administrated to MI/R rats *via* the femoral vein at 10 min prior to reperfusion, and the myocardial infract size was detected at 24 h after reperfusion. Scale bar: 1 mm. (C and D) Serum LDH and CK activities were determined using the LDH assay kit and CK assay kit. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

(Bax; Boster, Wuhan, China), Bcl-2 (Boster) and anti-β-actin (Boster) at 4 °C overnight. Subsequently, membranes were probed with HRP-conjugated secondary antibody (Boster) for 1 h at room temperature. Immunoblots were visualized using ECL reagents (Pierce, Rockford, IL, USA), and quantified using the Image J software.

2.11. Immunoprecipitation of FOXO3a and SOD2

Following treatment, tissue homogenate or cell lysates were washed with PBS, then lysed and centrifuged. Protein extracts were immunoprecipitated with IgG, anti-FOXO3a or anti-SOD2 antibody on a rotator at 4 °C overnight, followed by incubation with protein A and G-agarose beads (Santa Cruz, CA, USA) for 90 min at 4 °C. After washing three times with PBS, precipitated proteins were eluted by boiling the beads for 3 min in RIPA buffer. The samples were analyzed by immunoblotting.

2.12. Quantitative real-time polymerase chain reaction

Total RNA from H9C2 cells was isolate using Trizol reagent (Invitrogen) as instructed by the manufacturer. A total of 1 μg RNA was used to generate cDNA using a PrimeScript™ RT reagent kit (Takara, Dalian, China). Quantitative real-time polymerase chain reaction (qRT-PCR) assay was conducted using a SYBR Premix Ex Taq™ kit (Takara) on the iCycler iQ Real-Time Thermocycler Detection System (Bio-Rad, Hercules, CA, USA). The relative expression levels of SIRT3 was normalized to that of β-actin by the $2^{-\Delta\Delta Ct}$ method.

2.13. Detection of mitochondrial membrane potential

H9C2 cells were transfected with sh-SIRT3 or sh-NC and then subjected to OGD/R. After 4 h of OGD, H9C2 cells were treated with 0.5 μM TSC and then subjected to reoxygenation for 24 h. Mitochondrial membrane potential was monitored by a Mitochondrial Membrane Potential Kit (Sigma) according to the manufacturer's protocol. This kit utilizes JC-10, a cationic lipophilic dye, that is concentrated and forms reversible red-fluorescent JC-10 aggregates (λex = 540/λem = 590 nm) in the mitochondria of cells with a polarized mitochondrial membrane. Depolarization of mitochondrial membrane lead to the failure to maintain JC-10 in the mitochondria and a return of the dye to its monomeric, green fluorescent form (λex = 490/λem = 525 nm). The ratio of red/green fluorescence intensity is used to determine the mitochondrial membrane potential.

2.14. Measurement of ATP production

The level of mitochondrial ATP was determined using an ATP Bioluminescence Assay Kit (Sigma) according to the manufacturer's

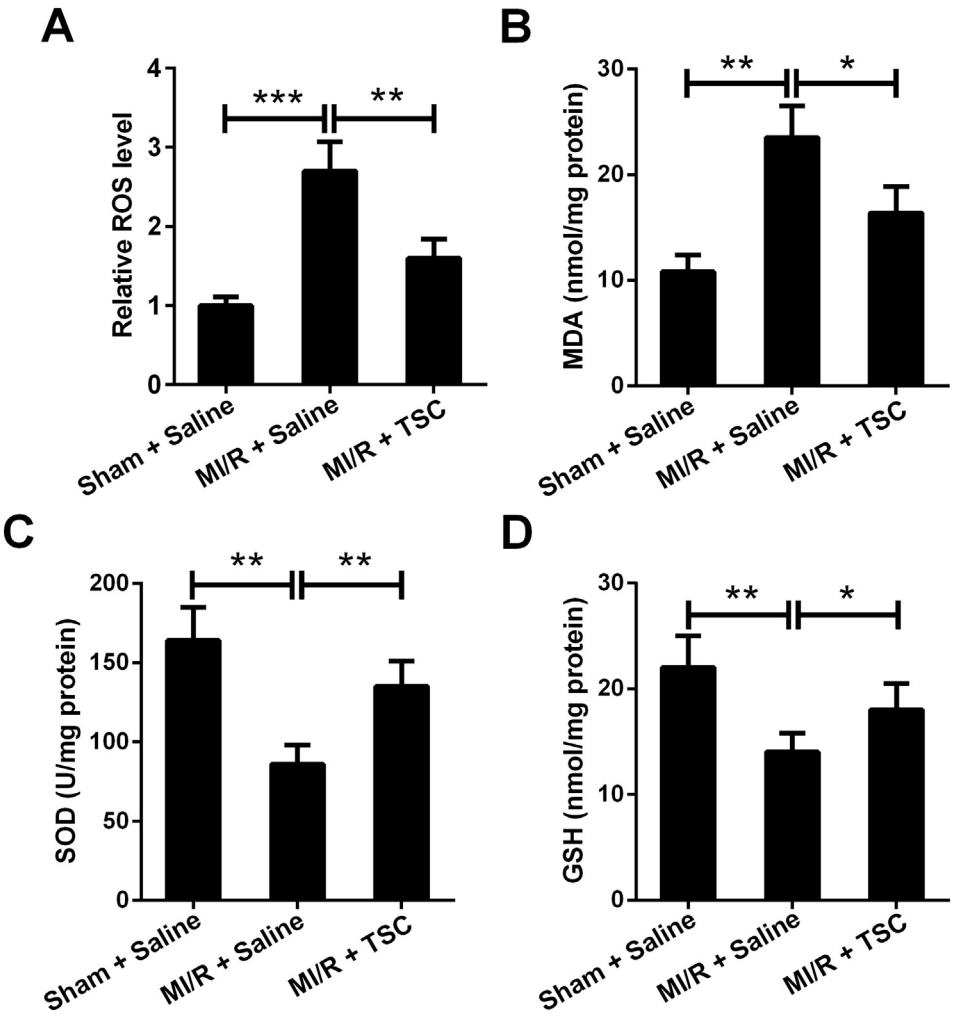


Fig. 2. TSC alleviates oxidative stress induced by MI/R. TSC treatment (25, 50, 100 μg/kg) was administrated to MI/R rats *via* the femoral vein at 10 min prior to reperfusion, and the levels of ROS (A), MDA (B), SOD (C) and GSH (D) were measured after 24 h of reperfusion. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

protocol. In brief, equal amounts of mitochondrial pellets (200 μg) were lysed, and centrifuged at 12,000*g* for 5 min at 4 °C. Then, the supernatant was mixed with the luciferase reagent and then the luminescence emission was detected with a microplate spectrophotometer (SpectraMax M5, Molecular Devices).

2.15. Statistical analysis

Results were shown as means ± standard deviation of the mean and analyzed by SPSS 20.0 software (Chicago, IL, USA). Statistical comparisons among groups were determined using one-way ANOVA test followed by Turkey's *post hoc* test. A P value < 0.05 was deemed a criterion for statistical significance.

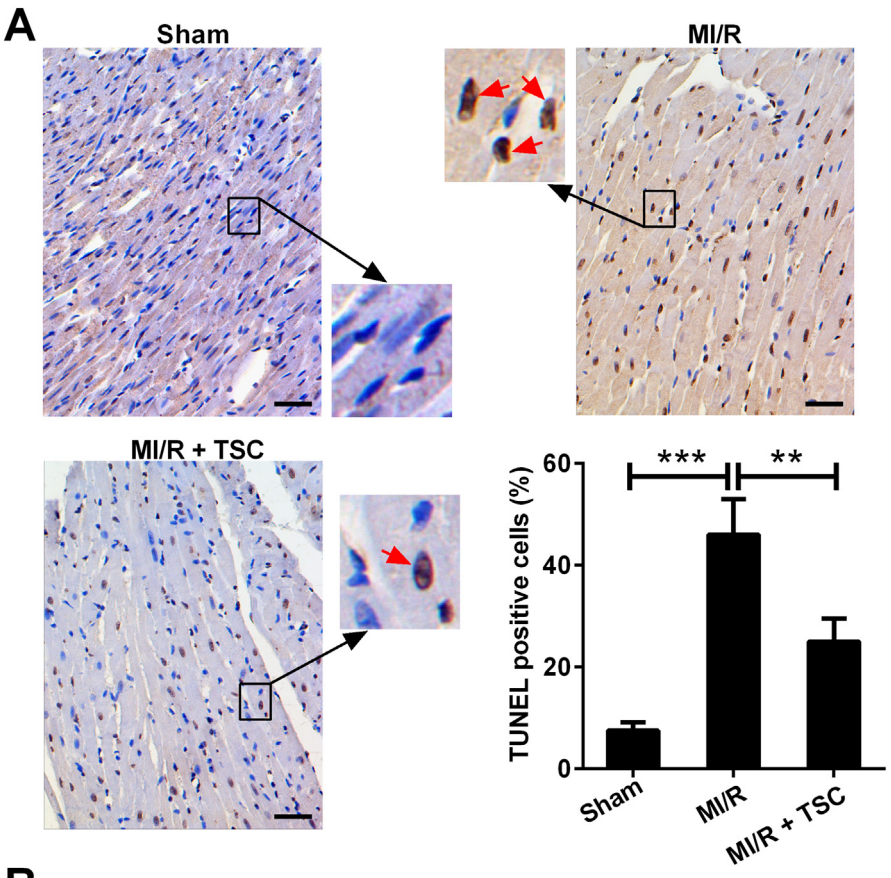
3. Results

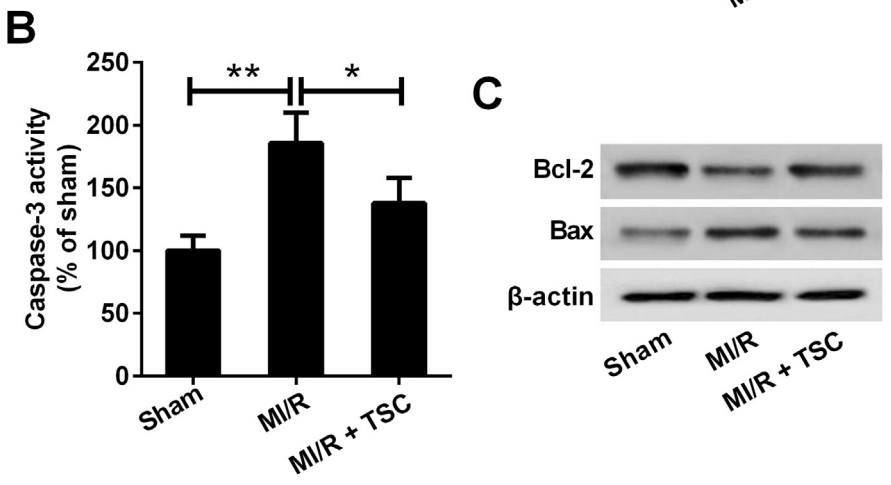
3.1. TSC alleviates MI/R injury

The chemical structure of TSC is represented in Fig. 1A. To determine the cardioprotective effect of TSC, TSC at doses of 25, 50 or 100 μg/kg was administered to MI/R rats *via* the femoral vein at 10 min prior to reperfusion, and the myocardial infract size was detected at 24 h after reperfusion. A middle one of cardiac tissue slices in each group was enlarged and exhibited in the upper-right part of Fig. 1. White tissues, as indicated by arrows, were the areas of injury. The non-infract area is in red color. Results of Image J-based quantitative analysis showed that the myocardial infract size in the MI/R + saline group was higher than that in the sham + saline group. TSC administration caused a dose-dependent reduction in the myocardial infract size in MI/R rats (Fig. 1C). Thus, a dose of 100 μg/kg of TSC was selected for all following experiments. Moreover, MI/R elevated the serum LDH and creatine kinase activities, which were blocked by TSC treatment (Fig. 1D and E). Together, these results indicated that TSC treatment alleviated MI/R injury, as shown by reduced myocardial infract size and decreased serum LDH and CK activities.

3.2. TSC alleviates oxidative stress induced by MI/R

To investigate the effect of TSC on oxidative stress in MI/R rats, the levels of ROS, MDA, SOD and GSH were measured after 24 h of reperfusion. As illustrated in Fig. 2A–D, compared with the sham + saline group, MI/R led to a marked increase in ROS and MDA levels, and decrease in SOD and GSH levels. Additionally, the levels of ROS and MDA were decreased in the MI/R + TSC group as compared with the MI/R group. While the levels of SOD and GSH were increased in the MI/R + TSC group as compared with the MI/R group. Thus, our findings revealed that TSC alleviated oxidative stress induced by MI/R, as showed by decreased ROS and MDA levels and increased SOD and GSH levels.





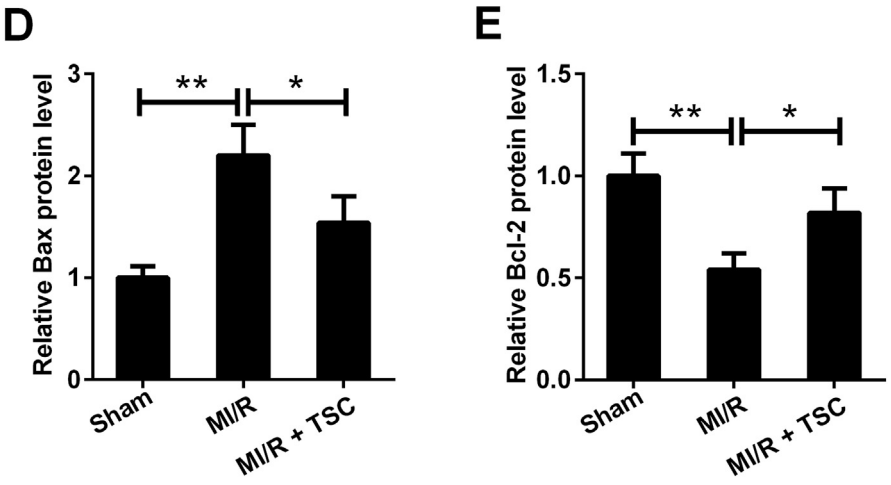


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Fig. 3. TSC alleviates apoptosis induced by MI/R. (A) Representative images of apoptotic cardiomyocytes and quantification of TUNEL-positive cells. Magnification, ×200. Scale bar: 100 μm. (B) The activity of caspase-3 was detected using a caspase-3 activity assay kit. (C) Representative images of western blot analysis of Bax and Bcl-2 expression. Quantification of Bax (D) and Bcl-2 (E) expression in Fig. 3C. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3.3. TSC alleviates apoptosis induced by MI/R

To assess the anti-apoptotic effect of TSC in MI/R rats, TUNEL assay was carried out. TUNEL-positive nuclei with brown granules were considered as apoptotic cells. As shown in Fig. 3A, the number of TUNEL positive cells was markedly increased in the MI/R + saline group as compared with the sham + saline group, but decreased in the MI/R + TSC group as compared with the MI/R + saline group. In line with this, the activity of caspase-3 and Bax protein level were higher in the MI/R + saline group than those in the sham + saline group, but lower in the MI/R + TSC group than those in the MI/R + saline group. Conversely, the protein level of Bcl-2 was decreased in the MI/R + saline group compared with that in the sham + saline group, but increased in the MI/R + TSC group compared with that in the MI/R + saline group (Fig. 3B–E). Therefore, our results suggested that TSC alleviated apoptosis induced by MI/R, as indicated by reduced number of TUNEL positive cells, decreased caspase-3 activity, decreased Bax protein level and increased Bcl-2 protein level.

3.4. The SIRT3/FOXO3a/SOD2 signaling pathway participates in the cardioprotective effects of TSC on MI/R injury

To study whether the SIRT3/FOXO3a/SOD2 signaling pathway participates in the cardioprotective effect of TSC on MI/R injury, we examined the influence of TSC on the expression of SIRT3, SOD2, and FOXO3a in MI/R rats. As a result, MI/R caused a striking decrease in SIRT3 activity, which was blocked by TSC treatment (Fig. 4A). As detected by western blotting, SIRT3 and SOD2 levels were decreased, while p-FOXO3a level was increased in the MI/R + saline group compared with those in the sham + saline group. However, these changes induced by MI/R could be abrogated by TSC treatment (Fig. 4B–E). Furthermore, the acetylated protein level of FOXO3a was notably increased in the MI/R + saline group as compared with that in the sham + saline group, but decreased in the MI/R + TSC group as compared with that in the MI/R + saline group (Fig. 4F). Compared to the sham + saline group, the acetylated protein level of SOD2 was increased in the MI/R + saline group. TSC administration decreased the acetylated protein level of SOD2 following MI/R (Fig. 4G). Taken together, these findings indicated that the cardioprotective effect of TSC on MI/R injury was probably mediated by, but definitely involving the SIRT3/FOXO3a/SOD2 signaling pathway.

3.5. TSC enhances SOD2 activity in OGD/R injured H9C2 cells by upregulating SIRT3

To evaluate the role of SIRT3 in the cardioprotective effect of TSC *in vitro*, we transfected sh-SIRT3_1, sh-SIRT3_2 or sh-NC into H9C2 cells, and the transfection efficiency was identified by qRT-PCR and western blotting. Compared with the sh-NC group, the expression of SIRT3 was remarkably decreased in the sh-SIRT3 group, especially in the sh-SIRT3_1 group (Fig. 5A and B). H9C2 cells were subjected to OGD/R and then incubated with a range of doses of TSC (0.025, 0.25, 0.5, 1 and 2 μM), and cytotoxicity of TSC was detected at 24 h after incubation. According to the cytotoxicity test, a dose of 0.5 μM TSC had no significant cytotoxicity and was selected to use in follow-up experiments. OGD/R reduced SIRT3 activity and SIRT3 and SOD2 protein levels, and enhanced phosphorylation of FOXO3a protein. TSC treatment markedly increased SIRT3 activity and SIRT3 and SOD2 protein levels, and decreased p-FOXO3a protein level. However, these changes induced by TSC treatment were obviously blocked by SIRT3 knockdown (Fig. 5C–G). Furthermore, OGD/R enhanced the acetylation of FOXO3a and SOD2 in H9C2 cells. TSC treatment reduced the acetylated protein levels of FOXO3a and SOD2 in H9C2 cells subjected to OGD/R, but this effect was abrogated by SIRT3 knockdown (Fig. 5H). Therefore, we concluded that TSC enhanced SOD2 activity by upregulating SIRT3.

3.6. SIRT3 knockdown blocks the anti-oxidative effect of TSC in OGD/R injured H9C2 cells

To address the role of SIRT3 in the anti-oxidative effects of TSC *in vitro*, H9C2 cells were transfected with sh-SIRT3 or sh-NC and then subjected to 4 h of OGD. After OGD, H9C2 cells were treated with 0.5 μM TSC and subjected to reoxygenation for 24 h, and the levels of ROS, MDA, SOD and GSH were measured. A dramatic increase in ROS (Fig. 6A) and MDA levels (Fig. 6B), and decrease in SOD (Fig. 6C) and GSH levels (Fig. 6D) were observed in H9C2 cells stimulated with OGD/R. TSC treatment reduced the levels of ROS and MDA, and increased the levels of SOD and GSH in H9C2 cells stimulated with OGD/R. However, SIRT3 knockdown could block the anti-oxidative effect of TSC in H9C2 cells stimulated with OGD/R. Accordingly, our findings suggested that the anti-oxidative effect of TSC depended on SIRT3.

3.7. SIRT3 knockdown blocks the anti-apoptotic effect of TSC in OGD/R injured H9C2 cells

We determined whether SIRT3 knockdown could block the anti-apoptotic effect of TSC on MI/R injury *in vitro*. As detected by TUNEL assay, OGD/R caused a marked increase in the number of TUNEL positive cells (green nuclear staining, as indicated by arrows), whereas TSC treatment reduced the number of TUNEL positive cells. However, SIRT3 knockdown reversed the anti-apoptotic effect of TSC in H9C2 cells stimulated with OGD/R (Fig. 7A). Consistently, the activity of caspase-3 was increased in the OGD/R group compared with that in the control group, but decreased in the OGD/R + TSC group compared with that in the OGD/R group. SIRT3 knockdown could abolish the anti-apoptotic effect of TSC by increasing the activity of caspase-3 (Fig. 7B). TSC treatment restrained OGD/R-induced upregulation of Bax protein expression and downregulation of Bcl-2 protein expression. Importantly, knockdown of SIRT3 strikingly abrogated the anti-apoptotic effect of TSC (Fig. 7C–E). Accordingly, these findings demonstrated that TSC exerted anti-apoptotic effect in OGD/R injured H9C2 cells by upregulating SIRT3.

3.8. SIRT3 knockdown blocks the protective effect of TSC on mitochondrial function of OGD/R injured H9C2 cells

To evaluate the effect of TSC on mitochondrial function of OGD/R injured H9C2 cells, we determined the mitochondrial membrane potential and mitochondrial ATP level. The results showed that OGD/R induced mitochondrial membrane potential disruption and reduction of mitochondrial ATP level in H9C2 cells, which was attenuated by TSC treatment. Knockdown of SIRT3 reversed the protective effect of TSC on mitochondrial function of OGD/R injured H9C2 cells (Fig. 8A and B). These data suggested that TSC improved OGD/R-induced mitochondrial dysfunction and SIRT3.

4. Discussion

Several studies over the past decades have revealed that TSC can protect against cerebral I/R injury. As an example, TSC administered by bolus-infusion-bolus led to a lower degree of brain edema and infract size in middle cerebral artery occlusion/reperfusion mice [21].

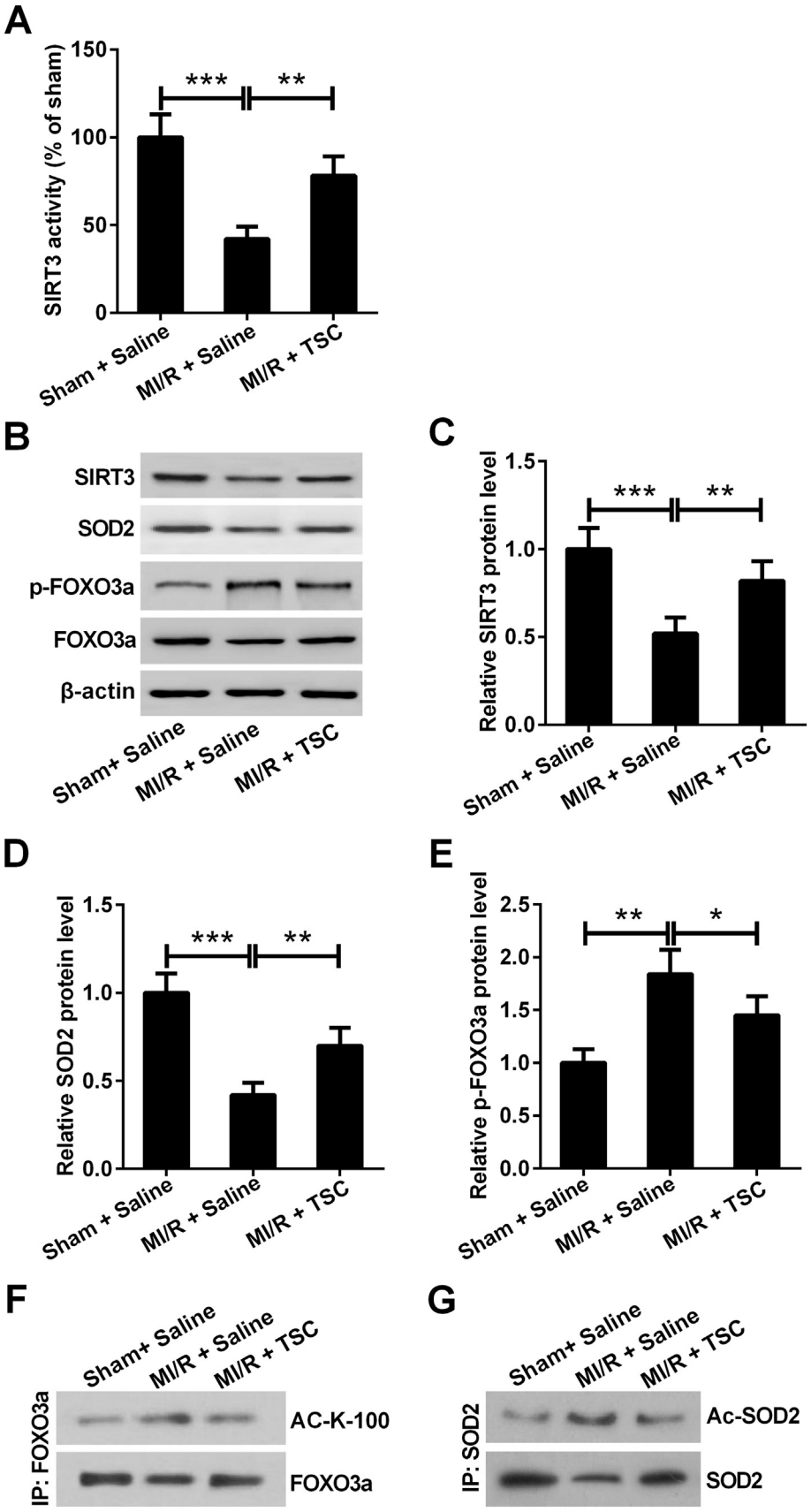

Fig. 4. The SIRT3/FOXO3a/SOD2 signaling pathway participates in the cardioprotective effects of TSC on MI/R injury. (A) The activity of SIRT3 was detected using a SIRT3 activity assay kit. (B) Representative images of western blot analysis of SIRT3, SOD2 and p-FOXO3a expression. Quantification of SIRT3 (C), SOD2 (D) and p-FOXO3a (E) expression in Fig. 4B. (F) Acetylated FOXO3a was detected by western blotting. (G) Acetylated SOD2 was detected by western blotting. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

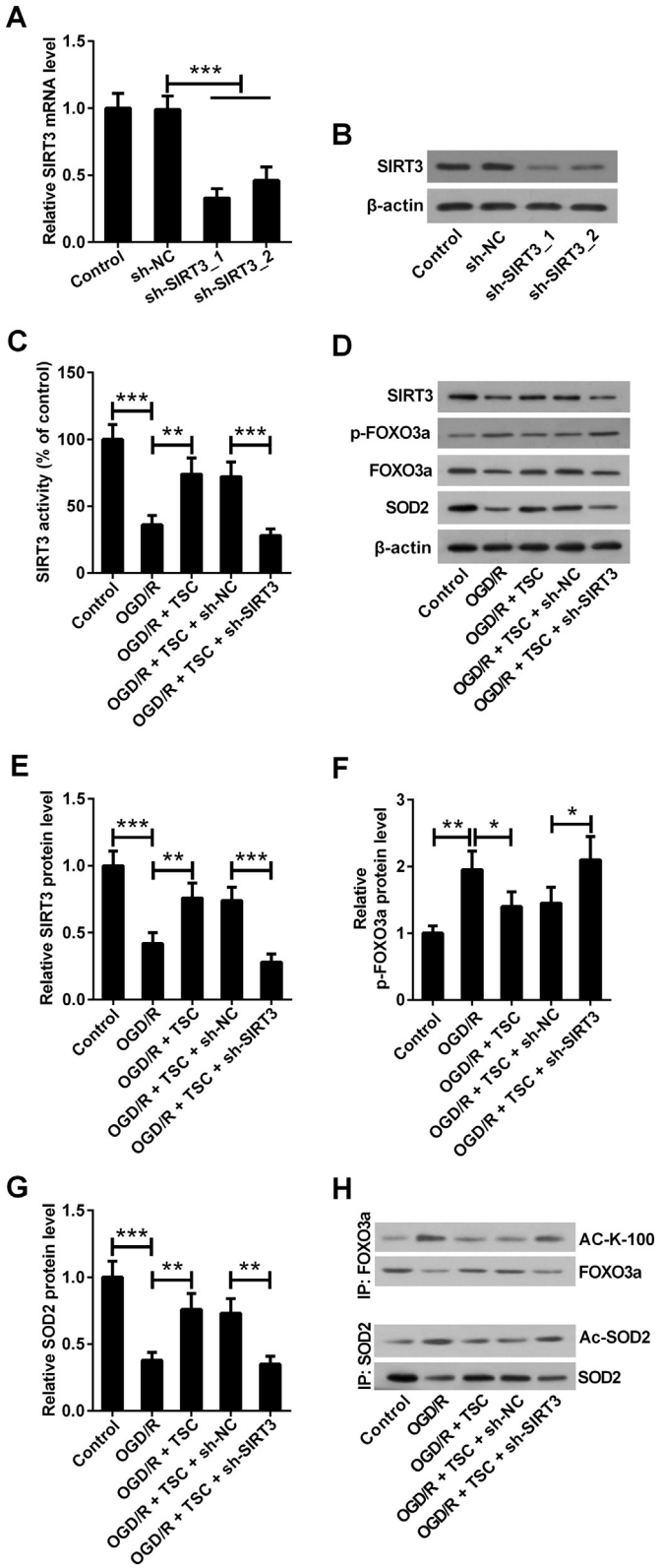

Fig. 5. TSC enhances SOD2 activity by upregulating SIRT3. H9C2 cells were transfected with SIRT3_1, sh-SIRT3_2 or sh-NC. The expression levels of SIRT3 mRNA and protein were evaluated by qRT-PCR (A) and western blotting (B). After 4 h of OGD insult, H9C2 cells were incubated with 0.5 μM TSC. (C) At 24 h after TSC treatment, the activity of SIRT3 was detected using SIRT3 activity assay kit. (D) Representative images of western blot analysis of SIRT3, SOD2 and p-FOXO3a expression. Quantification of SIRT3 (E), p-FOXO3a (F) and SOD2 (G) expression in Fig. 5D. (H) Acetylated FOXO3a and SOD2 were detected by western blotting. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Moreover, in a rat model of three-vessel occlusion stroke, TSC treatment reduced the degree of infract volume and increased the levels of oxygen in the ischemic penumbra tissue [22]. Furthermore, TSC treatment protected neurons against hemorrhagic injury as indicated by decreased hemispheric swelling and reduced content of tissue hemoglobin [23,24]. However, whether TSC could attenuate MI/R injury remain elusive. In the present study, we found that TSC treatment alleviated MI/R injury, accompanied by reduced myocardial infract size and decreased serum LDH and CK activities. TSC administration attenuated oxidative stress in MI/R rats and H9C2 cells exposed to OGD/R. TSC administration relieved I/R-induced myocardial apoptosis *in vivo* and *in vitro*. These results suggest that TSC exerts protective properties against MI/R injury *via* anti-oxidative stress and anti-apoptosis pathways.

Reperfusion injures cardiomyocytes by activating the inflammatory response and stimulating oxidative stress *via* generating ROS. Due to the heart is extremely susceptible to oxidative injury, I/R-stimulated oxidative stress can overwhelm antioxidant defenses leads to cell damage or death in the myocardium [25]. On the other hand, several ROS-dependent transcription factors can be induced in response to oxidative stress, resulting in dysregulation of important genes [26]. SIRT3 is capable of regulating the mitochondrial function and reducing oxidative stress by deacetylating several mitochondrial proteins [27]. Several studies have shown that SIRT3 functions as a tumor suppressor by modulating mitochondrial integrity [28]. Knockdown of SIRT3 increased the production of mitochondrial ROS, enhanced the cellular transformation, resulting in tumor growth [29]. SIRT3 was downregulated in MI/R rats [30]. Upregulation of SIRT3 mitigated MI/R injury through inhibition of mitochondrial permeability transition pore opening by deacetylating cyclophilin D [31]. Moreover, SIRT3 deficiency simulated I/R injury in H9c2 cardiac-derived cells and mouse Langendorff-perfused heart [32]. Besides, SIRT3 deletion exacerbated pre-existing coronary microvascular dysfunction and impaired cardiac recovery post myocardial ischemia [33]. In this study, we found that TSC treatment markedly increased SIRT3 activity and SIRT3 protein levels in MI/R rats and H9C2 cells exposed to OGD/R. Knockdown of SIRT3 blocked the effects of TSC on OGD/R -induced oxidative stress, apoptosis and mitochondrial dysfunction, suggesting that SIRT3 was a critical mediator of MI/R injury.

The FOXO3a translocation is important for its transactivation. Phosphorylation of FoxO3a at Ser253 leads to an inactivation form of FoxO3a that translocate to the cytoplasm, resulting in inhibition of its transcriptional activity [34]. Acetylation of FoxO3a results in the disruption of nuclear translocation, thereby inhibiting its transactivation and the transcription of FoxO3a-dependent genes. Furthermore, FoxO3a is considered as a substrate of SIRT3. SIRT3 interacts with FOXO3a, and induces FOXO3a translocation to the nucleus and then activates the FOXO3a-dependent antioxidant-encoding genes [35]. SIRT3 interacts with the daf-16 homolog FOXO3a in the mitochondria, as well as increases FOXO3a dependent gene expression [36]. SIRT3 also has been proved to protect mitochondria against oxidative damage by deacetylating FOXO3a [37]. SOD2 is a substrate of SIRT3 and the binding of SIRT3 with SOD2 lead to the deacetylation and activation of SOD2, thereby reducing ROS level [38]. In this study, we found that TSC treatment could restrain phosphorylation of FOXO3a protein, and could also attenuate the acetylation of FOXO3a protein in MI/R rats and H9C2 cells stimulated with OGD/R. Moreover, the anti-phosphorylation and anti-acetylation effects of TSC on FOXO3a were abrogated by knockdown of SIRT3 in OGD/R injured H9C2 cells. Here, our study demonstrated that TSC upregulated SIRT3 expression to modulate the post-translational protein modification of FOXO3a, thus mitigating the MI/R injury.

SOD2 acts as the primary mitochondrial oxidative scavenger that can clear mitochondrial ROS by transforming toxic superoxide into hydrogen peroxide and diatomic oxygen [39]. The dysregulation of SOD2 has been suggested to be closely associated with various diseases

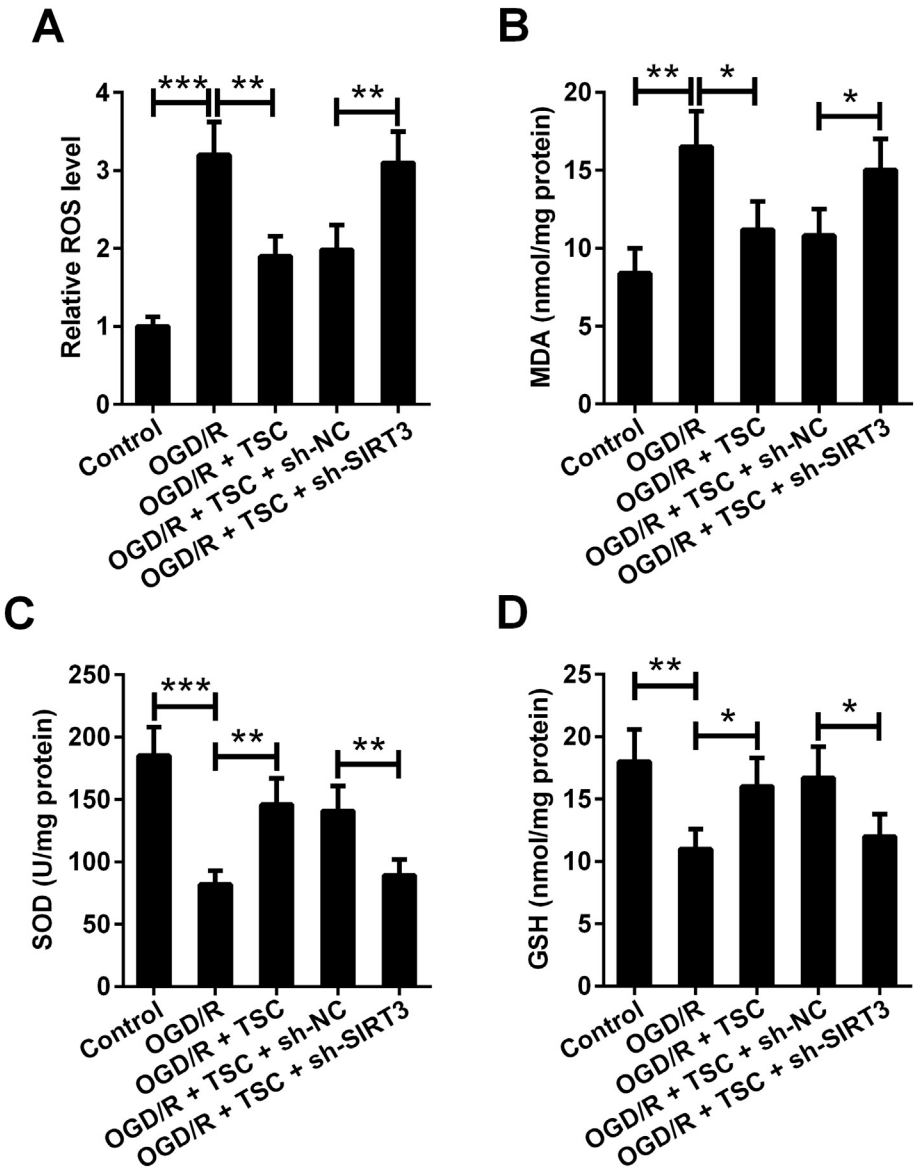

Fig. 6. SIRT3 knockdown blocks the anti-oxidative effect of TSC in OGD/R injured H9C2 cells. H9C2 cells were transfected with SIRT3 or sh-NC. After 4 h of OGD insult, H9C2 cells were treated with 0.5 μM TSC and then subjected to reoxygenation. At 24 after TSC treatment, the levels of ROS (A), MDA (B), SOD (C) and GSH (D) were measured using respective kits. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

[40]. Notably, SOD2 has been suggested to play a pivotal role in ischemic heart diseases [41]. Administration of irisin protected cardiomyocytes against I/R-induced oxidative stress and apoptosis *via* regulating the mitochondria-localization of SOD2 [42]. In this study, we found that TSC treatment markedly increased SOD2 protein levels, but decreased the acetylated protein level of FOXO3a in both *in vivo* and *in vitro* models of myocardial infarction. Nevertheless, downregulation of SIRT3 could block the effect of TSC on SOD2 expression. Consequently, the results of our study strongly indicate a regulatory function of TSC on the expression of SIRT3 and its downstream proteins FOXO3a and SOD2.

5. Conclusion

In general, the results of our study revealed that TSC exerts a cardioprotective effect in MI/R injury. TSC alleviated MI/R-induced myocardial oxidative stress, apoptosis and mitochondrial dysfunction *via* the SIRT3/FOXO3a/SOD2 signaling pathway. Our findings indicate that TSC might become an effective and viable therapeutic agent for the treatment of patients with myocardial infarction.

Author contribution statement

Guodong Chang: Designing the experiments, original draft preparation and project administration. Yingwei Chen: Performing the experiments. Hongwei Zhang: Performing the experiments. Wen Zhou: Data collection and analyzation.

Conflicts of interest

There is no conflict of interest regarding the publication of this paper.

Acknowledgement

This work was financially supported by the Specific Research Project of Science and Technology of Guangdong Provincial Hospital of Traditional Chinese Medicine (No. 2016KT1504, Wen Zhou).

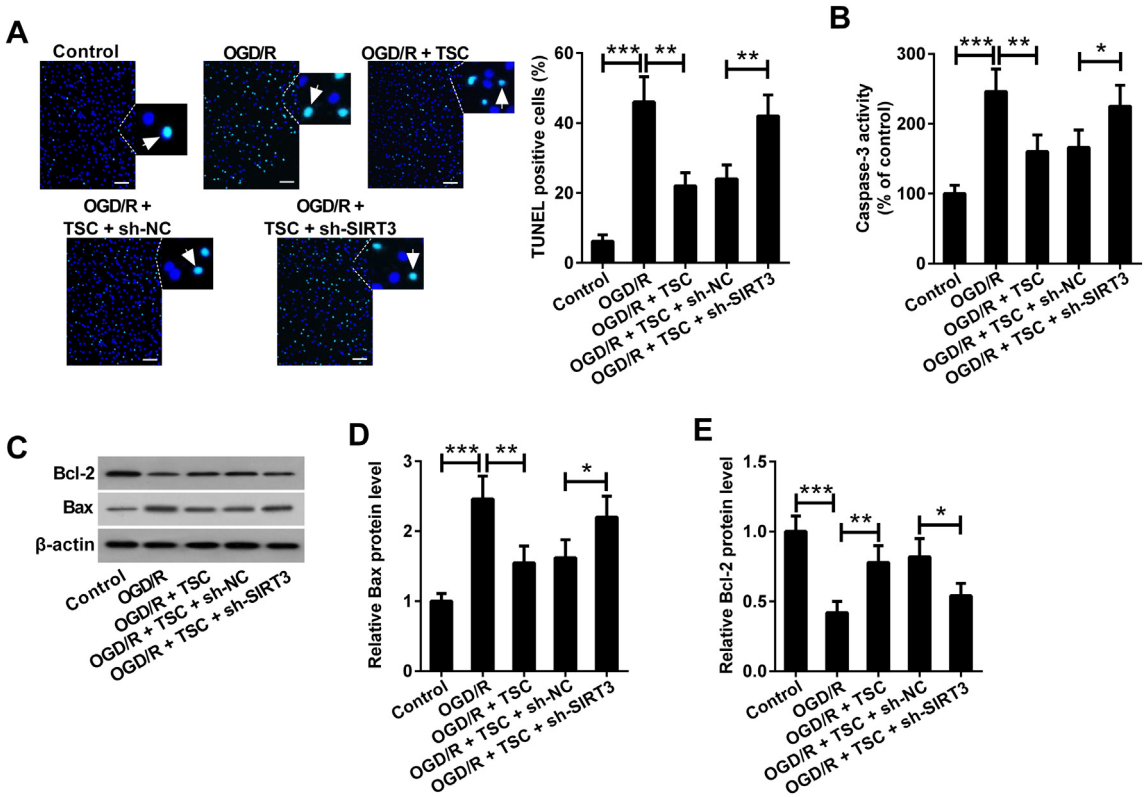

Fig. 7. SIRT3 knockdown blocks the anti-apoptotic effect of TSC in OGD/R injured H9C2 cells. H9C2 cells were transfected with sh-SIRT3 or sh-NC and then subjected to OGD/R. After 4 h of OGD, H9C2 cells were treated with 0.5 μM TSC and then subjected to reoxygenation for 24 h. (A) The apoptosis of H9C2 cells was determined by the TUNEL assay. Magnification, ×200. Scale bar: 100 μm. (B) The activity of caspase-3 was detected using a caspase-3 activity assay kit. (C) Representative images of western blot analysis of Bax and Bcl-2 expression. Quantification of Bax (D) and Bcl-2 (E) expression in Fig. 7C. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

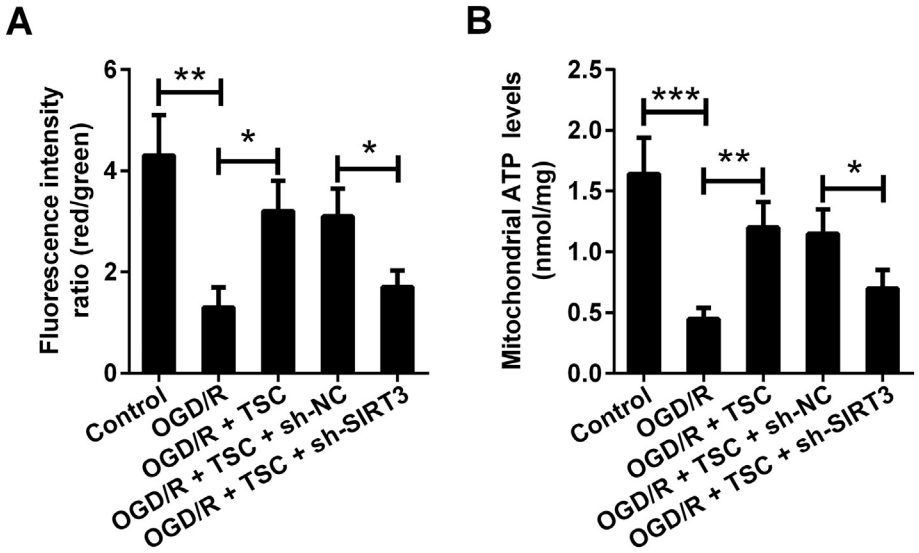

Fig. 8. SIRT3 knockdown blocks the protective effect of TSC on mitochondrial function of OGD/R injured H9C2 cells. (A) The loss of mitochondrial membrane potential was determined by JC-10 staining and represented as the ratio of red/green fluorescence intensity. (B) The level of mitochondrial ATP was detected by quantifying fluorescence produced by luciferase-catalyzed luciferin. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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