Abstract. Diabetic cardiomyopathy (DCM), one of the common diabetic complications, causes a high rate of mortality in patients with diabetes. Tanshinone IIA (TSIIA), one of the components of Salvia miltiorrhiza (Danshen), has anti-oxidative stress activity and is widely used to treat diabetes-associated diseases. However, its efficacy on DCM remains unclear. The present study aimed to investigate the potential therapeutic function of TSIIA on DCM in an experimental diabetic rat model. Streptozotocin (STZ)-induced diabetic rats were intraperitoneally injected with TSIIA for 6 weeks. The present results indicated that blood glucose concentration was slightly reduced in the low-dose TSIIA treatment group. TSIIA injection was also noted to improve cardiac function, and restore loss of mitochondrial cristae, swollen mitochondrial matrix and disorganized myofibrils in myocardial cells, which are thought to be characteristics of apoptosis. Furthermore, TSIIA injection could increase the activity of superoxide dismutase in STZ-induced diabetic rats, and suppress the endoplasmic reticulum (ER) stress signaling pathway via reducing the expression of glucose-regulated protein 78 and C/EBP homologous protein. These results provide evidence that TSIIA may ameliorate DCM in diabetic rats, possibly via suppressing oxidative stress and ER stress activation.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease that has become a major health problem worldwide. In 2014, an estimated 387 million individuals worldwide were diagnosed with DM and the prevalence is expected to rise to 592 million by 2035 (1). In addition, patients with DM have a high mortality rate that is often caused by diabetic cardiomyopathy (DCM) (2,3).

DCM is characterized by left ventricular hypertrophy and reduced diastolic function, and is a major complication in patients with either type 1 or type 2 DM (4). Previous studies have demonstrated that diverse pathogenic mechanisms contribute to DCM, including hyperglycemia, inflammation, fibrosis and apoptosis (5,6). Among these factors, cardiomyocyte apoptosis is thought to initiate cardiac remodeling and results in cardiac dysfunction (7). Therefore, it serves a key function in the pathogenesis and progression of DCM. Accumulating evidence has indicated that cardiomyocyte endoplasmic reticulum (ER) stress is involved in the pathogenesis of cardiac dysfunction in patients with DM and animal models (8,9). The early stages of ER stress are considered to be an adaptive response aiming at maintenance of ER homeostasis. This is known as the unfolded protein response (UPR), which is monitored by glucose-regulated protein 78 (Grp78), an ER chaperone (10). If it persists long-term, ER stress could induce the intrinsic pathway of apoptosis (11). C/EBP homologous protein (CHOP), a marker of ER stress, was identified to be elevated in multiple organs of a diabetic animal model, including myocardial tissue (9,12,13). Furthermore, CHOP knockout mice were observed to exhibit less pronounced hypertrophy and cardiac dysfunction in comparison with wild-type animals (14).

Tanshinone IIA (TSIIA), a phytochemical derived from the roots of Salvia miltiorrhiza, has the effect of suppressing apoptosis via multiple pathways (15,16). In previous studies, it has been demonstrated to inhibit ER-induced apoptosis in certain tissues (17,18). Therefore, it was hypothesized that TSIIA could improve cardiac function by inhibiting ER stress-induced apoptosis. The present study performed comparative studies on ER stress-associated signaling proteins Grp78 and CHOP to explore the mechanism of TSIIA on protecting cardiac function in a streptozotocin (STZ)-induced diabetic rat model.

Materials and methods

Animals. The experimental procedures were reviewed and approved by the Committee for the Care and Use of Laboratory
Animals at Zhejiang Chinese Medical University (Hangzhou, China). A total of 40 6-week-old male Sprague-Dawley rats (160-180 g; Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were individually housed in separately ventilated cages at a temperature of 23±2°C and humidity of 55±2% with a 12-h light/dark cycle, and were given free access to standard food and water.

Materials. TSIIA was purchased from Chiiatai Qingchunbao Pharmaceutical Co., Ltd., (Hangzhou, China). STZ was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The blood glucose test machine (FreeStyle Optium Neo) and strips were purchased from Abbott Laboratories (Lake Bluff, IL, USA). Grp78 (cat. no. 3813) and CHOP (cat. no. 2895) antibodies were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). The β-actin antibody (cat. no. AbDI214I) was purchased from Bio-Rad Laboratories, Inc., (Hercules, CA, USA).

Induction of diabetes in rats. A total of 40 male rats were randomized into the following groups (n=10 per group): Control group, DM group, DM rats treated with lower-dose TSIIA (L-TSIIA group) and DM rats treated with high-dose TSIIA (H-TSIIA group). DM was induced by a single intraperitoneal injection of STZ (dissolved in 10 mM citrate buffer, pH 4.5) at a dosage of 60 mg/kg body weight to induce diabetes. Rats in groups with TSIIA injection were intraperitoneally administered daily at a dose of 2 mg/kg (L-TSIIA group) and 4 mg/kg (H-TSIIA group) for 6 weeks, while the rats in the control and diabetes groups were injected with the same volume of vehicle (PBS). At day 2 after STZ injection, tail-vein blood glucose concentration was measured. Rats with blood glucose level >16 mmol/l were identified as diabetic model rats. Body weight and fasting blood glucose were determined at the end of week 6. TSIIA was dissolved in DMSO at a concentration of 10 mg/ml and then, diluted to 0.5 mg/ml with PBS prior to injection.

Blood and tissue sample preparation. At the end of week 6 following STZ injection, rats were anesthetized in an induction chamber with 2-3% isoflurane (Abbott Laboratories), then intraperitoneally injected with 1% pentobarbital sodium salt (30 mg/kg; Sigma-Aldrich; Merck KGaA). Blood was collected from carotid arteries and left at room temperature for 1 h. The blood samples were subsequently centrifuged at 1,500 x g for 15 min, followed by the separation of serum from the blood cells. Serum was preserved at -20°C for evaluating high-sensitivity superoxide dismutase (SOD), which was collected from blood cells. Serum was preserved at ‑20˚C for evaluating Mitochondrial subpopulation densities were determined within a defined region (100 µm²) with a minimum of 12 images in each group, which were taken at x5,900 magnification, as described previously (19). Analysis was performed using Image J software (v.1.46; National Institutes of Health, Bethesda, MD, USA).

Western blotting. Total protein from rat myocardium tissues was extracted using ice-cold RIPA buffer (cat. no. P0013B; Beyotie Institute of Biotechnology, Haimen, China). Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Inc.). Protein (30 µg) was separated by 12% SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 5% non-fat milk for 2 h at room temperature, then incubated overnight at 4˚C with primary antibodies (monoclonal anti-CHOP (cat. no. 2895; Cell Signaling Technology, Inc.; 1:1,000) and polyclonal GRP-78 (1:1,000; cat. no. 3177; Cell Signaling Technology, Inc.). After three washes with TBST, blots were incubated with horseradish peroxidase conjugated anti-mouse secondary antibodies (1:10,000; cat. no. BA1050; Wuhan Boster Biological Technology, Ltd., Wuhan, China) and horseradish peroxidase conjugated anti-rabbit secondary antibody (1:10,000; cat. no. BA2006; BOSTER Biological Tech Co., Ltd., Wuhan, China) for 1 h at room temperature. The immunoreactive bands were visualized using a chemiluminescent reagent as recommended by the supplied instructions.

RT-qPCR. Myocardial tissue preserved in liquid nitrogen was used for RT-qPCR analysis. Total RNA was isolated from the specimens using the TRIzol reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Tokyo, Japan). qPCR was performed using a 7300 Real Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). cDNA was treated daily at a dose of 2 mg/kg (L-TSIIA group) and 4 mg/kg (H-TSIIA group) for 6 weeks, while the rats in the control and diabetes groups were injected with the same volume of vehicle (PBS). At day 2 after STZ injection, tail-vein blood glucose concentration was measured. Rats with blood glucose level >16 mmol/l were identified as diabetic model rats. Body weight and fasting blood glucose were determined at the end of week 6. TSIIA was dissolved in DMSO at a concentration of 10 mg/ml and then, diluted to 0.5 mg/ml with PBS prior to injection.

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RT-qPCR. Myocardial tissue preserved in liquid nitrogen was used for RT-qPCR analysis. Total RNA was isolated from the specimens using the TRIzol reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Tokyo, Japan). qPCR was performed using Power SYBR Green qPCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). cDNA was then rinsed in 2.5% glutaraldehyde at 4°C for 4 h. The tissues were rinsed in buffer and post-fixed with 1% osmium tetroxide for 1 h at room temperature, dehydrated in graded alcohol, then transferred to propylene oxide. The tissues were embedded gradually in blocks of Epon 812 resin for 2 days at 60°C undergoing a graded ethanol dehydration series, and infiltrated using a mixture of 50% propylene oxide and 50% resin overnight. Then, 24 h later, the tissues were embedded in pure resin for 2 days at 60°C. Then, 120 nm-thick sections were cut using a histology diamond knife on an Ultracut E microtome (Leica Microsystems GmbH, Wetzlar, Germany) and stained with 4% uranyl acetate for 20 min and with 0.5% lead citrate for 5 min at room temperature. The ultrastructure of mitochondria and myofibrils was observed under TEM (Philips Tecnai 10; Philips Medical Systems B.V., Eindhoven, The Netherlands). Mitochondrial subpopulation densities were determined within a defined region (100 µm²) with a minimum of 12 images in each group, which were taken at x5,900 magnification, as described previously (19). Analysis was performed using Image J software (v.1.46; National Institutes of Health, Bethesda, MD, USA).

TEM. Following perfusion with 4% paraformaldehyde, the myocardial tissue from the left ventricle was dissected, then
TEM examination revealed that mitochondrial structure was
rescued in the diabetes group (P<0.05; Table I). TSIIA injection rescues injured mitochondrial structure and the diabetes group (20.78±2.88 mmol/l) at 6 weeks when compared with
the concentration was significantly decreased in the L-TSIIA groups (Table I). Furthermore, blood glucose was significantly decreased in the L-TSIIA group (20.78±2.88 mmol/l) at 6 weeks following STZ injection in the diabetes group as
compared with the control group (P<0.01; Table I). TSIIA application during treatment could reduce Grp78 and CHOP expression (P<0.05; Fig. 2). This suggested that
TSIIA treatment could reduce ER stress-asso-
ciation. Grp78 and CHOP expression was significantly enhanced in the diabetes group as
compared with the control group (P<0.05; Fig. 2). However, disordered myofibrils were observed in the myocardial cells of diabetic rats (Fig. 1B), but not the
control group. Administration of TSIIA was observed to attenuate the damage induced by hyperglycemia (Fig. 1C and D). Furthermore, it was observed that the mitochondrial density was significantly decreased in the diabetes group (P<0.01),
which was rescued by H-TSIIA treatment (P<0.05 vs. diabetes group; Table II).

Expression of ER stress-associated proteins. The mRNA
and protein expression levels of Grp78, an ER chaperone, and CHOP, a protein crucial to growth arrest and DNA damage, were measured. According to RT-qPCR
and western blot analysis results, the gene and protein levels of Grp78 and CHOP were significantly enhanced in the diabetes group as
compared with the control group (P<0.05; Fig. 2). However, TSIIA application during treatment could reduce Grp78 and CHOP expression (P<0.05; Fig. 2). This suggested that
ER stress was activated in myocardium tissue of diabetic rats, which could be suppressed by TSIIA treatment. These results indicate that TSIIA may be able to rescue injured

### Table I. Effect of TSIIA on body weight and blood glucose concentration.

<table>
<thead>
<tr>
<th>Group</th>
<th>Prior to STZ injection</th>
<th>2 days post-STZ injection</th>
<th>6 weeks post-STZ injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>133.27±3.67</td>
<td>141.59±4.92</td>
<td>404.85±31.48</td>
</tr>
<tr>
<td>Diabetes</td>
<td>134.16±4.03</td>
<td>142.02±5.63</td>
<td>299.26±47.14</td>
</tr>
<tr>
<td>L-TSIIA</td>
<td>136.40±4.23</td>
<td>144.20±5.51</td>
<td>311.99±49.92</td>
</tr>
<tr>
<td>H-TSIIA</td>
<td>130.08±5.39</td>
<td>138.68±6.55</td>
<td>289.50±42.18</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation (n=12). *P<0.01 vs. control group; **P<0.05 vs. diabetes group. TSIIA, Tanshinone IIA; STZ, streptozotocin; Diabetes, STZ-induced diabetic rats; L-TSIIA, diabetic rat treated with low dose TSIIA (2 mg/kg/day); H-TSIIA, diabetic rat treated with high dose TSIIA (4 mg/kg/day).

### Table II. Quantification of mitochondrial density under transmission electron microscopy.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mitochondrial density/100 µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47.56±12.90</td>
</tr>
<tr>
<td>Diabetes</td>
<td>28.12±8.39</td>
</tr>
<tr>
<td>L-TSIIA</td>
<td>28.14±8.39</td>
</tr>
<tr>
<td>H-TSIIA</td>
<td>40.62±14.47</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation (n=12). *P<0.01 vs. control group; **P<0.05 vs. diabetes group. TSIIA, Tanshinone IIA; Diabetes, streptozotocin-induced diabetic rats; L-TSIIA, diabetic rat treated with low dose TSIIA (2 mg/kg/day); H-TSIIA, diabetic rat treated with high dose TSIIA (4 mg/kg/day).
mitochondrial structure by hyperglycemia via inhibition of the ER stress response.

**TSIIA reduces the expression of SOD.** Oxidative stress could trigger ER stress activation (21). SOD, as an important antioxidant, was measured in the serum of rats to investigate the mechanism of TSIIA protective effects on DCM. As indicated in Fig. 3, SOD activity was significantly decreased in diabetic rats (22.64±8.18 U/ml) when compared with the control group (60.74±16.92 U/ml; P<0.001). TSIIA treatment significantly increased SOD activity in L-TSIIA (P<0.001) and H-TSIIA rats (P<0.05) when compared with non-treated diabetic rats.

**Discussion**

DCM, which is now recognized to have a high prevalence among patients with diabetes, leads to structural and functional changes of myocardial tissue (22). The aim of the current
study was to investigate the effect and mechanism of TSIIA on cardiac dysfunction in a diabetic rat model. The current results indicated that improved cardiac pathological changes were accompanied by rescued myocardial damage following TSIIA treatment. This suggested that TSIIA exerts a beneficial effect on DCM. The underlying mechanism was also investigated in the present study; to the best of our knowledge, this has not been reported previously.

As a key component of S. miltiorrhiza, TSIIA has been used to treat multiple diseases, including Alzheimer's disease, chronic kidney disease, cerebral ischemic injury and DCM (23-26). Furthermore, previous studies have demonstrated that TSIIA could attenuate myocardial ischemia reperfusion injury due to its antioxidant capacity (27). In the present study, it was observed that SOD activity was significantly reduced in the serum of diabetic rats compared with the control group, while TSIIA at different dosages could elevate the activity of SOD. SOD is known to be a major scavenger of superoxide radicals, which may attenuate oxidative stress (28). Therefore, it is proposed that TSIIA may alleviate the injury induced by hyperglycemia via increased SOD activity. In the current study, the ultrastructure of mitochondria and myofibrils was also observed under TEM. Previous studies have reported that STZ-induced hyperglycemia elevates free radicals and represses antioxidant defense mechanisms, which may lead to cell disruption, degenerated mitochondria and disordered myofibrils (29,30) and may induce DCM after 6 weeks (31). Consistent with these findings, in the current study, a lack of mitochondrial cristae and disordered myofibrils were observed in diabetic rats, which was accompanied by a reduction in mitochondrial density. However, it was identified that mitochondrial cristae were partially restored, and mitochondrial density was rescued, by application of TSIIA, which may be associated with increased activity of SOD. A previous study also reported that mitochondrial structure plays an important role in cardiac function (32). Degradation in mitochondrial structure results in a progressive increase in mitochondrial Ca²⁺ sequestration and mitochondrial depolarization, finally leading to cardiac dysfunction (33).

Previous research has identified direct associations between hyperglycemia, oxidative stress and protein folding (21). Alterations in redox state could result in unfolded/misfolded proteins accumulating in the ER and UPR being initiated, generally referred as ER stress (34). Accumulation of unfolded/misfolded proteins could also increase reactive oxygen species production and therefore exacerbate ER stress. One major goal of UPR/ER stress is to induce ER chaperones that promote protein folding, which is controlled by the ER chaperone, Grp78 (35). Activation of Grp78 protein expression is a widely used indicator of ER stress (21). As a key mediator of ER stress-induced apoptosis. This results suggested that ER stress was activated in myocardial tissue of the STZ-induced diabetic animal model. However, the expression of Grp78 was significantly decreased following TSIIA treatment when compared with that in the diabetic group. Furthermore, CHOP is involved in one of the ER stress-induced cell apoptosis signaling pathways that could trigger cell apoptosis (36). In the present study, TSIIA inhibited CHOP apoptosis signaling pathways. Therefore, it was suggested that TSIIA may alleviate cardiomyocyte ER stress-induced apoptosis.

The present results demonstrated that TSIIA may exert a therapeutic effect on DCM. TSIIA was observed to improve cardiac pathological changes, and this effect may be via inhibiting ER stress-mediated myocardial cell apoptosis.

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Availability of data and materials
All data generated or analyzed during the present study are included in this published article.

Authors' contributions
YZ designed the study. Diabetes animal model induction, blood and tissue sample preparation was performed by ST and LC. Sample preparation for TEM was done by JS, NZ and XS performed Western blot and real time PCR. RS and GG analyzed data. The manuscript was written by ST. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
The experimental procedures were reviewed and approved by the Committee for the Care and Use of Laboratory Animals at Zhejiang Chinese Medical University (Hangzhou, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


