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The antioxidant edaravone prevents cardiac dysfunction by suppressing oxidative stress in type 1 diabetic rats and in high glucose-induced injured H9c2 cardiomyoblasts

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1 The antioxidant edaravone prevents cardiac dysfunction by suppressing oxidative
2 stress in type 1 diabetic rats and in high glucose-induced injured H9c2
3 cardiomyoblasts

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Abstract

Edaravone, a radical scavenger, has been recognized as a potential protective agent for cardiovascular diseases. However, little is known about the effect of edaravone in cardiac complications associated with diabetes. Here, we have demonstrated that edaravone prevents cardiac dysfunction and apoptosis in the streptozocin induced-type 1 diabetic rat heart. Mechanistic studies revealed that edaravone treatment improved cardiac function and restored superoxide dismutase levels. In addition, treatment of diabetic animals by edaravone increased protein expressions of sirtuin-1 (SIRT-1), peroxisome proliferator activated receptor γ coactivator α (PGC-1 α), nuclear factor like-2 (NRF-2) and B-cell lymphoma 2 (Bcl-2), reduced protein expressions of Bax and Caspase-3 compared to the control group. High glucose incubation resulted in the production of reactive oxygen species (ROS) and cell death. Treatment of high glucose-incubated H9c2 cells by edaravone reduced ROS production and cell death. In addition, the treatment of high glucose-incubated H9c2 cells by edaravone increased the activity of anti-oxidative stress by increasing SIRT-1, PGC-1 α , and NRF-2 and this treatment also reduced apoptosis by increasing Bcl-2 expression and reducing Bax and Caspase-3 expressions. Knockdown SIRT-1 with small interferer RNA abolished the effects of edaravone. Overall, our data demonstrated that edaravone may be an effective agent against the development of diabetic cardiomyopathy.

Key words: edaravone, type 1 diabetes, oxidative stress, apoptosis.

1 **Introduction**
2

3 Cardiovascular disease is the leading cause of morbidity and mortality in patients
4 with type 1 diabetes (Huxley et al. 2015; Nathan 2015). Many etiological factors like
5 hyperglycemia, metabolic abnormalities may affect the physiology of the diabetic
6 heart. The overwhelming effects of all these factors lead to cardiac abnormalities
7 including myocardial dysfunction, myocardial hypertrophy and decreased left
8 ventricular compliance. The underlying molecular defect involved in the pathogenesis
9 of diabetic cardiomyopathy is not fully understood. Several mechanisms like
10 oxidative stress and cardiomyocytes apoptosis have been proposed for
11 hyperglycemia-induced cardiac injury in the diabetic heart (Kayama et al. 2015).
12 Oxidative stress is caused by imbalance of reaction oxygen species (ROS) generation
13 and ROS elimination in heart (Penckofer et al. 2002). A decrease in antioxidative
14 proteins is recognized to play an important role in genesis of diabetic cardiac
15 dysfunction and leads to apoptosis in cardiomyocytes, eventually induces heart failure
16 in diabetic heart(Hare 2001; Shen et al. 2007). These oxidative abnormalities may be
17 promising therapeutic targets for the treatment of diabetic cardiovascular
18 complications.

19

20 Edaravone (EDA), was approved in 2001 in Japan for the treatment of ischemia
21 stroke (Kikuchi et al. 2010). It has been shown that edaravone can diffuse into many
22 organs in addition to neurovascular system, edaravone exerts protective effects in a
23 number of tissue damage, including myocardial, muscle, lung, liver, pancreatic and

1 renal injury (Kikuchi et al. 2011; Liu et al. 2015; Naritomi et al. 2010; Song et al.
2 2015; Yang et al. 2010). The preventive effects of edaravone on cardiomyocytes
3 apoptosis, necrosis, as well as myocardial injury following ischemia reperfusion are
4 well documented (Kikuchi et al. 2012; Kikuchi et al. 2011; Tsujita et al. 2004). In
5 clinical studies, a reduction in antioxidant activity in heart occurs early after the
6 diagnosis of type 1 diabetes mellitus, which may result in the development of diabetic
7 cardiovascular complications (Marra et al. 2002). As an anti-oxidant regent, whether
8 edaravone protects heart function during the development of diabetes is unknown.
9 The mechanism of edaravone's ROS scavenger effects is still unclear. In the process
10 of ROS elimination, an intracellular regulatory protein Sirtuin-1 (SIRT-1) responds to
11 insulin sensitivity plays an important role in regulating anti-oxidant activity in
12 mitochondrial, SIRT-1 can bind to peroxisome proliferator activated receptor G
13 coactivator 1 α (PGC-1 α), which then can be deacetylated and activated. Activated
14 PGC-1 α can up-regulate the transcription of Nuclear factor (erythroid-derived 2)-like
15 2 (NRF-2), results in the induction of many antioxidant proteins (Nguyen et al. 2009;
16 Ping et al. 2015). In addition, edaravone reduces cardiomyocytes apoptosis by
17 increasing Bcl-2 and decreasing Bax and Caspase-3 (Rajesh et al. 2003), which may
18 also relate to its antioxidative effects (Hare 2001). In this study, we examined if
19 edaravone could attenuated cardiac dysfunction in type 1 diabetes rats. We showed
20 that edaravone improved cardiac function in type 1 diabetic rat heart and reduced
21 oxidative stress through its activation of SIRT-1/PGC-1 α /NRF-2 signal pathway and
22 in turn inhibited apoptosis by modulating Bcl-2/Bax /Caspase-3 signal pathway.

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Material and Methods

Materials

Edaravone was obtained from BoDa Pharmaceutical Factory (Jilin, China). Streptozotocin, Dulbecco's modification of Eagle's medium (DMEM), cell culture reagents, trypsin-EDTA solution, glucose, 3- (4,5-dimethylthiazol-2- yl)-2,5-diphenyl tetrazolium bromide (MTT) and reagents for Western blot analysis were obtained from Sigma-Aldrich (Beijing, China). Fetal bovine serum was obtained from Runsheng Biological Materials Co., Ltd. (Taiyuan, China). Polyclonal antibodies against SIRT-1, PGC-1 α , NRF-2, Bcl-2, Bax, secondary antibodies for Western blot analysis were obtained from Abcam (Beijing, China). Caspase-3 antibody was obtained from Cell signaling technology (Beijing, China). Polyvinylidene fluoride membranes were obtained from Millipore (Beijing, China). H9c2 rat cardiac myoblastic cells were obtained from Cell Resource Center (IBMS, Beijing).

Animals

Six week old male Wistar rats were housed individually in a room (constant temperature, 12/12 h light/dark cycles) and received standard laboratory rat diet and water ad libitum. All procedures were approved by the Ethics Committee for the Use of Experimental Animals of Jilin University.

Generation of type 1 diabetic animals

Type 1 diabetic model was induced with a single intraperitoneal injection of streptozocin (STZ) (60 mg/kg) in our rats as described previously (Vallejo et al. 2014). STZ was freshly dissolved in citrate buffer (0.1 M). Control animals received the buffer only. 7 days after the injection a drop of blood was collected from the tail vein and blood glucose concentration was determined by using a digital blood glucose meter (Accu-Chek® Sensor, Roche, Mannheim, Germany). Animals with a random blood glucose level >16.7 mmol/L were considered to be diabetic and were included into the study. Rats were divided randomly into three groups: vehicle-treated control (CON; $n = 12$), vehicle treated diabetic (Model; $n = 12$) and edaravone-treated diabetic (EDA; $n = 10$) groups. Animals were treated with saline or with edaravone (6 mg/kg/day) by intraperitoneal injection for 4 weeks.

Measurement of biological parameters

Body weight, Blood glucose (BG), Insulin (INS), malondialdehyde (MDA) and superoxide dismutase (SOD) in rat serum were determined using assay kits as per the manufacturer's instructions (Nanjingjiancheng Bio-engineering Institute, Nanjing, China)

Echocardiography

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Transthoracic two-dimensional M-mode echocardiogram pulsed wave Doppler spectral tracings were obtained from CON, Model and EDA animals using a Sequioa-512 Ultrasound System (Siemens AG, Munich, Germany) with a 15-MHz transducer in rats anesthetized using 0.3% Pentobarbital sodium (intraperitoneal injection) according to previously described (Chang et al. 2015). M-mode tracings were used to measure LV end-diastolic diameter (LVDD), percent ejection fraction (EF%) and interventricular septal thickness (IVS). Doppler flow velocities across the mitral valves were determined by measurement of early ventricular filling peak velocity (E wave) and late filling velocity (A wave) during the systolic and diastolic phase. E wave to A wave ratio (E/A) were used to determine diastolic function.

Hematoxylin and Eosin stain (H&E stain)

Hearts from CON, Model and EDA animals were fixed in 10% formalin and imbedded in paraffin. The morphology of myocardial was quantified in 4 mm paraffin sections after staining with Hematoxylin and Eosin stain. Transverse transnuclear widths (cardiomyocytes diameter) were measured of 100 longitudinally oriented, mono-nucleated cardiomyocytes. The cardiomyocyte diameter was calculated using IPP6.0 imaging software. 6 visual fields were randomly selected under a 40× magnification.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed to detect DNA strand breaks using a commercial kit (Roche, China). TUNEL positive cell nuclei were counted in 15 randomly selected fields of sections by IPP6.0.

Cell culture

H9c2 cells were maintained in low glucose DMEM with 10% fetal bovine serum, 2% L-glutamine, 10% sodium bicarbonate, 10% sodium pyruvate, 5% HEPES, 1% penicillin/streptomycin and 1% gentamycin in an incubator (37°C, 5% CO₂). For ROS fluorescence microscopy assay, H9c2 cells were plated on glass coverslips coated with poly-D-lysine in a 24-well plate. Cells were incubated with 35mM glucose plus or minus 30µM edaravone for 24h.

Cell viability assay

Cell viability was determined by MTT assay in 96-well tissue culture plates as described previously (Chang et al. 2013). After treatment, the culture medium was removed from the wells, and 200 µl of MTT reagent (Sigma) at a concentration of 1 mg/mL in PBS was added to each well. After 4 h incubation at 37°C, MTT reagent in PBS was removed and then the blue-colored formazan product was solubilized in 0.15 ml of Dimethyl Sulphoxide (DMSO) for 20 min. The absorbance of converted dye

1 was measured at a wave length of 570 nm.

2

3 *Reactive oxygen species measurements*

4

5 The level of intracellular ROS generation was assessed using the ELISA kit
6 (Shanghai Yanjing Biological Research Technology Co, LTD, China) according to
7 manufacturer's instruction. Furthermore, fluorescence microscopy was used to
8 confirm the results of the microplate ROS assay. Briefly, after the indicated
9 treatments, cells were washed twice with phosphate-buffered saline and then
10 incubated with serum-free DMEM containing 10 μ mol/l dichlorofluorescein diacetate
11 (DCFH-DA) at 37°C for 30 min. After that, cells were washed with PBS three times
12 in order to eliminate the residual DCFH-DA. Cells from each group were analyzed by
13 measuring the excitation and emission spectrum at 488 and 525 nm, respectively. The
14 fluorescence was visualized using a fluorescence microscope coupled with an image
15 analysis system Olympus IX71 system biological microscope.

16

17 *siRNA knockdown of SIRT-1*

18

19 H9c2 cells were seeded at 6 wells plate. Then transfections were carried out
20 using scramble small interfere RNA or sirtuin-1 small interfere RNA (SIRT-1 siRNA,
21 Santa Cruz Biotechnology) and transfection reagent (Inventrigen, Thermo Fisher
22 Scientific) according to the manufacturer's instructions. The transfection solution
23 (volume 400 μ l) was prepared in serum- and antibiotic-free DMEM (low glucose)

9

1 medium containing 0.5µg/well siRNA-SIRT-1 or scrambled siRNA and transfection
2 reagent. The mixture was incubated for 20 min at room temperature and then diluted
3 with 600 µl of DMEM medium and then added to the cells for 6 h. This was followed
4 by the addition of 1 ml DMEM containing 20% FBS to the cells and incubation for
5 another 10 h. Then cells were incubated with 35µM high glucose plus or minus EDA
6 for 24h.

7

8 *Western blot analysis*

9

10 Hearts from CON, Model and EDA animals were homogenized and protein
11 samples were prepared with ice-cold RIPA lysis buffer; in vitro western blot
12 experiments, upon completion of the experiments, cells were harvested using ice-cold
13 RIPA lysis buffer. Protein concentrations were measured using the bicinchoninic acid
14 assay (BCA assay, Pierce BCA Protein Assay Kit). For western blot detection of
15 SIRT-1, PGC-1α, NRF-2 and Bcl-2, Bax, Caspase-3 protein expressions, 70µg
16 proteins were used, all proteins were separated by 12 % SDS polyacrylamide gel and
17 electro transferred onto polyvinylidene fluoride membranes. Membranes were
18 blocked with 5 % (w/v) skim milk for 2 h at room temperature and then incubated
19 with primary antibodies with gentle agitation overnight at 4 °C. The membranes were
20 washed 3 times for 10 min each with 15 ml of TBST (10 mM Tris-HCl, 150 mM
21 NaCl and 0.1 % (v/v) Tween-20 and then incubated with secondary antibody at room
22 temperature for 2 h. Protein was visualized with enhanced chemiluminescence

10

1 solution and images generated with GENE Imaging system. The images were
2 quantified using Image Analysis Software (Quantity One).

3

4 *Statistical analysis*

5

6 Values are presented as mean $\pm$ S.E.M. An unpaired Student's *t* test was
7 performed for single comparisons between two groups. $P < 0.05$ was considered
8 statistically significant.

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Results

Edaravone treatment increases insulin and superoxide dismutase levels, but does not alter body weight and blood glucose

Type 1 diabetic rats were killed after 4 weeks with or without EDA treatment.

No difference in body weight was observed between Model and EDA rats (Table 1).

In contrast, serum insulin (INS) was increased in EDA treated animals compared to

Model. Serum glucose levels were unaltered between Model and EDA animals.

Serum lipid peroxidation was assessed by analysis of MDA and SOD, biomarkers of

oxidative stress. Serum MDA levels were decreased in EDA animals compared to

Model animals, serum SOD levels were increased in EDA animals compared to

Model. Thus, EDA treatment increases serum insulin and SOD levels but does not

alter body weight and serum glucose in type 1 diabetic animals.

Edaravone treatment attenuates cardiac dysfunction induced by type 1 diabetes

To assess the effect of edaravone on cardiac function in type 1 diabetic rats,

echocardiography was performed in CON, Model and EDA groups one week prior to

sacrifice. Systolic and diastolic dysfunction developed in type 1 diabetic animals

compared to CON, EDA treatment attenuated these changes (Fig. 1A, B). E/A, EF%

and LVDD were significantly decreased in Model group compared to CON (Fig.1C,

D, F). Treatment of Model animals with edaravone restored E/A, EF% and LVDD to

that of CON animals. In addition, IVS was significantly increased in Model animals

1 compared to CON (Fig.1E), treatment of Model animals with EDA restored IVS to
2 that of the control group. Thus, EDA treatment improves cardiac function in type 1
3 diabetic rats.

4

5 *Edaravone reduces cardiomyocytes hypertrophy and apoptosis in type 1 diabetic rats*

6

7 To evaluate the effect of edaravone on cardiac hypertrophy, cardiomyocytes
8 diameter were measured in H&E staining. When compared with controls, the
9 cardiomyocytes diameters in Model animals were significantly increased, indicating
10 cardiomyocytes hypertrophy occurred. However, this increase was prevented by EDA
11 treatment (Fig.2A).

12

13 Apoptosis of cardiomyocytes were measured by DNA fragmentation in TUNEL
14 staining. TUNEL-positive nuclei were rare or absent in the hearts of control animals,
15 whereas the number of TUNEL positive nuclei was significantly increased in diabetic
16 animals (Fig.2B). Treatment with edaravone significantly decreased the number of
17 TUNEL-positive nuclei in the myocardium.

18

19 *Edaravone treatment increases cardiac antioxidant protein expressions and reduces*
20 *apoptotic protein expressions*

21

22 To explore the possible mechanisms of EDA in protection of myocardium injury
23 in type 1 diabetic rats, several key antioxidant transcriptional gene proteins were

13

1 examined including SIRT-1, PGC-1 α and NRF-2. As shown in Figure 3, SIRT-1,
2 PGC-1 α and NRF-2 protein expressions were significantly decreased in diabetic
3 animals compared to CON, treatment of diabetic animal with EDA increased SIRT-1,
4 PGC-1 α and NRF-2 protein expressions compared to diabetic animals.

5
6 We further examined expressions of apoptotic related proteins in CON, Model
7 and EDA animals. Bcl-2, which was an anti-apoptotic protein, were decreased in
8 diabetic animals compared to control, treatment of diabetic animals with EDA
9 reversed this reduction. Bax and Caspase-3 were increased in Model animals
10 compared to CON, treatment of diabetic animals with EDA significantly decreased
11 the expressions of Bax and Caspase-3. Thus, EDA treatment increases SIRT-1,
12 PGC-1 α , NRF-2 and Bcl-2 and reduces Bax and Caspase-3 (Fig.4A, B, C).

13
14 *Edaravone treatment attenuates high glucose-induced cardiomyocytes cytotoxicity*
15 *and ROS generation*

16
17 High glucose level is believed to be a major contributing factor in the
18 pathogenesis of diabetic complications. As an in-vitro model, we examined if
19 edaravone treatment altered cellular apoptosis and reactive oxygen species generation
20 in high glucose incubated H9c2 cardiac myocytes. High glucose (35mM)
21 concentration in cell culture medium was used to mimic the high glucose in our type 1
22 diabetic rat model. H9c2 cells were incubated for 24 h with EDA (30 μ M) or high
23 glucose with EDA, and cytotoxicity was examined. Incubation of cells with 0-30 μ M

1 EDA has no cytotoxicity (Fig.5A). In contrast, incubation of cells with 35mM glucose
2 resulted in significant cytotoxicity. EDA (30µM) prevented the cell death induced by
3 high glucose incubation in H9c2 cells (Fig.5B). We next examined the ROS
4 generation in high glucose treated H9c2 cells. Incubation of H9c2 cells with 35mM
5 glucose resulted in a remarkable increase in ROS generation compared to untreated
6 cells (Fig.5C, D), EDA treatment significantly reduced ROS generation induced by
7 high glucose. Thus EDA treatment reduces high glucose-induced cell cytotoxicity and
8 ROS generation.

9

10 *Edaravone treatment increases SIRT-1, PGC-1α, NRF-2 and Bcl-2 and reduces Bax*
11 *and Caspase-3.*

12

13 Since high glucose incubation of H9c2 cells resulted in ROS generation and cell
14 cytotoxicity, EDA treatment reduces ROS generation and increases cell viability. We
15 next examined if antioxidant and apoptosis proteins was playing a role in the
16 regulation effect of EDA on high glucose induced H9c2 cell injury. Treatment of
17 H9c2 cells with high glucose resulted in the reduction in SIRT-1, PGC-1α and NRF-2
18 protein expression compared to control. EDA treatment in high glucose-treated cells
19 significantly increased the expression of SIRT-1, PGC-1α and NRF-2 (Fig.6A, B, C).
20 In addition, Bcl-2 was reduced in high glucose treated H9c2 cells compared to
21 untreated control, treatment of high glucose treated H9c2 cells with EDA increased
22 Bcl-2 protein levels. Bax and Caspase-3 were increased in high glucose treated H9c2

15

1 cells compared to control, EDA treatment significantly reduced the expression of Bax
2 and Caspase-3 in high glucose treated H9c2 cells (Fig.7A, B, C). Thus, EDA
3 treatment increases the expression of SIRT-1, PGC-1 α , NRF-2 and Bcl-2 and reduces
4 the expression of Bax and Caspase-3 in high glucose-induced cardiomyocytes injury.

5

6 *Knockdown SIRT-1 diminished edaravone-regulated protein expression in high*
7 *glucose-incubated H9c2 cardiomyoblasts.*

8

9 As SIRT-1 regulates cell growth and survival in response to oxidative stresses,
10 we next examined if knockdown SIRT-1 mRNA could abolish the protective effects
11 of EDA. The results showed that knockdown SIRT-1 mRNA by small interfere RNA
12 caused a reduction in SIRT-1 protein expression, accompanied by decrease of NRF-2
13 expression in high glucose treated H9c2 cells. In addition, after knockdown SIRT-1,
14 the expressions of Bax and Caspase-3 were markedly increased and the expression of
15 Bcl-2 was reduced in EDA treated cells (Fig.8). Thus, EDA treatment attenuates high
16 glucose induced apoptosis in H9c2 cells by increasing SIRT-1 and NRF-2.

17

1 **Discussion**

2

3 Diabetes leads to cardiac structural and functional disturbances in the
4 myocardium. It is widely recognized that cardiomyocyte hypertrophy, fibrosis and
5 apoptosis are present in diabetic heart (Fuentes-Antras et al. 2015; Trachanas et al.
6 2014). Several studies have described that increased oxidative stress may be primarily
7 responsible for the development of diabetic cardiovascular diseases (Liu et al. 2014;
8 Trachanas et al. 2014; Wold et al. 2005). Many compounds and drugs with
9 antioxidant properties prevent development and progression of diabetes-induced
10 cardiovascular injury (Abdel-Hamid and Firgany Ael 2015; Call et al. 2015; Kajstura
11 et al. 2001; Suzuki et al. 2015; Umbarkar et al. 2015). Recently, the protective effects
12 of edaravone on cardiovascular disease have attracted much attention. Edaravone has
13 preventive effects on myocardial injury following ischemia and reperfusion, as well as
14 myocarditis-induced dilated cardiomyopathy and has beneficial effects on the
15 impaired oxidant/antioxidant status of heart in VPA-induced toxicity
16 (Emekli-Alturfan et al. 2015; Tsujita et al. 2004; Yamawaki et al. 2004; Yamazaki et
17 al. 2009). As a free radical scavenger, whether edaravone protects against
18 diabetes-related myocardium injury is still unknown. In the present study, type 1
19 diabetes induced diabetic rats exhibited systolic and diastolic dysfunction and
20 increased in cardiomyocytes diameter. Treatment of these animals with EDA
21 prevented cardiac functional loss and cardiomyocytes hypertrophy. Also, treatment of
22 high glucose treated H9c2 cells with EDA reduced ROS generation. The mechanism
23 for the improvement of cardiac functional in edaravone treated rats was related the

1 increase of SIRT-1, PGC-1 α and NRF-2 expressions. Apoptosis of cardiomyocytes is
2 another important outcome of hyperglycemia-induced inflammation and oxidative
3 stress in the heart. In current study, edaravone also protected cardiomyocytes from
4 apoptosis in diabetic animals and in high glucose-induced apoptotic H9c2 cells by
5 reduction of Bax and Caspase-3 and increased of Bcl-2 expression, these observations
6 indicated that mechanism of action of edaravone attributed to anti-apoptosis in
7 addition to its anti-oxidant activity.

8

9 SIRT-1, a histone deacetylase, plays an important role in the regulation of ROS
10 generation in the heart (Lai et al. 2013). Deacetylation reflects its main task, this
11 deacetylase protects from endothelial dysfunction, atherothrombosis, diet-induced
12 obesity, type 2 diabetes and myocardial infarction (Breitenstein et al. 2011; Guarani et
13 al. 2011; Hsu et al. 2010). SIRT-1 directly deacetylated PGC-1 α and activated
14 PGC-1 α , overexpression of PGC-1 α in vascular endothelial cells increased
15 mitochondrial antioxidant enzyme expression, and decreased oxidative stress and cell
16 death (Lu et al. 2010b). Activated PGC-1 α can bind to NRF-2, in turn positively
17 regulates the expression of several ROS detoxifying enzymes such as superoxide
18 dismutase (SOD) and decreased oxidative stress (Nojiri et al. 2006; Nonn et al. 2003).
19 The preventive effect of PGC-1 α /NRF-2 activation on cardiovascular and diabetic
20 cardiovascular diseases has been reported by several groups (Ping et al. 2015).
21 Enhanced SIRT-1 and NRF-2 protected the heart from exhaustive exercise-induced
22 injury and increased NRF-2 prevent diabetes-induced high blood pressure and cardiac

dysfunction as well as cardiac hypertrophy, fibrosis, oxidative damage, and inflammation (Cheng et al. 2013; Nguyen et al. 2009; Uruno et al. 2013). In contrast, NRF-2 knockout mice exhibited cardiac hypertrophy and left ventricular dysfunction (Velmurugan et al. 2013). In the current study, edaravone treatment reduced ROS generation in high glucose treated H9c2 cells and inhibited cardiomyocytes hypertrophy via activation of SIRT-1, PGC-1 α and NRF-2.

Cardiomyocyte apoptosis also plays an important role in the development of diabetes-induced decreased cardiac function and myocardial hypertrophy (Cai et al. 2002). As a result of diabetes progression and high glucose induced cell apoptosis, a progressive rise in DNA damage levels that was accompanied by increasing activation of proteins involved in the mitochondria-mediated apoptotic pathway. Crucial events in apoptosis are the translocation of the pro-apoptotic Bax protein from the cytoplasm to the mitochondria where it forms an oligomeric pore in the outer membrane, and the release of Bcl-2, an anti-apoptotic protein that prevents Bax oligomerization. The formation of oligomeric pore in mitochondrial outer membrane release cytochrome C, in turn activates caspase 3, which are responsible for apoptosis within cell (Ivanovic-Matic et al. 2014; Lu et al. 2010a; Ryu et al. 2015). In our present study, edaravone treatment reduces TUNEL stained DNA damage via activation of Bcl-2 and inhibition of Bax and Caspase-3. In addition, numerous experimental studies demonstrate that ROS are involved in cardiomyocyte apoptosis. Doxorubicin-derived ROS is responsible for its toxicity to myocardium and lead to cardiomyocytes

1 apoptosis, and suppressing the elevation of reactive oxygen species improve high
2 glucose-induced cardiomyocytes apoptosis in mouse myocardium (Cai et al. 2002). In
3 our experiments, treatment of high glucose-treated H9c2 cells with edaravone reduced
4 ROS generation and this was accompanied by reducing Bax, Caspase-3 and increasing
5 Bcl-2. In addition, knockdown SIRT-1 resulted in a decreased NRF-2, hence
6 increased apoptotic protein expression. These data suggest that anti-apoptotic effect of
7 edaravone in the diabetic heart may be regulated, in part, through its anti-oxidant
8 action.

9 In summary, our findings demonstrate that edaravone treatment improves cardiac
10 dysfunction in diabetic rats and reduces oxidative stress and apoptosis in high
11 glucose-induced apoptotic H9c2 cardiomyocytes. The mechanisms underlying these
12 beneficial effects are increased activities of SIRT-1, PGC-1 α , NRF-2 and Bcl-2 and
13 reduced activities of Bax and Caspase-3. The present study suggested that edaravone
14 may be a candidate drug for the treatment of diabetes-induced cardiovascular
15 diseases.

16

17

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Draft

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14

1 Table 1. Body weight and blood biochemical parameters in diabetic rats.

	Body weight (g)	Blood Glucose (mmol/L)	INS (mIU/L)	MDA (nmol/ml)	SOD (U/ml)
Control	344±11.3	6.6±0.10	6.02±0.29	6.12±0.62	306.6±6.35
Model	249±10.5 ^a	30.2±1.16 ^a	2.89±0.27 ^a	8.85±1.25 ^a	256.4±6.49 ^a
EDA	259±9.5	27.2±1.33	5.08±0.18 ^b	6.07±0.78 ^b	292.9±9.22 ^b

2

3 Body weight and blood biochemical parameters were determined in Control, Model
 4 and EDA(6mg/kg, ip) animals as described in Materials and Methods. Values
 5 represent the mean±S.E.M ($n=8$). ^a $P<0.05$ vs. Control, ^b $P<0.05$ vs. Model.

6

7

1 Figure legends

2

3 Figure 1. Echocardiographic analysis in diabetic rats.

4

5 Echocardiography was performed on Control, Model and EDA animals one week
6 prior to sacrifice as described in Material and Methods. A. Mitral valvular inflows
7 showing E wave and A wave (E wave: LV early-filling wave; A wave: filling from
8 atrial contraction). B. Left ventricular end-diastolic diameter (D). C. Ratio of the early
9 to late peak diastolic trans-mitral flow velocity (E/A). D. Percentage of ejection
10 fraction(EF%). E. Inter-ventricular Septum thickness (IVS). F. Left ventricular
11 internal dimension-diastolic (LVDD). Data represent the mean±S.E.M ($n=8$). * $P<0.05$,
12 ** $P<0.01$ vs Control, # $P<0.05$, ## $P<0.01$ vs Model.

13

14 Figure 2. Edaravone reduces cardiomyocytes diameters and apoptosis.

15

16

17 A. Hematoxylin and eosin staining of left ventricular tissue slices depicting
18 cardiomyocyte hypertrophy and bar graph showing the average cardiomyocyte
19 diameter of 100 cells per field ($\times 40$). Values given are from three independent
20 measurements. B. TUNEL stained myocardial tissue sections for apoptotic nuclei and
21 bar graph showing the average number of TUNEL positive apoptotic nuclei/field.
22 * $P<0.05$, ** $P<0.01$ vs Control, # $P<0.05$, ## $P<0.01$ vs Model.

Figure 3. Edaravone treatment increases SIRT-1, PGC-1 α and NRF-2 expression.

Expression of SIRT-1 (A), PGC-1 α (B) and NRF-2 (C) were determined in Control, Model and EDA animals as described in Materials and Methods. Representative Western blots are depicted. Data represent the mean $\pm$ S.E.M ($n=6$). * $P<0.05$, ** $P<0.01$ vs Control, # $P<0.05$ vs Model.

Figure 4. Edaravone treatment reduces Bax, Caspase-3 and increases Bcl-2 expression.

Expression of Bax (A), Bcl-2 (B) and Caspase-3 (C) were determined in Control, Model and EDA animals as described in Materials and Methods. Representative Western blots are depicted. Data represent the mean $\pm$ S.E.M ($n=6$). * $P<0.05$, ** $P<0.01$ vs Control, # $P<0.05$, ## $P<0.01$ vs Model.

Figure 5. Edaravone treatment restores cell viability in high glucose-cultured H9c2 cardiomyoblasts.

A. H9c2 cells were incubated with various concentrations of edaravone and cell viability determined as described in Materials and Methods. B. H9c2 cells were

1 incubated with high glucose plus or minus EDA (30 μ M) and cell viability determined
2 as described in Materials and Methods. C. H9c2 cells were incubated with high
3 glucose (HG) plus or minus EDA and ROS generation determined by fluorescence (C)
4 and ELISA (D) as described in Materials and Methods. A representative micrograph
5 is depicted. Data represent the mean $\pm$ S.E.M ($n=6$). * $P<0.05$, ** $P<0.01$ vs control, #
6 $P<0.05$, ## $P<0.01$ vs HG.

7

8 Figure 6. Edaravone treatment increases SIRT-1, PGC-1 α and NRF-2 expressions in
9 high glucose-incubated H9c2 cardiomyoblasts.

10

11 H9c2 cells were incubated in the absence or presence of high glucose(HG) with or
12 without edaravone and expression of SIRT-1(A), PGC-1 α (B), NRF-2 (C) were
13 determined as described in Materials and Methods. Representative Western blots are
14 depicted. Data represent the mean $\pm$ S.E.M ($n=6$). * $P<0.05$, ** $P<0.01$ vs Control,
15 # $P<0.05$, ## $P<0.01$ vs HG.

16

17 Figure 7. Edaravone treatment reduces Bax, Caspase-3 and increases Bcl-2
18 expressions in high glucose-incubated H9c2 cardiomyoblasts.

19

20 H9c2 cells were incubated in the absence or presence of high glucose(HG) with or
21 without edaravone and expression of Bax(A), Bcl-2(B), Caspase-3(C) were
22 determined as described in Materials and Methods. Representative Western blots are

1 depicted. Data represent the mean±S.E.M ($n=6$). * $P<0.05$, ** $P<0.01$ vs Control,
2 # $P<0.05$, ## $P<0.01$ vs HG.

3

4 Figure 8. Knockdown SIRT-1 diminished edaravone-regulated protein expression in
5 high glucose-incubated H9c2 cardiomyoblasts.

6

7 Knockdown SIRT-1siRNA were performed as described in Materials and Methods,
8 after knockdown SIRT-1, the cells were incubated in the absence or presence of high
9 glucose (HG) or high glucose with edaravone (HG+EDA) or siSIRT-1 with high
10 glucose and edaravone (siSIRT-1+HG+EDA), then expression of SIRT-1 (A), NRF-2
11 (B), Bax (C), Bcl-2 (D) and Caspase-3 (E) determined as described in Materials and
12 Methods. Data represent the mean±S.E.M of three separate experiments, * $P<0.05$
13 compared to control, # $P<0.05$ compared to HG, ϕ $P<0.05$ compared to HG+EDA.

14

1 **Abbreviations:** Bcl-2, B-cell lymphoma 2; BG, Blood glucose; EDA, edaravone; EF%,
2 percent ejection fraction; INS, Insulin; IVS, interventricular septal thickness; LVDD,
3 left ventricular end-diastolic diameter; MDA, Malondialdehyde; NRF-2, nuclear
4 factor like-2; PGC-1 α , peroxisome proliferator activated receptor γ coactivator α ;
5 ROS, reaction oxygen species; SIRT-1, sirtuin-1; STZ, streptozocin; SOD, superoxide
6 dismutase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling
7 assay.

Draft

Figure 1. Echocardiographic analysis in diabetic rats.
262x258mm (300 x 300 DPI)

Figure 2. Edaravone reduces cardiomyocytes diameters and apoptosis.
186x346mm (300 x 300 DPI)

Figure 3. Edaravone treatment increases SIRT-1, PGC-1α and NRF-2 expression.
384x127mm (300 x 300 DPI)

Figure 4. Edaravone treatment reduces Bax, Caspase-3 and increases Bcl-2 expression.
397x127mm (300 x 300 DPI)

Figure 5. Edaravone treatment restores cell viability in high glucose-cultured H9c2 cardiomyoblasts.
341x237mm (300 x 300 DPI)

Figure 6. Edaravone treatment increases SIRT-1, PGC-1α and NRF-2 expressions in high glucose-incubated H9c2 cardiomyoblasts.
465x153mm (300 x 300 DPI)

Figure 7. Edaravone treatment reduces Bax, Caspase-3 and increases Bcl-2 expressions in high glucose-incubated H9c2 cardiomyoblasts.
417x146mm (300 x 300 DPI)

Figure 8. Knockdown SIRT-1 diminished edaravone-regulated protein expression in high glucose-incubated H9c2 cardiomyoblasts.
360x209mm (300 x 300 DPI)