Beneficial effects of edaravone, a novel antioxidant, in rats with dilated cardiomyopathy

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Abstract

Edaravone, a novel antioxidant, acts by trapping hydroxyl radicals, quenching active oxygen and so on. Its cardioprotective activity against experimental autoimmune myocarditis (EAM) was reported. Nevertheless, it remains to be determined whether edaravone protects against cardiac remodelling in dilated cardiomyopathy (DCM). The present study was undertaken to assess whether edaravone attenuates myocardial fibrosis, and examine the effect of edaravone on cardiac function in rats with DCM after EAM. Rat model of EAM was prepared by injection with porcine cardiac myosin 28 days after immunization, we administered edaravone intraperitoneally at 3 and 10 mg/kg/day to rats for 28 days. The results were compared with vehicle-treated rats with DCM. Cardiac function, by haemodynamic and echocardiographic study and histopathology were performed. Left ventricular (LV) expression of NADPH oxidase subunits (p47[phox], p67[phox], gp91[phox] and Nox4), fibrosis markers (TGF-β_1 and OPN), endoplasmic reticulum (ER) stress markers (GRP78 and GADD 153) and apoptosis markers (cytochrome C and caspase-3) were measured by Western blotting. Edaravone-treated DCM rats showed better cardiac function compared with those of the vehicle-treated rats. In addition, LV expressions of NADPH oxidase subunits levels were significantly down-regulated in edaravone-treated rats. Furthermore, the number of collagen-III positive cells in the myocardium of edaravone-treated rats was lower compared with those of the vehicle-treated rats. Our results suggest that edaravone ameliorated the progression of DCM by modulating oxidative and ER stress-mediated myocardial apoptosis and fibrosis.

Keywords: edaravone ● oxidative stress ● endoplasmic reticulum stress ● apoptosis ● fibrosis ● dilated cardiomyopathy

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Introduction

Inflammation and autoimmunity are involved in many cardiac diseases among which myocarditis, an inflammatory heart disease, causes both acute and chronic heart failure as a result [1]. A model of rat experimental autoimmune myocarditis (EAM) resembles human giant-cell myocarditis, and the recurrent form of EAM leads to dilated cardiomyopathy (DCM) [2]. Neurohumoral factors such

as cytokines and chemokines, and myocardial remodelling including myocardial apoptosis, play important roles in the progression of EAM [3, 4]. Excessive production of reactive oxygen species (ROS) such as superoxide induced by inflammatory stimuli is an important observation in failing hearts and produces myocardial injury in autoimmune-mediated heart failure [5] and it has also been reported that the patients with heart failure were found with excess levels of ROS in their plasma [6]. Myocardial fibrosis probably plays an important role in both diastolic and systolic dysfunctions and has adverse clinical consequences that result in increases in mortality because of progressive heart failure [7]. Many kinds of cytokines, such as basic fibroblast growth factor, Angiotensin (Ang)-II, transforming growth factor (TGF)-β_1, and collagen-III, have been suggested to play an important role in structural remodelling of the non-myocyte compartment of the myocardium after heart failure [8].

Edaravone, a novel free radical scavenger, may be an effective agent for myocardial inflammation [9] by combating oxidative stress. It was reported by various studies about the protective effect of edaravone against myocardial injury. Edaravone significantly reduced myocardial infarct size and improved cardiac function and left ventricular (LV) remodelling 14 days after infarction [10]. It was also reported that edaravone significantly attenuates pressure overload-induced cardiac hypertrophy mediated through its antioxidative function [11]. We have also reported the protective effects of edaravone against EAM induced by cardiac myosin, focusing on oxidative stress, endoplasmic reticulum (ER) stress, inflammatory cytokines and myocardial apoptosis [12]. But it is now of interest to identify whether treatment with edaravone can inhibit the progression of EAM into DCM.

Thus, the purpose of the present study was to test the effect of edaravone treatment against porcine cardiac myosin-induced chronic heart failure model, focusing on its inhibitory effects on oxidative stress, myocardial apoptosis and fibrosis.

Materials and methods

Materials

Edaravone was obtained from Mitsubishi Research, Japan and Lewis rats (male, 8 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). All the chemicals and reagents were purchased from Sigma Aldrich, Tokyo, Japan unless otherwise mentioned.

Experimental design

All experiments were carried out using 8-week-old male Lewis rats and were performed in accordance with the guidelines of our institute.

Lewis rats ($n = 30$) were injected in the footpads with antigen-adjuvant emulsion in accordance with a procedure described previously [13]. In brief, porcine cardiac myosin was dissolved in phosphate-buffered saline at 5 mg/ml and emulsified with an equal volume of complete Freund's adjuvant with 11 mg/ml *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA). Experimental autoimmune myocarditis in rats was induced by immunization with 0.1 ml of emulsion once by subcutaneous injection into the rear footpads (0.1 ml to each footpad). The morbidity of EAM was 100% in rats immunized by this procedure. Twenty-eight days after immunization, the surviving Lewis rats were divided into three groups and received an intraperitoneal injection of edaravone [3 and 10 mg/kg/day; Group Ed 3 ($n = 9$) and Group Ed 10 ($n = 9$)] or vehicle [Group V ($n = 8$)] for 28 days. These doses were selected as per the previous report suggesting its cardioprotective effects [11]. Age-matched Lewis rats without immunization were used as normal controls [Group N ($n = 6$)].

Haemodynamic study

Eight weeks after immunization, cardiac function of each rat was measured by haemodynamic study, and cardiac pressure changes were recorded as described previously [14]. The rats were anaesthetized with 2% halothane in O_2 during the surgical procedures preceding haemodynamic measurements; this concentration was then reduced to 0.5% to minimize the anaesthetic effect.

Transthoracic echocardiographic analysis

Echocardiographic studies were carried out with a 7.5-MHz transducer (Aloka Inc., Tokyo, Japan) under 0.5% halothane anaesthesia, which has produced no effect on measurement. The LV dimensions in diastole (LVDd) and systole (LVDs), percentage ejection fraction (%EF) and percentage fractional shortening (%FS) were estimated using M-mode measurements. Doppler analysis was carried out to estimate the E/A ratio [15].

Histopathology

The body weight (bw) of rats was noted just before the surgical procedure. After the echocardiographic analysis, the rats were killed, and the wet heart was isolated and weighed to calculate the ratio of heart weight (HW) to bw. The excised myocardium was kept in formalin and the mid-ventricle sections were then embedded with paraffin.

Analysis of cardiac apoptosis by terminal transferase-mediated dUTP nick-end labelling (TUNEL) assays

The TUNEL assay was performed as specified in the instructions for the *in situ* apoptosis detection kit (Takara Bio Inc., Shiga, Japan). Sections embedded in paraffin were mounted and examined using light microscopy. Digital photomicrographs were obtained by using a colour image analyser (CIA-102; Olympus, Tokyo, Japan) at 200× magnification, and 25 random fields from each heart were chosen and the number of TUNEL-positive nuclei was quantified in a blinded manner. For each group, three sections were scored for apoptotic nuclei. Only nuclei that were clearly located in cardiac myocytes were considered [16].

Azan-Mallory staining for myocardial fibrosis

The area of myocardial fibrosis in LV tissue sections stained with Azan-Mallory was quantified by using a colour image analyser (CIA-102; Olympus) and the blue fibrotic areas were measured as opposed to the red myocardium at 200x magnification. The results were presented as the ratio of the fibrotic area to the whole area of the myocardium [17, 18].

Immunohistochemical assay

Immunohistochemical staining was performed [19] with the formalin-fixed, paraffin-embedded cardiac tissue sections of the rats from different groups. After deparaffinization and hydration, the slides were washed in Tris-buffered saline (TBS; 10 mmol/l Tris HCl, 0.85% NaCl, pH 7.5) containing 0.1% bovine serum albumin. Endogenous peroxidase activity was quenched by incubating the slides in 0.6% H_2O_2 in methanol. To perform antigen retrieval, the sections were pre-treated with trypsin for 15 min. at 37°C. After overnight incubation with the goat polyclonal anti-collagen type III antibody (diluted 1:100) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C, the slides were washed in TBS and horseradish peroxidase-conjugated secondary antibody was then added and the slides were further incubated at room temperature for 45 min. The slides were washed in TBS and incubated with diaminobenzidine tetrahydrochloride as the substrate, and counterstained with haematoxylin. A negative control without primary antibody was included in the experiment to verify the antibody specificity. Measurement of myocardial immunoreactivity for collagen-III was performed in 100 randomly selected fields in heart sections in 200x magnification by light microscopy.

In situ detection of superoxide production in hearts

Superoxide generation was estimated by dihydroethidium (DHE) staining as previously described [20]. To evaluate *in situ* superoxide production from hearts, unfixed frozen cross-sections of the specimens were stained with DHE (Molecular Probes, Eugene, OR, USA) according to the previously validated method. In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen LV tissues were cut into 10-μm-thick sections and incubated with DHE 10 μM at 37°C for 30 min. in a light-protected humidified chamber. Fluorescence images (200x magnification) were obtained by using a fluorescence microscope equipped with a rhodamine filter.

Western immunoblotting analysis

This analysis was carried out as per the earlier method by Thandavarayan *et al.* [21]. The myocardial tissue samples obtained from different groups were homogenized with lysis buffer. Protein concentrations in these homogenized samples were measured by the bicinchoninic acid method. For Western blots, proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and identified with the following polyclonal antibodies to quantify their myocardial levels: rab-bit polyclonal anti-p47^{phox}, goat polyclonal anti-p67^{phox}, goat polyclonal anti-gp91^{phox}, goat polyclonal anti-nox4, mouse polyclonal anti-osteopontin (OPN), rabbit polyclonal anti-TGF-β_1, goat polyclonal anti-glucose regulated protein (GRP)78, mouse polyclonal anti-growth arrest and DNA damage inducible gene (GADD)153 and rabbit polyclonal anti-cleaved caspase-3 antibodies and rabbit polyclonal anti-glyceraldehyde 3 phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology or Cell Signaling) (diluted 1:100). The cytosolic fraction was separated from the homogenate as per the previous method [22]. Briefly, the tissues were minced and added to ice-cold homogenizing buffer (250 mM sucrose, 20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin). After 30 min. incubation on ice, cells were homogenized with polytron homogenizer (single stroke for 20 sec.). The homogenates obtained were then subjected to a series of centrifugations at $100,000 \times g$ for 60 min. to collect the cytosolic fractions for the estimation of cytochrome C level. According to the molecular weight of the primary antibody, we have used 7.5%, 10% and 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA), and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk or 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in TBS-T (20 mmol/l Tris, pH 7.6, 137 mmol/l, NaCl, and 0.05% Tween).

After incubation with primary antibody, the bound antibody was visualized with respective horseradish peroxidase-coupled secondary antibody (Santa Cruz Biotechnology) and chemiluminescence-developing agents (Amersham Biosciences, Buckinghamshire, UK). The level of GAPDH was estimated in every sample. Films were scanned and band densities were quantified with densitometric analysis by using Scion Image program (Epson GT-X700, Tokyo, Japan). Finally, Western blot data were normalized with cardiac GAPDH.

Statistical analysis

All the values are expressed as means ± S.E.M. Statistical analysis of differences between the groups was performed by one-way analysis of variance, followed by Dunnett's or Tukey's test. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of edaravone on myocardial dimensions

Although heart rate was not different among the four groups of rats, transthoracic echocardiographic studies of group V rats showed evidence for the cardiac remodelling with increased LVDs (7.42 ± 0.67 *versus* 3.44 ± 0.19 mm, $P < 0.01$), reduced %FS (15 ± 0.68 *versus* 49.2 ± 1.9%, $P < 0.01$) and %EF (28.8 ± 3.71 *versus* 85.1 ± 1.6%, $P < 0.01$) and reduced E/A ratio. These results indicate the impairment of cardiac function in the DCM rats of group V compared with that in group N. Treatment with edaravone (3 or 10 mg/kg) significantly prevented all of these changes compared with those in group V (Table 1 and Fig. 1).

Table 1 Changes in haemodynamic and echocardiographic parameters in rats with dilated cardiomyopathy (DCM) after treatment with edaravone

Parameter	Group N	Group V	Group Ed 3	Group Ed 10
Histopathology				
bw (g)	412 ± 8	329 ± 5**	338 ± 7**	337 ± 8**
HW (g)	0.96 ± 0.01	1.24 ± 0.03**	1.06 ± 0.03	1.08 ± 0.01*$^{\#}$
HW/bw (g/kg)	2.32 ± 0.03	3.62 ± 0.14**	3.20 ± 0.11**	3.27 ± 0.12**
Haemodynamic data				
HR (beats/min)	378 ± 19	329 ± 8	349 ± 14	364 ± 7
Mean BP (mmHg)	99 ± 6	82 ± 4*	75 ± 2**	75 ± 3**
CVP (mmHg)	1.0 ± 0.4	4.2 ± 0.4*	3.3 ± 0.9	3.2 ± 0.8
LVP (mmHg)	128 ± 5	101 ± 4**	118 ± 5$^{\#}$	106 ± 1**
LVEDP (mmHg)	19.7 ± 1.4	40.4 ± 2.3**	33.8 ± 3.7*	35.9 ± 4.4*
+dP/dt (mmHg/min)	6446 ± 881	4205 ± 77*	4769 ± 211	4530 ± 404
−dP/dt (mmHg/min)	7291 ± 1124	3933 ± 413**	4054 ± 191**	3730 ± 274**
Echocardiographic data				
LVDd (mm)	6.7 ± 0.31	7.62 ± 0.32	7.56 ± 0.22	7.5 ± 0.1
LVDs (mm)	3.44 ± 0.19	7.42 ± 0.67**	5.44 ± 0.12**$^{,\#\#}$	5.48 ± 0.11**$^{,\#\#}$
FS (%)	49.2 ± 1.9	15 ± 0.68**	32.7 ± 1.49**$^{,\#\#}$	25.8 ± 1.4**$^{,\#\#,\dagger}$
EF (%)	85.1 ± 1.6	28.8 ± 3.71**	64.4 ± 1.58**$^{,\#\#}$	56.3 ± 2.29**$^{,\#\#}$
E/A ratio	2.94 ± 0.05	0.32 ± 0.01**	1.73 ± 0.10**$^{,\#\#}$	2.05 ± 0.16**$^{,\#\#}$

Group N: age-matched intact rats; Group V: rats with DCM treated with vehicle; Group Ed3: rats with DCM treated with edaravone (3 mg/kg); Group Ed10: rats with DCM treated with edaravone (10 mg/kg); bw: body weight; HW: heart weight; CVP: central venous pressure; LVP: left ventricular pressure; LVEDP: left ventricular end-diastolic pressure; dP/dt: rate of intra-ventricular pressure rise and decline; LVDd: left ventricular dimension in diastole; LVDs: left ventricular dimension in systole; FS: fractional shortening; EF: ejection fraction. **$P < 0.01$ and *$P < 0.05$ *versus* Group N, $^{\#\#}P < 0.01$ and $^{\#\#}P < 0.05$ *versus* Group V, $^{\dagger}P < 0.05$ *versus* Group E3. Values are mean ± S.E.M. (one-way ANOVA followed by Tukey's multiple comparison test).

Effect of edaravone on cardiac hypertrophy

Dilated cardiomyopathy is a condition where there will be increased HW with respect to bw, indicating the cardiac hypertrophy and dilatation of the cardiac chambers. Here, in the present study, myocarditis was confirmed with the increased HW of the group V rats. The ratio (HW/bw) was found to be significantly higher (3.62 ± 0.14, $P < 0.01$) when compared with group N (2.32 ± 0.03). In the edaravone-treated rats, this value was less than that of group V rats (Table 1).

Effect of edaravone on myocardial fibrosis and its marker molecules

Progression of EAM to DCM involves myocardial remodelling in the form of extensive replacement fibrosis, which can be identified by the measurement of its marker proteins. The hearts from group V DCM

rats showed massive fibrosis and increased expressions of OPN and TGF-β_1 compared with those from group N. Azan-Mallory staining of the myocardial tissue sections revealed the extensive fibrosis in the myocardium of group V rats, whereas the edaravone-treated (3 or 10 mg/kg) rats were significantly protected from these changes (Fig. 2A). This antifibrotic effect has also been confirmed by immuno-histochemical studies for deposition of collagen-III in the myocardial tissue sections of edaravone-treated rats (Fig. 2B). In addition, treatment with edaravone significantly reduced the myocardial expression of OPN and TGF-β_1 than in group V DCM rats (Fig. 3A and B).

Effect of edaravone on myocardial apoptosis

Apoptotic cell death plays a major role in several cardiovascular disorders and is also an important myocardial remodelling process leading to cardiac dysfunction *via* cardiomyocyte loss. TUNEL staining
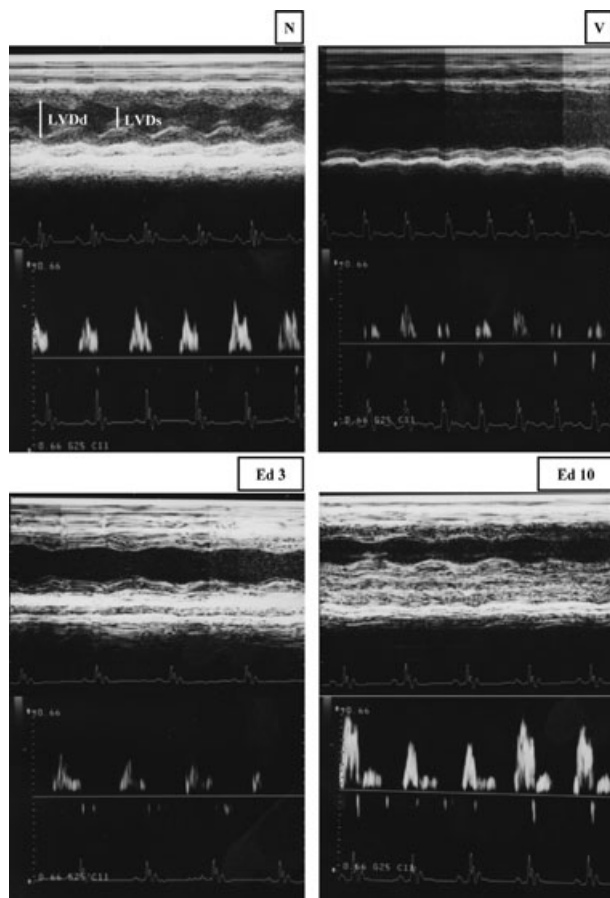
Fig. 1 Echocardiographic data. Group N, age-matched intact rats; Group V, rats with dilated cardiomyopathy (DCM) treated with vehicle; Group Ed 3, rats with DCM treated with edaravone (3 mg/kg); Group Ed 10, rats with DCM treated with edaravone (10 mg/kg).

reveals the number of apoptotic cells present in the stained area, and increased apoptotic cell count indicates the disease progression. In the present study, the number of TUNEL positive nuclei was significantly higher in myocardial tissue sections from group V rats than in group N. However, treatment with edaravone (3 or 10 mg/kg) significantly decreased their number compared with that in group V (Fig. 2C). In addition, the cytosolic level of cytochrome C, an apoptosis marker, was significantly elevated in the group V rats, whereas treatment with edaravone (3 or 10 mg/kg) significantly prevented this change (Fig. 3C).

Effect of edaravone on oxidative stress

Oxidative stress is involved in the pathogenesis of various cardiovascular disorders, which has also been reported to be involved in the progression of EAM to DCM. In the present study, the rats of group V suffered from the excess oxidative stress as identified by DHE stain-

ing and Western blotting for its marker molecules. Involvement of superoxides in the progression of EAM has been confirmed by the DHE staining of the myocardial tissue sections of group V rats compared with the group N rats, whereas the treatment with edaravone (3 or 10 mg/kg) significantly protected from these changes (Fig. 2D). The cardiac expressions of NADP(H) oxidase subunits like $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$ and Nox4 were significantly higher in group V rats compared with that in group N, and these changes were significantly reversed by the treatment with edaravone (Fig. 4A and D).

Effect of edaravone on ER stress marker proteins

Various cellular stresses are reported to be increased during myocardial injury, of which ER stress is the one which directly leads to myocardial cell loss *via* apoptosis involving caspases. The elevated levels of various ER stress markers were identified in the DCM hearts of group V rats. The myocardial expressions of GRP78 and GADD153 were significantly increased in the group V rats. However, these changes were significantly attenuated by the treatment with edaravone (3 or 10 mg/kg) as demonstrated by the decreased levels of these above-mentioned markers (Fig. 5A and B). Prolonged ER stress stimulates apoptotic signalling *via* caspases, and the present study with edaravone has provided significant suppression in the myocardial levels of caspase-3, which is the downstream of the caspase-mediated apoptotic signalling when compared with the group V rats (Fig. 5C).

Discussion

The present findings clearly suggest that edaravone, a novel free radical scavenger, reduced the severity of EAM and also prevented its progression to DCM. The cardioprotection offered by edaravone treatment may be partly as a result of the suppression of oxidative and ER stresses.

Edaravone treatment has protected the hearts from functional deterioration as demonstrated by haemodynamic study. There was a significant improvement in the cardiac haemodynamics of the edaravone-treated rats, as the LV pressure parameters were significantly improved when compared with those in the group V. Similarly, echocardiography also confirmed the improved cardiac performance in the edaravone-treated rats as their %FS and %EF values were improved significantly when compared with the group V rats. No significant change in the HR was observed; however, the HW/bw ratio of the edaravone-treated rats was less when compared with the group V rats. Thus, our present study confirmed the effect of edaravone in maintaining the cardiac function against DCM derived from EAM induced by immunization with porcine cardiac myosin.

Oxidative stress and inflammation are thought to play important roles in the progression of cardiovascular diseases. An increase in the level of oxidative stress is implicated in the pathogenesis of heart failure and various autoimmune disorders including DCM mediated by cardiac myosin [23]. Investigations suggest that free radicals may be important contributors to the deterioration of the decompensating
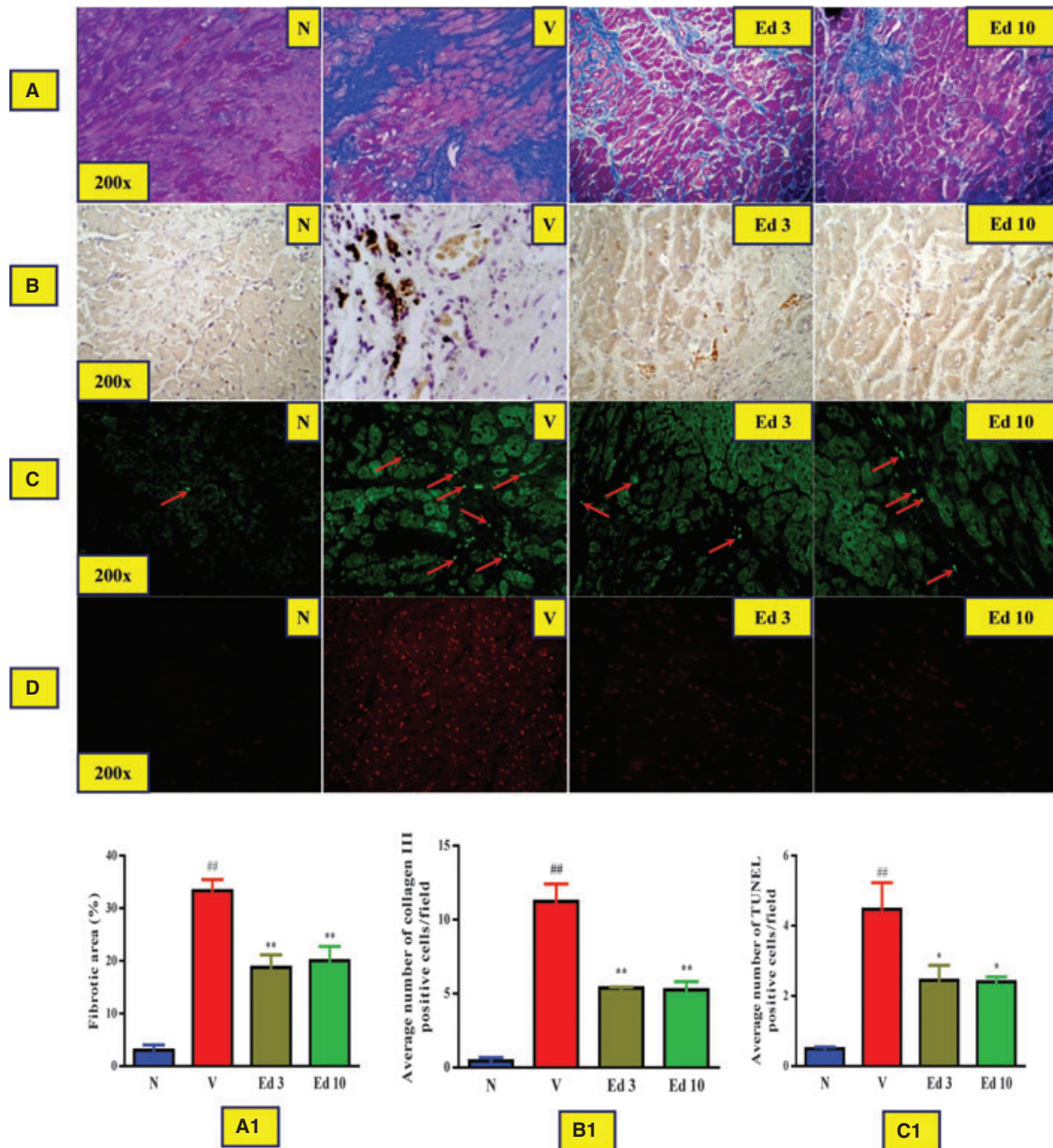
Fig. 2 (**A** and **A1**) Azan-Mallory staining for fibrosis of the cross-sectional tissue slices of hearts (Fibrosis is indicated by blue area as opposed to the red myocardium and bar graph showing the percentage fibrotic area, respectively). (**B** and **B1**) Immunohistochemical stained myocardial tissue sections for collagen III and bar graph showing the average number of collagen-III positive cells/field, respectively. (**C** and **C1**) TUNEL stained myocardial tissue sections for apoptotic nuclei and bar graph showing the average number of TUNEL positive apoptotic nuclei/field, respectively. (**D**) Di-hydroethidium-stained myocardial tissue sections for superoxide production. Group N: age-matched intact rats; Group V: rats with dilated cardiomyopathy (DCM) treated with vehicle; Group Ed 3: rats with DCM treated with edaravone (3 mg/kg); Group Ed 10: rats with DCM treated with edaravone (10 mg/kg); $^{##}P < 0.01$ *versus* Group N, $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* Group V (one-way ANOVA followed by Dunnett's test). Values are expressed as mean $\pm$ S.E.M., $n = 3$.

myocardium [24]. Treatments that reduce the levels of oxidative stress or inflammation have thus been found to improve myocardial function in patients with advanced heart failure as well as in animal models of this condition. The NADPH oxidase system, present in cardiac and vascular tissues, is also a candidate for the source of the superoxide [25]. The NADPH oxidase complex is a cluster of proteins
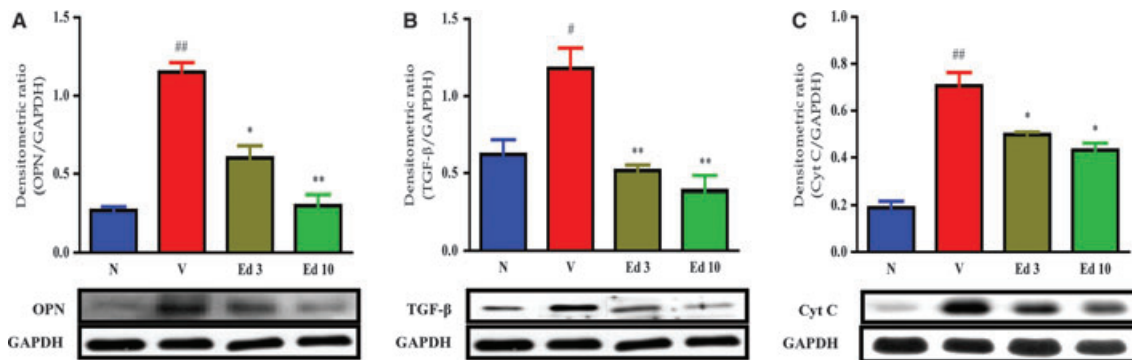
Fig. 3 Myocardial expressions of osteopontin (OPN), transforming growth factor-β_1 (TGF-β_1) and cytochrome (cyt)-C. (**A–C**) Densitometric data of protein analysis. The mean density values of OPN, TGF-β_1 and cyt-C (Expressed as a ratio relative to that of GAPDH) with representative Western blots showing specific bands for OPN, TGF-β_1 and cyt-c. GAPDH was used as an internal control. Group N: age-matched intact rats; Group V: rats with dilated cardiomyopathy (DCM) treated with vehicle; Group Ed 3: rats with DCM treated with edaravone (3 mg/kg); Group Ed 10: rats with DCM treated with edaravone (10 mg/kg). $^{##}P < 0.01$ *versus* Group N, $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* Group V (one-way ANOVA followed by Dunnett's test). Values are expressed as mean ± S.E.M., $n = 3$.
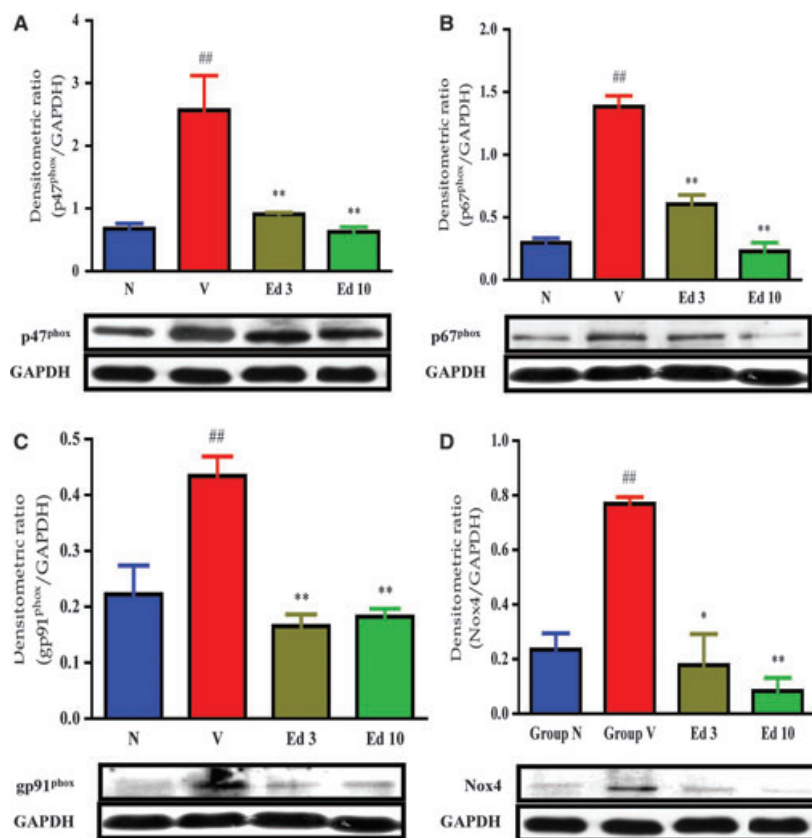

Fig. 4 Myocardial expressions of p47[phox], p67[phox], gp91[phox] and Nox4. (**A–D**) Densitometric data of protein analysis. The mean density values of p47[phox], p67[phox], gp91[phox] and Nox4 (Expressed as a ratio relative to that of GAPDH) with representative Western blots showing specific bands for p47[phox], p67[phox], gp91[phox] and Nox4. GAPDH was used as an internal control. An equal amount of protein sample obtained from whole ventricular homogenate was applied in each lane. These bands are representative of three separate experiments. Group N: age-matched intact rats; Group V: rats with dilated cardiomyopathy (DCM) treated with vehicle; Group Ed 3: rats with DCM treated with edaravone (3 mg/kg); Group Ed 10: rats with DCM treated with edaravone (10 mg/kg). $^{##}P < 0.01$ *versus* Group N, $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* Group V (one-way ANOVA followed by Dunnett's test). Values are expressed as mean ± S.E.M., $n = 3$.

that promote donation of an electron from NADPH to molecular oxygen to produce superoxide. Activation of electron transfer from NADPH to molecular oxygen requires recruitment of the cytosolic subunits p47[phox], p67[phox] and other subunits [26, 27]. Thus, estimation of these proteins is an important mode for the identification of oxidative stress during various disorders. Our study has confirmed that the DCM rats suffer from oxidative stress as demonstrated by the increased levels of myocardial NADPH oxidase subunits like p47[phox],
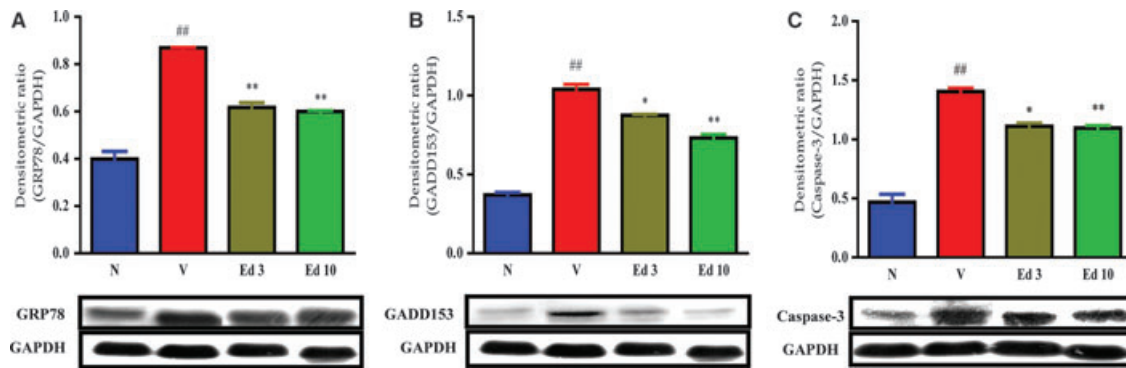
Fig. 5 Myocardial expressions of GRP78, GADD153, and caspase-3. (**A–D**) Densitometric data of protein analysis. The mean density values of GRP78, GADD153 and caspase-3 (expressed as a ratio relative to that of GAPDH) with representative Western blots showing specific bands for GRP78, GADD 153, Cyt C and caspase-3. GAPDH was used as an internal control. Group N: age-matched intact rats; Group V: rats with DCM treated with vehicle; Group Ed 3: rats with dilated cardiomyopathy (DCM) treated with edaravone (3 mg/kg); Group Ed 10: rats with DCM treated with edaravone (10 mg/kg). $^{\#\#}P < 0.01$ *versus* Group N, $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* Group V (one-way ANOVA followed by Dunnett's test). Values are expressed as mean ± S.E.M., $n = 3$.

p67[phox], gp91[phox] and Nox4. The results from edaravone treatment showed that the treated rats have been protected from myocardial oxidative stress as observed with their reduced myocardial levels of the above-mentioned NADPH oxidase subunits. This effect was expected as edaravone has been reported to provide protection against oxidative stress in various animal models. Zhang *et al.* [28] reported the protective effect of edaravone against acute myocardial ischaemia/reperfusion injury where they have suggested its antioxidative role in reducing the infarct size. It has also been reported that e-daravone at 10 mg/kg dose provided significant cardioprotection and reduced the ROS production in pressure overload-induced cardiac hypertrophy [11]. Consistent with these reports and from the present results, we can suggest that edaravone treatment can provide significant cardioprotection in rats with DCM, at least in part *via* the inhibition of oxidative stress.

Oxidative stress is known to induce cardiomyocyte apoptosis, an important contributor to hypertrophic remodelling and cell dysfunction [29] in a variety of cell types by activating intracellular cell death signalling cascades [30]. Apoptosis is the key contributor to cell loss during heart failure causing the loss of contractile muscle mass, which is largely irreplaceable [31]. There are various reports regarding the involvement of apoptosis during myocardial injury and damage. Most of them suggest that activation of caspases is the main finding during this process and blocking these caspases was reported to be effective against apoptosis-mediated myocardial functional loss [32, 33]. Activation of these caspases is mainly *via* the functional stress in the ER. The ER is classically characterized as an organelle that participates in the folding of membrane and secretory proteins. Stimuli such as ischaemia, hypoxia, heat shock, genetic mutation, oxidative stress and increased protein synthesis that cause ER dysfunction are collectively known as ER stress [34]. Any disturbance in its function causes ER stress, leading to up-regulation of ER chaperones such as GRP78. GRP78 serves as the master modulator of unfolded protein response network by binding to various ER stress sensors.

Increased GRP78 was reported in ER stress-associated apoptosis of cardiocytes in the heart failure [35]. In our present study, the protein levels of GRP78 and GADD153 were markedly up-regulated in the group V rats confirming the ER stress, whereas the treatment with edaravone significantly attenuated this change, suggesting its protective effect against ER stress. Similarly, the change in the cytosolic level of cytochrome C was also reversed by the treatment with edaravone. When ER stress is excessive and/or prolonged, however, apoptotic signals are initiated by the ER, proceed through various routes involving caspases and finally induce apoptosis by activation of caspase-3 [34, 36]. Similarly, we have also observed the elevated myocardial level of cleaved caspase-3, which has confirmed the activation of apoptosis in the group V rats that could have been mediated *via* oxidative stress and/or ER stress. Interestingly, the rats treated with edaravone showed significant suppression of caspase-3, suggesting its antiapoptotic role in DCM. Apart from this, the antiapoptotic role of edaravone is also confirmed by the reduction in the number of TUNEL positive apoptotic cells in the myocardial sections of the DCM rats treated with edaravone. From these results, we can suggest that treatment with e-daravone is effective in preventing myocardial apoptosis possibly *via* modulation of oxidative and ER stresses.

Cardiac adaptation in response to intrinsic or external stress involves a complex process of chamber remodelling and myocyte molecular modifications and growing evidence highlights oxidative stress as an important mechanism for this maladaptation [37]. ROS generated by NADPH oxidase plays a key role in cardiac remodelling [38]. Myocardial fibrosis, the replacement of fibrous tissue in place of damaged myocardium is the hallmark of DCM, was observed in DCM hearts as measured by Azan–Mallory staining and increased concentrations of its marker molecules (TGF-β_1 and OPN). Excessive expressions of TGF-β_1 and collagen-III are involved in the myocardial fibrosis, which promote the synthesis of extracellular matrix constituents such as proteoglycans and fibronectin. In addition, they suppress the degradation of extracel-

lular matrix. It was reported that, in a rat model of dimethylnitrosamine-induced liver cirrhosis, treatment with 10 mg/kg of edaravone produced significant reduction in the fibrotic area [39]. Similarly, in a pressure overload-induced LV hypertrophic rat model, cardiac fibrosis was significantly attenuated by the treatment with the same dose of edaravone [11]. Consistent with those reports, in the present study, treatment with edaravone has reduced the percentage area of fibrosis and reduced myocardial levels of TGF-β_1, OPN and collagen III. From these results, we can confirm that edaravone, a novel antioxidant is effective in preventing the myocardial fibrosis in the rats with DCM.

Conclusion

There are several reports regarding the protective role of edaravone against cardiac impairment in various immune-mediated cardiac inflammatory conditions [40, 41] and ischaemia-reperfusion injury [42] including the one which we have published earlier

regarding its protective effect against EAM in rats [12], but its effect on progression of EAM to chronic cardiac complications like DCM is not available. The present study has provided lines of evidence for the protective effects of edaravone against the deterioration of cardiac architecture during EAM-mediated DCM.

Acknowledgements

Conflict of interest

The authors declare that there are no conflicts of interest with this work.

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