Research article

Edaravone protects rats against oxidative stress and apoptosis in experimentally induced myocardial infarction: Biochemical and ultrastructural evidence

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Objectives: The present study was designed to evaluate the cardioprotective potential of edaravone on oxidative stress, anti-apoptotic, anti-inflammatory and ultrastructure findings in isoproterenol (ISO) induced myocardial infarction (MI) in rats.
Methods: Rats were pretreated with edaravone (1, 3, 10 mg/kg body weight-1 day-1) intraperitoneally. MI was induced by subcutaneous administration of ISO (85 mg/kg body weight-1) at two doses with 24h interval.
Results: ISO treated rats showed significant increase in the levels of thiobarbituric acid reactive substances (TBARS) and decreased levels of reduced glutathione, glutathione perdoxidase, glutathione reductase and glutathione-S- transferase in the cardiac tissues. Moreover, significant increase in the levels of lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), C - reactive protein and caspase-3 activity was observed in ISO treated group. Pretreatment of ISO intoxicated rats with edaravone showed significant decrease in the level of TBARS, increased activities of antioxidant enzymes and significantly decreased levels of LDH and CK-MB. Moreover, results also showed decreased C-reactive protein level, caspase-3 activity and maintained ultrastructure of the myocardial cells.
Discussion: Our study suggests that edaravone possess strong cardioprotective potential. Edaravone may have exhibited cardioprotective effects by restoring antioxidant defense mechanism, maintaining integrity of myocardial cell membrane, reducing apoptosis and inflammation against ISO induced MI and associated oxidative stress.

Keywords: Edaravone, Oxidative stress, Caspase-3, C-reactive protein, Isoproterenol, Myocardial infarction, Ultrastructure evaluation

Introduction

Oxidative stress is mainly associated with increased formation of reactive oxygen species (ROS) that play a crucial role in cardiovascular pathophysiology. Cardiovascular diseases (CVDs) are the most alarming of the disease predictions across the globe. Following myocardial infarction (MI), the progressive condition of heart failure is caused by remodeling of the cardiac tissue due to an imbalance between coronary blood supply and myocardium demand. According to a report of the World Health Organization (WHO), 17.3 million people died from CVDs in 2008 and 23.3 million people would die annually from CVDs by 2030.[1]

Isoproterenol (ISO) is a synthetic catecholamine that causes rigorous stress to the myocardium, resulting in an infarct-like necrosis of cardiac tissue.[2] Biochemical and morphological changes are seen in the cardiac tissue of ISO-treated experimental rats that are similar to those observed in humans.[3] Adameova et al.[4] have reported that oxidative products of ISO play a critical role in the progression of contractile dysfunction leading to MI. Additionally, it leads to contractile irregularities as well as ultra-structural alterations in the cardiac tissue.[5] The ISO-induced MI experimental model is widely used to assay drugs intended to be used in CVDs.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is an effective and strong scavenger of free radicals. Edaravone has been broadly used in patients with acute ischemic stroke, due to its established neurovascular

protective effects.[6,7] Edaravone prevents free radical-initiated lipid peroxidative damage.[8,9] Edaravone has been reported to have protective effects against experimental models of myocardial ischemia-reperfusion injury by reducing the myocardial necrotic area.[10,11] Though ISO itself generates ROS, it may also be activating biochemical pathways responsible for altering cellular functions through elevation of oxidative stress that plays a crucial role in the progression of MI.[12] Based on the above facts, the present study was designed to evaluate the effect of edaravone in ISO-treated MI in rats.

Materials and methods

Experimental animals

The study was approved by the Institutional Animal Ethics Committee, Jamia Hamdard, New Delhi; Approval no. 943. Wistar rats weighing 200–220 g were obtained from the Central Animal House Facility, Jamia Hamdard, New Delhi, housed in polypropylene cages (six in each cage) lined with rice husk, changed every 24 hours. They had free access to commercial pellet diet and water *ad libitum*. The animal house temperature was maintained at 25 ± 2°C.

Drugs and chemicals

Edaravone was procured from Tocris Bioscience, Bristol, United Kingdom; caspase-3 and C-reactive protein (CRP) kits were purchased from Immunology Consultant Laboratory, Inc., USA and Biovision Research Product, USA, respectively. Creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) kits were purchased from Crest Biosystems Ltd., India. All other chemicals used were of analytical grade purchased from Sigma Aldrich Chemicals Pvt. Ltd.

Selection of dose

Doses were selected on the basis of the available literature. We found that the majority of the published research was done with 1, 3, or 10 mg/kg doses.[8,13] However, some research also has been done with 3 or 6 mg/kg doses.[14]

Experimental design

Rats were randomly divided into six groups having six rats each. Group 1 served as the normal control group and received normal saline for 28 days; Group II received only edaravone (10 mg/kg of body weight, i.p.) and served as *per se* group; Group III was considered as toxic control group and rats were injected ISO (85 mg/kg of body weight, s.c.) in two doses with a 24-hour interval. Groups IV, V, and VI were pretreated with edaravone (1, 3, and 10 mg/kg of body weight, i.p.)[13] respectively for 28 days and then two doses of ISO with a 24-hour interval. After the end of this schedule, rats were anesthetized by anesthetic ether and euthanased by cervical decapitation. Blood samples were collected from tail vein and serum was separated by centrifugation at 3000 *g* for 30 minutes. The separated serum was preserved at −20°C for estimation of serum LDH, CK-MB, and CRP. Hearts were immediately excised, washed in ice-cold normal saline, and preserved in liquid nitrogen at −80°C for biochemical estimations.

Biochemical analysis in the myocardial tissue

The level of thiobarbituric acid reactive substances (TBRAS) in the cardiac tissue was estimated by the method of Okhawa *et al.*[15] The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) were assayed by the methods of Marklund and Marklund,[16] Clairborne,[17] Wheeler *et al.*,[18] Calberg and Mannervik,[19] Habig *et al.*,[20] respectively. The level of reduced glutathione in cardiac tissue was estimated by the method of Ellman.[21] The content of protein in the cardiac tissue was determined by the method of Lowry *et al.*[22]

Biochemical analysis of cardiac marker enzymes in serum

Activities of CK-MB[23] and LDH[24] were measured in the serum as per the reported procedures.

Biochemical analysis of caspase-3 protease in myocardial tissue

Caspase-3 protease was determined as per the instructions provided in the kit.[25]

Biochemical analysis of inflammatory marker in serum

CRP in rat serum was determined as per the procedure described in the kit.[26]

Transmission electron microscopic evaluation of myocardium

Small pieces, approximately 1.0–1.5 mm, from the ventricle tissue of rat heart were taken and rinsed in 0.1 M phosphate buffer (pH 7.2) several times and immediately fixed in 2.5% glutaraldehyde and 2% paraformaldehyde solution in 0.1 M sodium phosphate buffer (pH 7.2) and kept at 4°C for 12 hours. The grids containing ultrathin sections were stained with 2% uranyl acetate and 0.2% lead acetate. Tissue processing for transmission electron microscope (TEM) (FMI-Margagani Operated 268 Netherland) studies was carried out and evaluated under TEM (10 000×).[27]

Statistical analysis

Data were expressed as the mean ± SEM. Multiple comparisons against a control group were made using a one-way analysis of variance (ANOVA) followed by Dunnett's test. Results were considered significant if $P < 0.05$. All statistical evaluations were performed using the Prism software package (version 4, GraphPad, San Diego, CA, USA).

Results

Effect of edaravone on TBARS and GSH

Table 1 shows the levels of TBARS and GSH in the cardiac tissues of experimental groups. The ISO-treated group showed a significant ($P < 0.01$) increase in the levels of TBARS and a significant ($P < 0.01$) decrease in the levels of GSH in the cardiac tissues as compared to the normal control group. Pretreatment with edaravone (10 and 3 mg/kg) in ISO-treated rats significantly ($P < 0.01$) decreased the level of TBARS and significantly ($P < 0.01$) increased the level of GSH in the cardiac tissue as compared to the ISO-treated group.

Effect of edaravone on SOD and CAT

Table 1 depicts the activities of SOD and CAT in cardiac tissue. ISO treatment caused a significant ($P < 0.01$) reduction in the activities of SOD and CAT in the cardiac tissue as compared to the control group. Pretreatment with edaravone (10 mg/kg) in the ISO-treated group resulted in a significant ($P < 0.01$) increase in the levels of SOD and CAT in the cardiac tissue as compared to the ISO-treated group. Pretreatment with edaravone (3 mg/kg) in ISO-treated rats showed significantly ($P < 0.05$) improved activity of CAT in the cardiac tissue as compared to the ISO-treated group.

Effect of edaravone on antioxidant enzymes

Table 2 shows the activities of antioxidant enzymes, namely GST, GPx, and GR, in the myocardium. The ISO-treated group had significantly ($P < 0.01$) decreased activities of these enzymes in the cardiac tissue as compared to the control group. Pretreatment with edaravone (10 mg/kg) in the ISO-treated group showed significantly ($P < 0.01$) increased activities of these antioxidant enzymes in the cardiac tissue as compared to the ISO-treated group. Pretreatment with edaravone (3 mg/kg) in the ISO-treated group showed significantly ($P < 0.01$) increased activities of GST and GPx, while edaravone (3 mg/kg) led to a significant ($P < 0.05$) increase in the activity of GR in the cardiac tissue as compared to the ISO-treated group.

Effect of edaravone on CK-MB and LDH level

The effects of edaravone on CK-MB and LDH are shown in Fig. 1. Rats injected with ISO showed a significant ($P < 0.01$) increase in the level of serum LDH and CK-MB as compared to the normal control group. Pretreatment with edaravone (10 and 3 mg/kg) significantly ($P < 0.01$) reduced the levels of LDH in serum compared with the ISO-treated group. Pretreatment with edaravone (10 mg/kg) significantly ($P < 0.01$) reduced the level of CK-MB in serum as compared to the ISO-treated group. Edaravone (3 mg/kg) significantly ($P < 0.05$)

Table 1 Effects of edaravone on the activities of thiobarbituric acid reactive substance (TBARS), superoxide dismutase (SOD), and catalase (CAT) in the cardiac tissue of experimental groups

Experimental groups	TBARS (nmol MDA/mg prot)	SOD (unit/mg prot)	CAT (nmol H_2O_2/minute/mg prot)
Normal control	0.08 ± 0.008	3.79 ± 0.48	7.49 ± 0.33
Edaravone per se	0.08 ± 0.009	3.93 ± 0.53	7.27 ± 0.43
Isoproterenol (ISO) (85 mg/kg)	0.39 ± 0.033[†]	1.49 ± 0.25[†]	1.89 ± 0.08[†]
Edaravone (1 mg/kg) + ISO	0.30 ± 0.027	2.59 ± 0.21	2.54 ± 0.71
Edaravone (3 mg/kg) + ISO	0.23 ± 0.050**	2.62 ± 0.19*	4.19 ± 0.36**
Edaravone (10 mg/kg) + ISO	0.20 ± 0.020**	3.18 ± 0.14**	5.23 ± 0.49**[‡]

Each value is mean ± SEM for six rats in each group.
*$P < 0.05$ vs. ISO toxic group.
**$P < 0.01$ vs. ISO toxic group.
[†]$P < 0.01$ vs. normal control.
[‡]$P < 0.01$ vs. edaravone (1 mg/kg) + ISO.

Table 2   Effects of edaravone on the activities of reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione reductase (GR) in the cardiac tissue of experimental groups

Experimental groups	GSH (µmol GSH/mg prot)	GST (nmol NADPH/minute/mg prot)	GPx (nmol CDNB/minute/mg prot)	GR (nmol NADPH/minute/mg prot)
Normal control	1.33 ± 0.12	8.11 ± 0.29	5.83 ± 0.32	6.53 ± 0.32
Edaravone *per se* (10 mg/kg)	1.09 ± 0.13	7.04 ± 0.46	5.38 ± 0.39	5.21 ± 0.82
Isoproterenol (85 mg/kg)	0.23 ± 0.19†	2.10 ± 0.24†	1.67 ± 0.28†	1.58 ± 0.15†
Edaravone (1 mg/kg) + ISO	0.63 ± 0.07*	3.81 ± 0.53*	2.73 ± 0.19*	2.98 ± 0.17
Edaravone (3 mg/kg) + ISO	0.98 ± 0.05**	4.19 ± 0.48**	3.32 ± 0.20**	3.38 ± 0.35*
Edaravone (10 mg/kg) + ISO	1.02 ± 0.09**‡	4.72 ± 0.33**	3.51 ± 0.18**	3.73 ± 0.41**

Each value is mean ± SEM for six rats in each group.
*$P < 0.05$ vs. ISO toxic group.
**$P < 0.01$ vs. ISO toxic group.
†$P < 0.01$ vs. normal control.
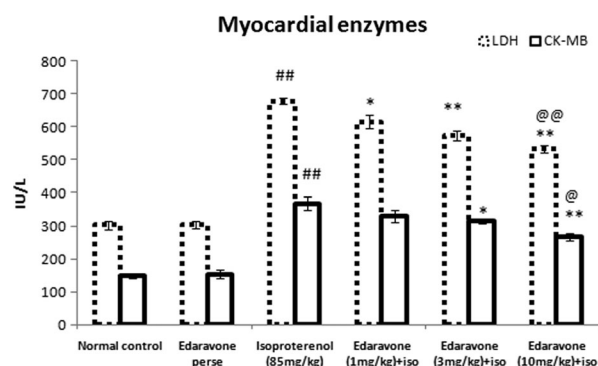‡$P < 0.05$ vs. edaravone (1 mg/kg) + ISO.

Figure 1   LDH and CK-MB in serum of experimental groups. Values are mean ± SEM from a group of six animals. ##$P < 0.01$ when control group compared with toxic group. *$P < 0.05$, **$P < 0.01$, when pretreated group compared with toxic group. @$P < 0.05$, @@$P < 0.01$, when high dose pretreated group compared with low dose pretreated group.

decreased the level of CK-MB in serum as compared with the ISO-treated group.

Effect of edaravone on CRP
Figure 2 shows the effect of edaravone on the serum level of CRP. The ISO-treated group showed a significant ($P < 0.01$) increase in the level of serum CRP as compared to the normal control rats. Pretreatment with edaravone (10 and 3 mg/kg) significantly ($P < 0.01$) reduced the level of serum CRP as compared with the ISO-treated group, while edaravone (1 mg/kg) significantly ($P < 0.05$) reduced the level of CRP.

Effect of edaravone on caspase-3
Figure 3 shows the effect of edaravone on the myocardial activity of caspase-3. The ISO-treated group showed significantly ($P < 0.01$) increased expression of activated caspase-3 in myocardial cells of rats as compared to normal control rats. Pretreatment with edaravone (10 mg/kg) in ISO-treated animals significantly ($P < 0.01$) decreased the levels of activated caspase-3 as compared with the ISO-treated group.
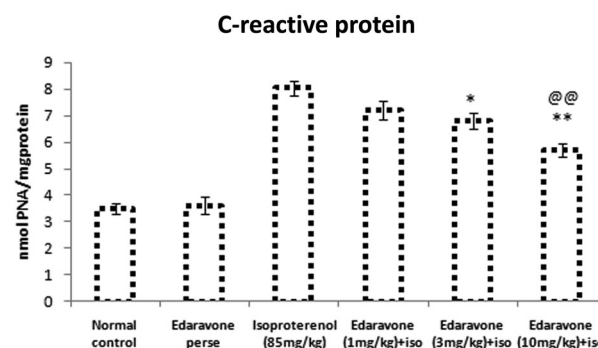

Figure 2   C-reactive protein in serum of experimental groups. Values are mean ± SEM from a group of six animals. ##$P < 0.01$ when control group compared with toxic group. *$P < 0.05$, **$P < 0.01$, when pretreated group compared with toxic group. @@$P < 0.01$, when high dose pretreated group compared with low dose pretreated group.
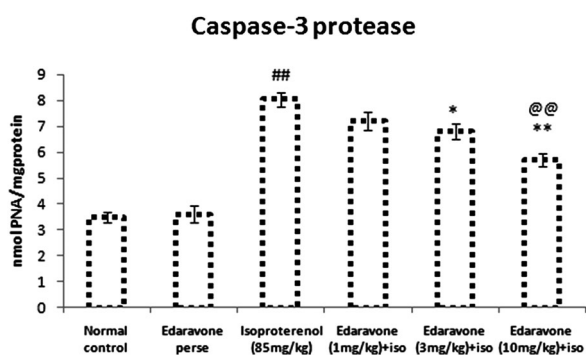
Caspase-3 protease

Figure 3 Caspase-3 protease in cardiac tissue of experimental groups. Values are mean ± SEM from a group of six animals. ##P < 0.01 when control group compared with toxic group. *P < 0.05, **P < 0.01, when pretreated group compared with toxic group. @@P < 0.01, when high dose pretreated group compared with low dose pretreated group.

Pretreatment with edaravone (3 mg/kg) significantly (P < 0.05) decreased the expression of activated caspase-3 as compared with the ISO-treated group.

Effect of edaravone on the ultrastructure of myocardial tissue

Figure 4 represents the ultrastructure of myocardial tissue, with clearly elaborated mitochondria and myofibrils. Ultrastructural analysis of the myocardial tissue in the ISO-treated group showed significant alteration of the integrity of mitochondrial membranes, disruption of cristae with vacuolation (Fig. 4C). Pretreatment with edaravone (10 and 3 mg/kg) in the ISO-treated group showed well preserved mitochondria and less evidence of myocyte injury, as well as preservation of the integrity of mitochondrial membranes and architecture of the cristae (Fig. 4E and F). Pretreatment with edaravone (1 mg/kg) in the ISO-treated group showed moderate loss of mitochondrial membrane and disruption of cristae with vacuolation (Fig. 4D). Control and *per se* groups showed normal architecture of the myocardial mitochondria and myofilaments as shown in Fig. 4A and B, respectively.

Discussion

Oxidative stress generates ROS, which play a crucial role in the pathogenesis of MI. In the present study, the ISO-treated experimental model of MI was used. Cardiotoxic actions of ISO on cardiac tissues are directly produced by the activation of beta-adrenoceptors, and the indirect effects are progressed by the formation of ROS and oxyradicals.[28] A study has shown that adrenochromes and oxyradicals are produced during the oxidation of catecholamines.[29] Oxidative products of catecholamines induce functional and subcellular changes in the rat myocardium, leading to cardiomyopathy.[28,30] Edaravone is one of the most potent scavengers of free radicals and prevents lipid peroxidative damage.[8] Edaravone prevents the increased levels of hydroxyl radicals and superoxide radicals in ischemia models.[31] It also has been reported to reduce myocardial infarct size and improve cardiac function and left ventricular remodeling, possibly due to reduced free radical generation in myocardium.[32] In the present study, the protective effects of edaravone on ISO-treated MI were evaluated.

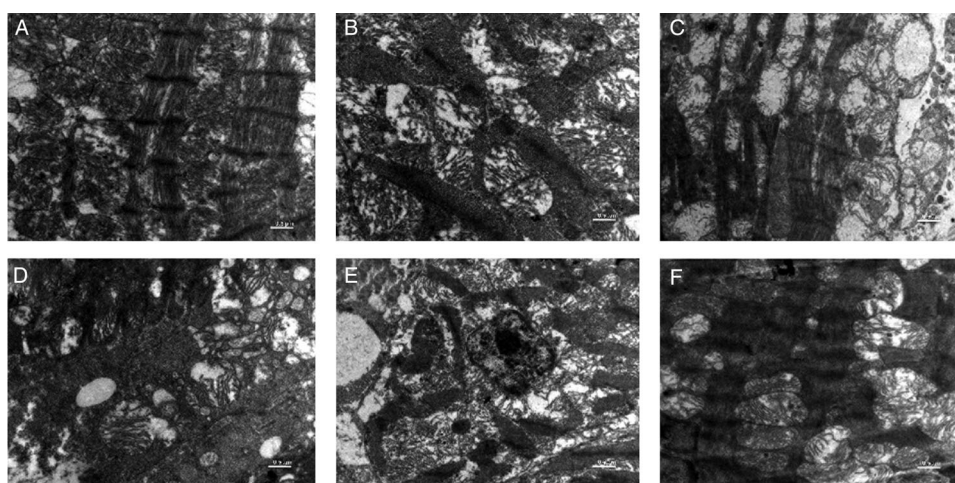ISO treatment significantly increased the levels of TBARS in heart tissue when compared to the

Figure 4 Transmission electron microscopic images of rat heart mitochondria. (A) Normal control group showing normal architecture of the heart mitochondria. (B) *per se* group (edaravone 10 μg/kg, i.p.) rats showing normal mitochondria along with good appearance of myofilaments of the heart mitochondria. (C) ISO-treated (85 mg/kg, s.c.) rat heart mitochondria showing ruptured mitochondrial cell membrane, bulging of mitochondria and disorder of cristae with vacuolation. (D) Edaravone (1 mg/kg, i.p. + ISO) treated heart mitochondria showing disarrangement of cristae with some swelling and vacuolation. (E) Edaravone (3 mg/kg, i.p. + ISO) treated heart mitochondria showing maintained architecture and slight fibrinolysis of mitochondria space. (F) Edaravone (10 mg/kg, i.p. + ISO) treated heart mitochondria showing maintained architecture and no fibrinolysis of mitochondrial space. (Scale bar: 0.5 μm).

control group, which is consistent with earlier reports.[33] This may be due to the formation of free radicals and the activation of lipid peroxidation, resulting in irreversible myocardial membrane injury. It also has been reported that oxyradicals lead to lipid peroxidation, and cause the alteration of membrane integrity and permeability.[34] Pretreatment with edaravone along with ISO showed a significant decrease in the TBARS levels when compared with the ISO-treated group, which may be due to the anti-oxidant and free radical scavenging activities of edaravone.

Inhibition of the activities of SOD and CAT leads to production of superoxide ions and hydroxyl radicals, which damage the myocardium. In the ISO-treated group, the activities of SOD and CAT were significantly decreased, which corroborates a previous study.[35] Pretreatment with edaravone along with ISO led to a significant increase in the activities of SOD and CAT when compared to the ISO-treated group.

The steady ratio of glutathione (GSH) to oxidized glutathione (GSSH) in cells depends on the activities of GPx and GR. These activities are reduced by the consumption of GSH, as reported earlier.[36] In the present study, it was found that the level of GSH and the activities of GPx, GR, and GST in cardiac tissue were significantly reduced in the ISO-treated group. Pretreatment with edaravone in the ISO group significantly increased the concentration of GSH and the activities of GPx, GR, and GST. The results suggested that edaravone might protect the cellular antioxidant defense system against oxidative stress.

Oxidative products of ISO damage myocardial cells leading to the loss of membrane integrity, and as a consequence, the cytosolic enzymes CK-MB and LDH leak into the blood and serve as markers of the MI.[37] Serum levels of the myocardial damage markers CK-MB and LDH were significantly increased in the ISO-treated group in our study also. Figure 1 illustrates that ISO treatment increased the levels of myocardial markers, which was significantly decreased by pretreatment with edaravone, in accordance with Tsujita *et al.*[38] This may possibly be due to the scavenging property of edaravone preserving the myocardium from oxyradicals generated by ISO. Thus, the antioxidant property of edaravone might have participated in maintaining the myocardial cell membrane integrity.

Studies have reported that ROS stimulate an inflammatory cascade by inducing cytokine expression, which has the potential to damage myocardium and endothelial cells.[39] CRP is a highly specific and sensitive systemic marker of inflammation and tissue damage.[40] CRP is a good indicator of the inflammatory cascade that propagates the pathology of MI.[41]

The present study demonstrates that serum CRP levels were significantly increased in the ISO-treated group. Pretreatment with edaravone in the ISO-treated group significantly reduced the levels of CRP in the serum.

Caspase-3 is an important apoptotic factor that propagates the pathway of apoptosis protease cascade.[42] Reports are available in the literature that oxidative stress coexists with apoptosis in the remote non-infarcted rat myocardium after MI.[43] The ISO treatment increases work load on the heart by the activation of beta-adrenergic receptors and its oxidative metabolites (adrenochrome and oxyradicals) cause remodeling and apoptosis in the myocardium.[44] In our study, a significant increase in caspase-3 activity was observed in ISO-treated rats indicating increased apoptosis in the myocardium. Pretreatment with edaravone in the ISO-treated group led to a significantly reduced activation of caspase-3 protease. These results indicate that edaravone decreased myocardial apoptosis by reducing the expression of activated caspase-3. These findings also suggest that there is a link between oxidative stress and apoptosis in MI induced by ISO, which may have been attenuated by the antioxidant property of edaravone.

Transmission electron microscopic examination showed significant alteration in the integrity of mitochondrial membranes and disruption of cristae structure, which may have been caused by the elevation of free oxygen radicals produced by oxidation of ISO. Pretreatment with edaravone preserved the ultra-structure of myocardial cells as well as the shape and architecture of mitochondria and cristae.

Thus it can be concluded that edaravone may have prevented ISO-induced cardiotoxicity in rats by attenuating oxidative stress and apoptosis in ISO-induced myocardial infarction.

Disclaimer statements

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