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# Edaravone protects against ischemia/reperfusion-induced oxidative damage to mitochondria in rat liver

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**Abstract**

This study investigated the effects of edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, MCI-186), a potent free radical scavenger, on the prevention of mitochondrial injury induced by hepatic ischemia and reperfusion. Mature male rats were subjected to 70 min of hepatic ischemia and 2 h of reperfusion. The rats received vehicle or edaravone (10 mg/kg body weight) intravenously prior to ischemia, before reperfusion and 1 h after reperfusion. In the vehicle-treated animals, the respiratory control index, ADP/O, State 3 respiration and dinitrophenol-induced uncoupled respiration decreased markedly after ischemia/reperfusion and were restored by edaravone administration. Mitochondrial lipid peroxidation was elevated in the vehicle-treated group, which was attenuated by edaravone, while mitochondrial glutathione peroxidase activity decreased in the vehicle-treated group, which was similarly abrogated by edaravone treatment. Electron microscopic observation demonstrated that treatment with edaravone restored the ischemia/reperfusion-induced disorganization of mitochondrial structures. Edaravone protects against mitochondrial injury, which prevents mitochondrial oxidative stress and improves ischemia/reperfusion-induced hepatic energy metabolism.


*Keywords:* Edaravone; Antioxidant; Fee radical; Mitochondrion; Ischemia/reperfusion

## 1. Introduction

Hepatic ischemia/reperfusion is a common problems encountered in many clinical conditions, such as liver transplantation, hepatic failure after shock and liver surgery. Ischemia/reperfusion causes functional and structural damage to liver cells (Mittnacht and Farber, 1981). Considerable evidence suggests that the role of oxygen-derived free radicals is involved in the pathogenesis of hepatic ischemia/reperfusion injury (Hirata et al., 1996; Kobayashi et al., 1991). Pharmacological evidence, such as the beneficial effects of the xanthine oxidase inhibitor allopurinol (Jeon et al., 2001), or of enzymes that metabolize reactive oxygen such as superoxide dismutase and catalase (Romani et al., 1988), support the oxygen radical hypothesis. Also other antioxidants, e.g. α-tocopherol, Trolox, a soluble analog of vitamin E and co-enzyme $Q_{10}$, have been used to protect the liver from ischemia/reperfusion injury (Marubayashi et al., 1985; Wu et al., 1991).

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, MCI-186), a synthetic antioxidant, is a ubiquitously acting direct free radical scavenger that inhibits lipoxygenase activity (Watanabe et al., 1994; Watanabe and Egawa, 1994)); while being highly efficient in detoxifying the devastating reactive •OH, edaravone also directly neutralizes peroxyl radicals ($LOO^{\cdot}$) (Watanabe et al., 1994; Yamamoto et al., 1996). The product of this interaction is 2-oxo-3-(phenylhydrazono)-butanoic acid (Yamamoto et al., 1996). Edaravone reportedly diminishes free radical-mediated lipid peroxidation in vitro (Watanabe et al., 1994). Edaravone also ameliorates cerebral edema and tissue injury after recirculation following ischemia in rats (Abe and Kogure, 1988). Edaravone has been clinically prescribed in Japan since 2001 to treat patients with cerebral ischemia. A number of studies have shown its virtual absence of serious toxicity, even when given in massive doses (90 mg/body, twice per day for 14 days) and it has no known interactions with drugs (Otomo et al., 1998; Shibata et al., 1998).

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Reperfusion of the ischemic tissue has been associated with oxidative modifications and functional impairment of mitochondria (Hirata et al., 1996; Kobayashi et al., 1991). However, no study investigated the effect of edaravone on the mitochondrial respiratory function in the liver subjected to ischemia/reperfusion. Accordingly, we designed this study to investigate the effects of edaravone on the post-ischemic deterioration of hepatic mitochondrial function.

## 2. Materials and methods

### 2.1. Chemicals

ADP was obtained from Sigma (St. Louis, USA). Dinitrophenol, succinate, glutamate, tetramethoxypropane and thiobarbituric acid were purchased from Wako (Osaka, Japan). Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) was a gift from Mitsubishi Pharma (Osaka, Japan). All other chemicals in this study were of reagent grade and were locally and commercially available.

### 2.2. Animals

Male Sprague–Dawley rats (350 ± 20 g body weight) were housed in plexiglas cages with three animals per cage. The animal room was windowless with automatic temperature (22 ± 2 °C) and lighting controls (light on at 0700 h and off at 1900 h). A solid diet (CE-2, Clea, Tokyo, Japan) and water were provided ad libitum. The Animal Research Committee of the Kochi Medical School approved all research protocols. Edaravone was dissolved in 1 M NaOH and adjusted pH at 7 and thereafter diluted to 6 mg/ml in sterile water.

### 2.3. Experimental procedure

The animals were used after 14 days of acclimation to the animal room. The surgical procedure was performed with the rats under anesthesia [rodent cocktail given ip at a dose of 0.2 ml/250 g body weight (BW); the cocktail consisted of ketamine (60 mg/ml) and xylazine (8 mg/ml)]. Throughout of the experiment, body temperature was maintained at 36–38 °C with the aid of a heating pad. The animals were divided into four groups: the control group; hepatic ischemia (70 min); hepatic ischemia plus 2-h reperfusion; hepatic ischemia plus 2-h reperfusion and three injections of edaravone. Edaravone (10 mg/kg BW) or saline was injected at three time points: the first before ischemia, the second before reperfusion and the third 1 h after reperfusion. The sham-operated control animals received the same surgical procedure as the other groups without the ischemia/reperfusion protocol, and were given an equivalent amount of sterile saline only. As for the dose of edaravone, it was shown that the intravenous administration of edaravone, at 0.3, 1 and 3 mg/kg body weight, to rats attenuated post-ischemic brain edema (Watanabe and Egawa, 1994) and myocardial reperfusion (Yanagisawa et al., 1994) injury in a dose-dependent manner. Following the intravenous injection of sodium heparin (200 U/kg BW), all vessels (hepatic artery, portal vein and bile duct) on the left and the median liver lobes were occluded for 70 min with a vascular clamp. Thereafter, the clamp was removed and blood flow was reperfused for 2 h in each group. During the period of ischemia, 0.5 ml of saline was given ip every 20 min to maintain hemodynamic stability and to replace losses due to portal stasis. After 2 h, animals were killed by perfusing with ice-cold saline via the inferior vena cava.

### 2.4. Preparation of mitochondria

Mitochondria were prepared from fresh livers essentially by the method of Hogeboom (1985). The isolated mitochondria were suspended in 0.25 M sucrose, 10 mM Tris–HCl (pH 7.2) and 0.1 mM ethylenediaminetetraacetic acid (EDTA) at 20–30 mg protein/ml. Mitochondrial protein was determined by Lowry's method (1951) using bovine serum albumin as a standard.

### 2.5. Measurement of mitochondrial respiratory activity

Oxygen consumption was measured polarographically at 25 °C using 1.0–2.5 mg protein from the fresh mitochondrial fraction in 2.0 ml of incubation medium consisting of 100 mM KCl, 0.05 mM EDTA, 10 mM Tris–HCl and 0.1 M sucrose, at pH 7.4, using a Clark-type electrode. Mitochondrial respiration was initiated by adding 150 μM ADP with 5 mM glutamate or 5 mM succinate as the respiratory substrate. Oxygen consumption measured in the presence of added ADP was defined as State 3 respiration, while that measured following the consumption of ADP was defined as State 4 respiration (Chance and Williams, 1956). The respiratory control index was calculated as ratio of State 3 respiration to State 4 respiration, and used as a marker of mitochondrial respiratory activity. The ADP/O ratio was calculated as the ratio of the added ADP concentration to the consumption of oxygen during State 3 respiration. Uncoupled respiration was induced by adding 25 μM dinitrophenol. Mitochondrial respiration was calculated as the nanomoles of $O_2$ per minute per milligram of protein.

### 2.6. Measurement of glutathione peroxidase activity and lipid peroxidation

Glutathione peroxidase activity in the mitochondria was measured using a GPx-340 kit (Bioxytech, Paris, France) based on the method described by Paglia and Valentine (Paglia and Valentine, 1967). The change in absorbance at 340 nm, which results from the oxidation of NADPH, is the basis for quantitating cellular glutathione peroxidase activity. Mitochondria (10 μl) was mixed with 75 μl of assay buffer (0.05 M Tris–HCl, 5 mM EDTA) and 75 μl of NADPH

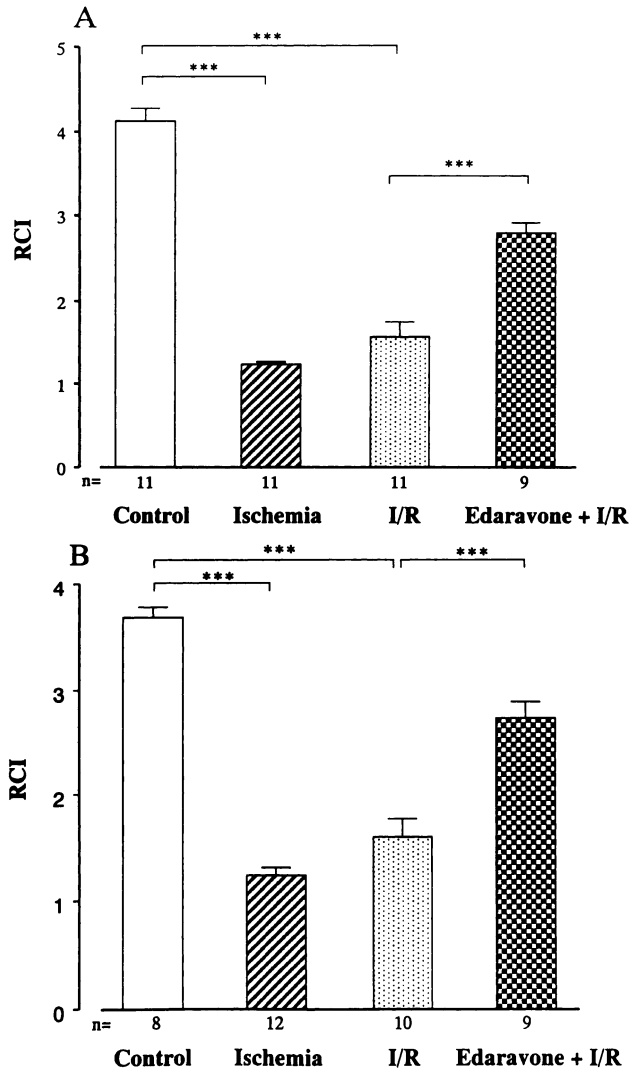


Fig. 1. Effect of edaravone (10 mg/kg BW, iv) on the hepatic mitochondrial respiratory control index (RCI) after 70-min liver ischemia (I) followed by 2-h reperfusion (R). The substrate was glutamate (A) or succinate (B).Values are means ± S.E.M. from *n* (at the base of each column) rats. **P* < 0.0001.

reagent containing 3.2 mM glutathione, 1600 U/l glutathione reductase, and 640 μM betanicotinamide–adenine dinucleotide. Kinetic specrophotometric analysis was started by adding 75 μl of 0.007% tetra-butyl hydroperoxide at 340 nm. The sample was replaced with water in the blanks. The rate of decrease in $A_{340}$ per minute was determined by averaging the rate of change at 30-s intervals between 30 and 180 s.

Concentrations of thiobarbituric acid-reactive substances were determined according to the method of Ohkawa et al. (1979), as described before (Wakatsuki et al., 1999). In brief, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of a 0.8% thiobarbituric acid solution were added to the mitochondria, and the volume was brought to 4.0 ml with distilled water. The mixture was heated in an oil bath at 95 °C for 60 min. After the mixture was cooled with tap water, 5.0 ml of butyl alcohol and pyridine (15:1, v/v) was added, and the sample was shaken gently for 5 min. After centrifugation at 1500 × *g* for 10 min, the butyl alcohol-pyridine phase containing thiobarbituric acid-reactive substances was separated, and absorbance measured at 532 nm. The results were expressed as molar equivalents of malondialdehyde per milligram of protein, using malondialdehyde from tetramethoxypropane as the standard.

### 2.7. Electron microscopic observations

Mitochondrial pellets were pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 2 h and post-fixed in 1.5% osmium tetraoxide at 4 °C for 2 h. The samples were dehydrated and embedded in epoxy resin. Ultra-thin sections were cut by Ultrcut-E ultramicrotome (Reichert-Jung, Germany), stained with uranyl acetate and lead citrate, and observed with an electron microscope (JEM-100S; JEOL, Tokyo).

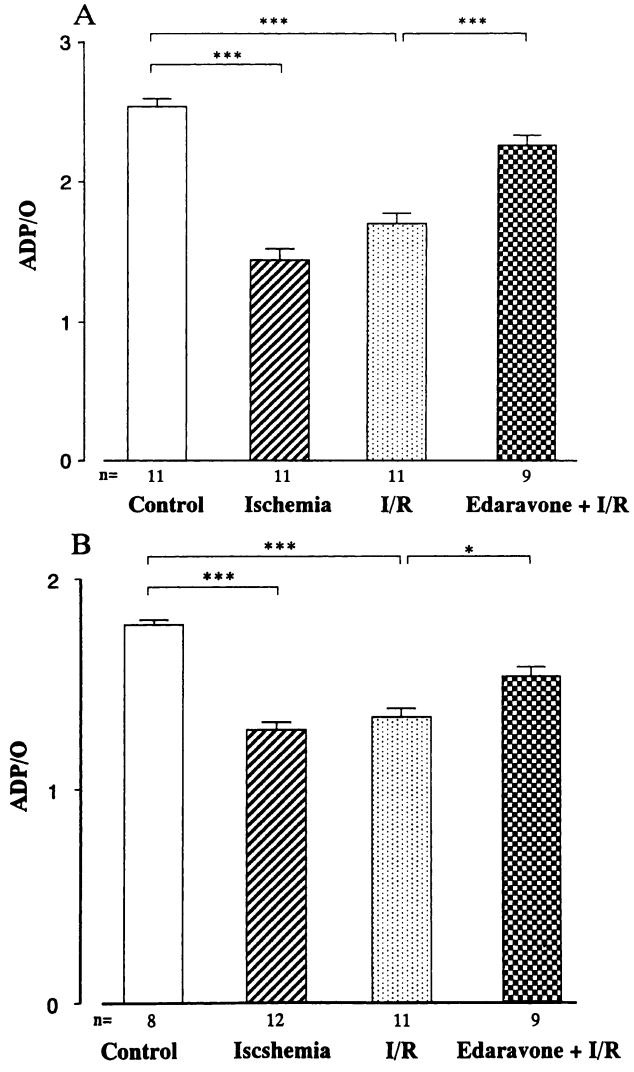


Fig. 2. Effect of edaravone ( 10 mg/kg BW, iv) on ADP/O in hepatic mitochondria after 70-min liver ischemia (I) followed by 2-h reperfusion (R). The substrate was glutamate (A) or succinate (B). Values are means ± S.E.M. from *n* (at the base of each column) rats. ****P* < 0.001.

### 2.8. Statistics

All data are expressed as the mean ± S.E.M. Data were analyzed by one-way analysis of variance, and Scheffe's test was applied to determine differences between the groups. A level of $P<0.05$ was accepted as indicating statistical significance.

## 3. Results

### 3.1. Oxidative phosphorylation

The results of mitochondrial respiration in the presence of glutamate or succinate and inorganic phosphate are presented in Figs. 1–4. Respiratory control index and ADP/O deceased significantly after ischemia. The mean values of respiratory control index and ADP/O tended to increase after reperfusion, but no significant increase was found during reperfusion (Figs. 1 and 2). The decreases in respiratory control index and ADP/O during ischemia/reperfusion were significantly increased to baseline levels by edaravone administration. State 3 respiration also decreased markedly after ischemia and tended to increase during reperfusion. Edaravone significantly increased State 3 respiration compared with ischemia/reperfusion alone (Fig. 3). State 4 respiration increased significantly after ischemia in the presence of glutamate and remained elevated during ischemia/reperfusion (Fig. 4). State 4 respiration tended to decrease by edaravone treatment, and was similar to that in glutamate in the presence of succinate, but no significant difference was found between the groups.

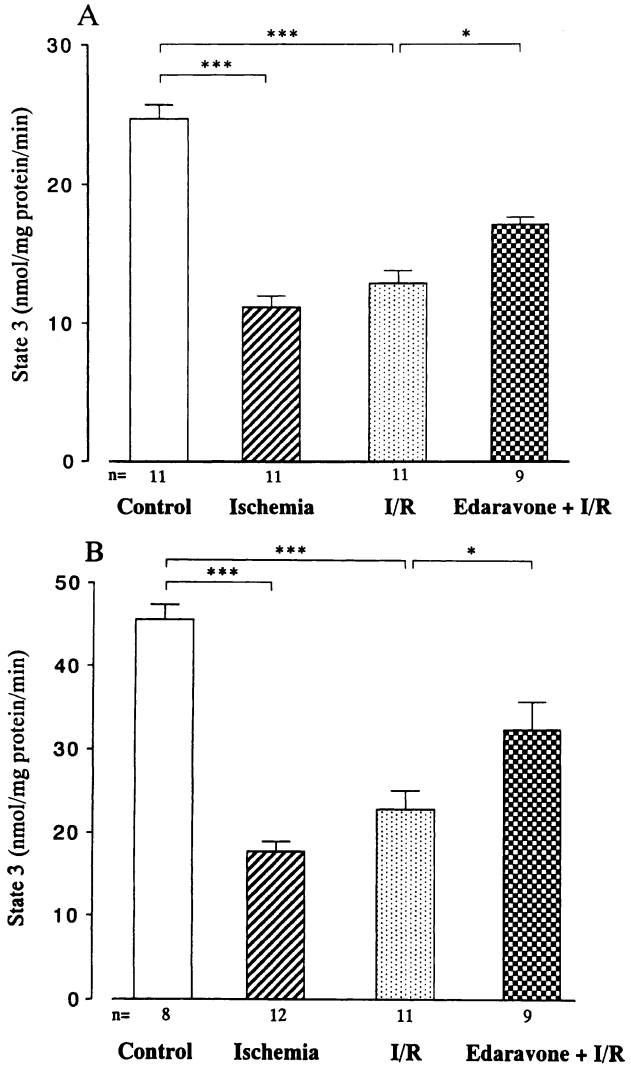


Fig. 3. Effect of edaravone (10 mg/kg BW, iv) on State 3 respiration in hepatic mitochondria after 70-min liver ischemia (I) followed by 2-h reperfusion (R). The substrate was glutamate (A) or succinate (B). Values are means ± S.E.M. from *n* (at the base of each column) rats. *$P<0.05$, ***$P<0.001$.

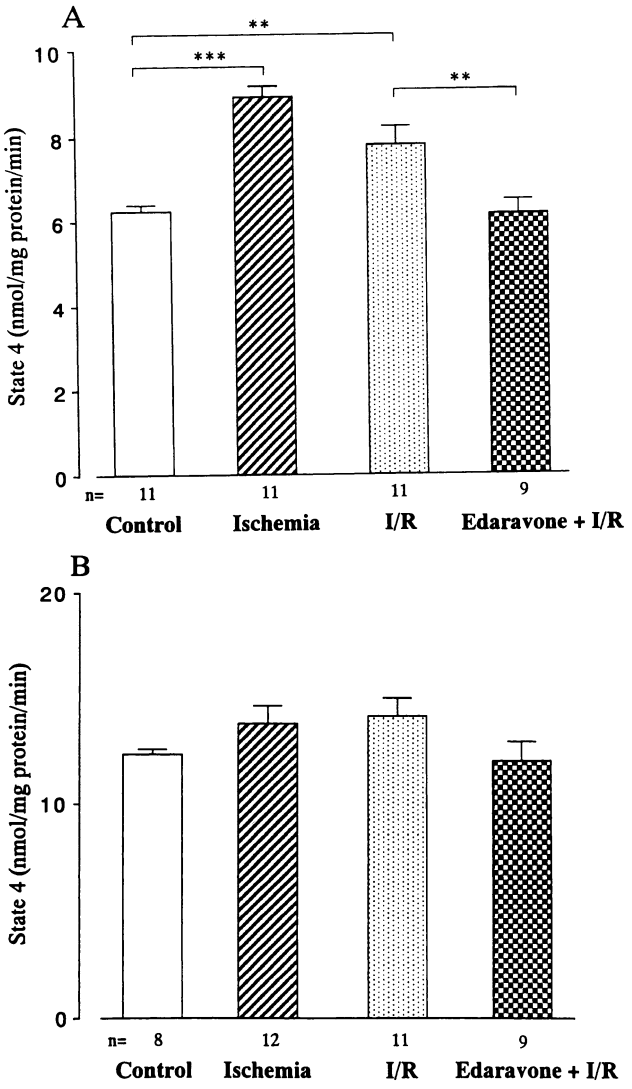


Fig. 4. Effect of edaravone (10 mg/kg BW, iv) on State 4 respiration in hepatic mitochondria after 70-min liver ischemia (I) followed by 2-h reperfusion (R). The substrate was glutamate (A) or succinate (B). Values are means ± S.E.M. from *n* (at the base of each column) rats. **$P<0.01$, ***$P<0.0001$.

### 3.2. Uncoupled respiration

Respiration in mitochondria is uncoupled by dinitrophenol and an increase in oxygen consumption, reflecting an accelerated electron transport activity. Dinitophenol-induced uncoupled respiration showed similar patterns and values to those for State 3 respiration (Fig. 5). Dinitrophenol-induced uncoupled respiration decreased markedly after ischemia and remained decreased during reperfusion. Treatment with edaravone significantly restored dinitrophenol-induced uncoupled respiration.

### 3.3. Lipid peroxidation and glutathione peroxidase activity

The levels of mitochondrial thiobarbituric acid-reactive substances increased as a result of ischemia/reperfusion with the increase being abolished by edaravone administration (Fig. 6), and glutathione peroxidase activity remained unchanged after ischemia, but decreased significantly during reperfusion (Fig. 7). The decrease in glutathione peroxidase activity after ischemia/reperfusion was significantly restored by edaravone treatment.

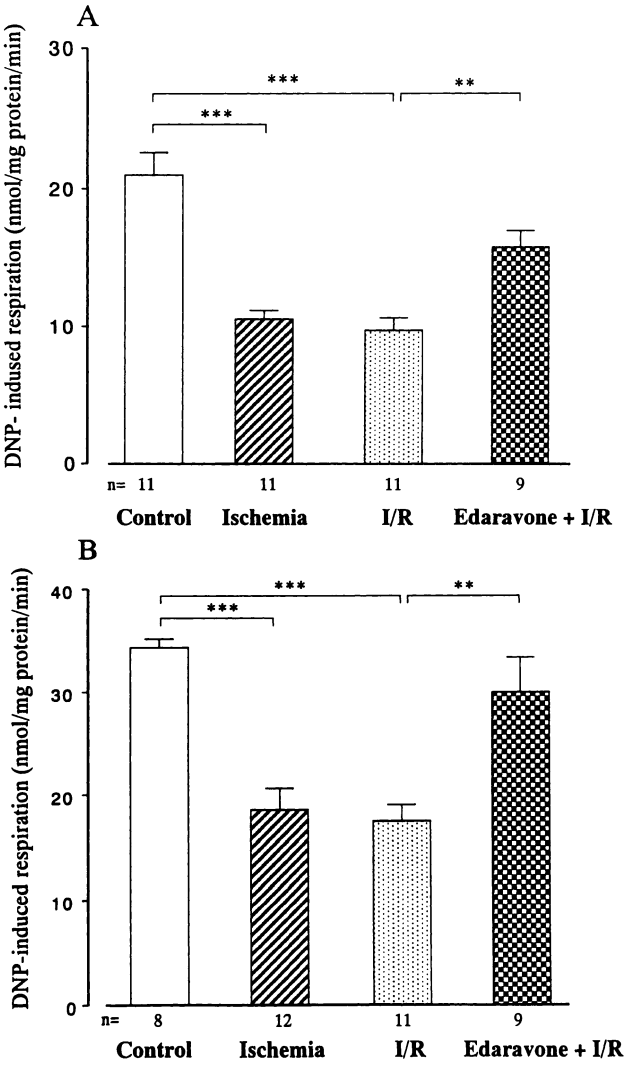


Fig. 5. Effect of edaravone (10 mg/kg BW, iv) on dinitrophenol (DNP)-induced uncoupled respiration in hepatic mitochondria after 70-min liver ischemia (I) followed by 2-h reperfusion (R). The substrate was glutamate (A) or succinate (B). Values are means ± S.E.M. from *n* (at the base of each column) rats. $^{**}P<0.01$, $^{***}P<0.0001$.

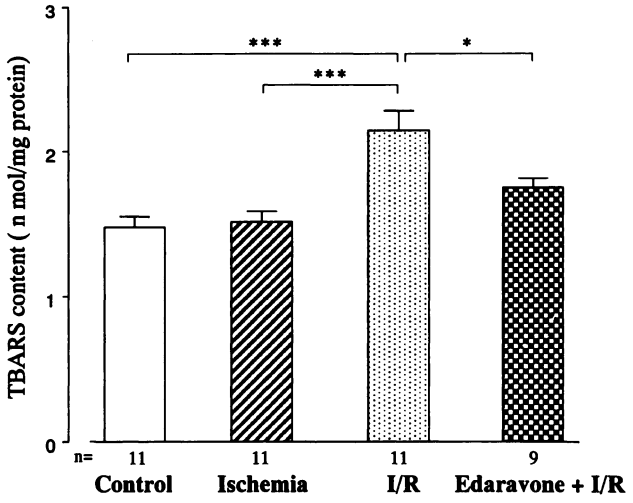


Fig. 6. Effect of edaravone (10 mg/kg BW, iv) on thiobarbituric acid reactive substance (TBARS) in hepatic mitochondria after 70-min liver ischemia (I) followed by 2-h reperfusion (R). Values are means ± S.E.M. from *n* (at the base of each column) rats. $^{*}P<0.05$, $^{***}P<0.0001$.

### 3.4. Ultrastructural observations

As shown in Fig. 8A, most of the mitochondria from the sham-operated animals were in a highly condensed form. The matrix was tightly packed and rather heavily stained.

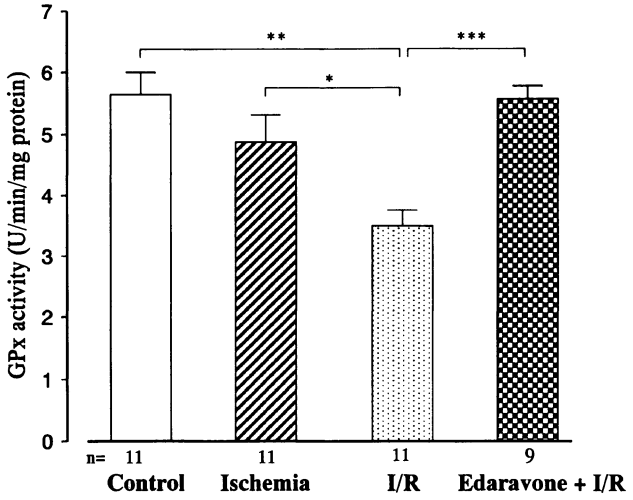


Fig. 7. Effect of edaravone (10 mg/kg BW, iv) on glutathione peroxidase (GPx) activity in hepatic mitochondria after 70-min liver ischemia (I) followed by 2-h reperfusion (R). Values are means ± S.E.M. from *n* (at the base of each column) rats. $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$.

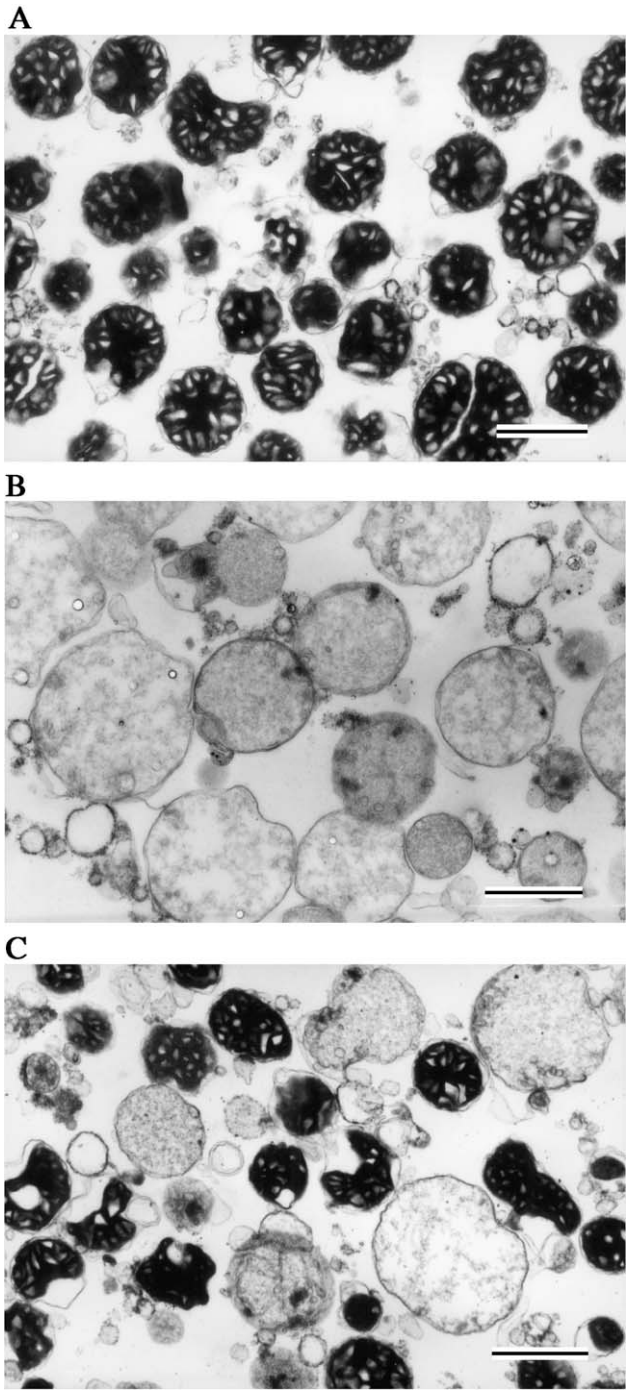


Fig. 8. Conformational changes in rat liver mitochondria induced by ischemia/reperfusion. (A) Vehicle-treated control rat. (B) Ischemia/reperfusion without edaravone treatment. (C) Ischemia/reperfusion treated with edaravone; magnification, × 10 000. Bars in figures indicate 1 μm.

However, the mitochondria from ischemia/reperfusion-treated animals lost their swelling–contraction cycle and appeared in a swollen state (Fig. 8B). In contrast, in mitochondria from edaravone-treated animals, some were swollen while others were in a relatively contracted state (Fig. 8C).

## 4. Discussion

Reactive oxygen species, such as $O_2^{-}\cdot$, $H_2O_2$, HOCl, may be involved in the tissue destructive effects of reperfusion after ischemia (De Groot, 1994). During ischemia/reperfusion, most oxygen free radicals are produced in mitochondria as well as activated granulocytes, monocytes and macrophage (Jaeschke, 1991). Lipid peroxidation may cause alterations in biomembrane-associated functions and structure and may disrupt the function of the cell or subcellular organelle (McCord, 1985). In the present study, mitochondrial lipid peroxidation remained unchanged in vehicle-treated ischemic rats, but significantly increased during reperfusion.

Reduced glutathione level, the most important endogenous antioxidant, reportedly decreases in liver after a period of ischemia followed by reperfusion (Sewerynek et al., 1996). The drop in reduced glutathione levels during ischemia/reperfusion was probably due to its consumption during oxidative stress. Furthermore, the present study showed that the activity of glutathione peroxidase, which detoxifies $H_2O_2$ while oxidizing reduced glutathione to oxidized glutathione, was depressed during ischemia/reperfusion, possibly contributing to the resulting oxidative damage by making $H_2O_2$ available for conversion to •OH.

The present study demonstrates that respiratory control index in hepatic mitochondria from ischemia/reperfusion showed a marked reduction, as evidenced by reduced State 3 respiration and an increased State 4 respiration. The decrease in ADP/O in mitochondria from ischemia/reperfusion may be due to uncoupling as a result of membrane damage. The increase in State 4 respiration in mitochondria from ischemia/reperfusion may also be explained by their uncoupling. Similar to State 3 respiration, the increased rate of oxygen consumption induced by dinitrophenol in the ischemia/reperfusion animals was markedly reduced. This also suggests that the coupling mechanism for energy transfer reactions of the electron transport system may be altered during ischemia/reperfusion. Higher levels of oxygen free radicals are also found when respiratory chain is inhibited (Livrera et al., 1997).

A large body of evidence suggests that a channel formed in the mitochondrial membranes, called the permeability transition pore, is involved in cell damage associated with ischemia/reperfusion (Crompton, 1999; Fiskum et al., 1999). This channel increases the permeability of the mitochondrial inner membrane to solutes (Bernardi et al., 1994; Zoratti and Szabo', 1995). The permeability transition pore opening is triggered by the association of calcium overload with an inducer, such as oxidative stress or high phosphate concentration, conditions encountered during ischemia/reperfusion. The opening of this pore leads to the destruction of the mitochondrial membrane potential and mitochondrial swelling, resulting in mitochondrial uncoupling and inhibition of ATP synthesis. In this study, we did not determine mitochondrial membrane potential;

however, ischemia/reperfusion resulted in disorganization of mitochondrial structures characterized by swelling as shown in Fig. 8B.

The most important finding in the present study is that edaravone protects against ischemia/reperfusion-induced impairment of mitochondrial respiration, swelling and lipid peroxidation. This indicates that mitochondria are probably the main pharmacologic targets of edaravone. This effect of edaravone may be related to its potent free radical scavenger action and/or inhibition of the permeability transition pore opening. Ischemia/reperfusion is closely associated with $Ca^{2+}$ overload, an overproduction of oxygen free radicals, an increase in phosphate and a decrease in cellular ATP concentration (Bernardi et al., 1994; Zoratti and Szabo', 1995). Permeability transition pore is highly sensitive to the oxidative-reduced state of mitochondria and it has been shown that oxidative stress triggers permeability transition pore. Edaravone is a potent scavenger of free radicals, which is highly effective in scavenging .OH species (Watanabe et al., 1994). Edaravone reportedly diminishes free radical-mediated lipid peroxidation in vitro (Watanabe et al., 1994; Yamamoto et al., 1996). We also found the protective effect of melatonin, a potent free radical scavenger, against an impairment of cerebral damage of lipids, DNA and mitochondrial respiratory function induced by ischemia/reperfusion in fetal rat in vivo (Wakatsuki et al., 1999, 2001). Edaravone is highly lipophilic, readily accessible to tissue and effective tissue level can be maintained with one intravenous bolus injection. Furthermore, edarvone reportedly improves portal flow, hepatic enzyme release into the perfusate, total bile production and histologic alteration following the hepatic ischemia/reperfusion in the rat (Ninomiya et al., 2002).

It is known that polymorphonuclear neutrophils are also involved in the pathogenesis of hepatic ischemia/reperfusion injury because they stimulate inflammatory mediators such as tumor necrosis factor and interleukin-1 (Suzuki et al., 1994). Activation of the cerebral arachidonate cascade is one of the major causes of edema and tissue injury in cerebral ischemia, particularly after reperfusion (Dempsey et al., 1986). Edaravone ameliorates cerebral edema and tissue injury especially exacerbated after reperfusion following ischemia in rats (Abe and Kogure, 1988). The site of action of edaravone may be mediated by the ability to inhibit lipoxygenase metabolism (Watanabe and Egawa, 1994). However, further studies are needed to clarify the protective effect of edaravone against ischemia/reperfusion-induced mitochondrial dysfunction and the association of cytokines or components of the arachidonate cascade.

We conclude that exogenous edaravone is capable of preserving the mitochondrial function and energetic status during ischemia/reperfusion. This improvement is most likely by scavenging endogenously produced oxygen free radicals. Pharmacologically, edaravone may have clinical applicability in ischemia/reperfusion injury.

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