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# The radical scavenger edaravone counteracts diabetes in multiple low-dose streptozotocin-treated mice

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**Abstract**

Edaravone is a potent scavenger of hydroxyl radicals and attenuates oxidative damage-related neurodegenerative diseases. Previous studies suggest that oxidative stress plays a key role in the pathogenesis of diabetes. The present study examined the effect of edaravone on diabetes in multiple low-dose streptozotocin-treated mice. Mice treated with low-doses of streptozotocin for five consecutive days showed progressive hyperglycemia and an increased incidence of diabetes. Daily treatment with edaravone during the streptozotocin injections counteracted the multiple low-dose streptozotocin-induced hyperglycemia in a dose-dependent manner. Edaravone protected against the multiple low-dose streptozotocin-induced reduction in pancreatic insulin. The suppressive effects of edaravone were also observed when it was administered after the last injection of streptozotocin. Histochemical examination showed that multiple low-dose streptozotocin treatment caused mononuclear cell infiltration in pancreatic islets, followed by hyperglycemia, and that edaravone significantly inhibited the multiple low-dose streptozotocin-induced insulitis. Multiple low-dose streptozotocin treatment also increased the lipid peroxidation product thiobarbituric acid reactive substance in pancreatic tissues of mice, and this effect was completely inhibited by edaravone. These findings suggest that edaravone, even after streptozotocin treatment, counteracts the development of multiple low-dose streptozotocin-induced diabetes by scavenging free radicals, which are possible mediators of the immune destruction of islet β cells.


*Keywords:* Edaravone; Multiple low-dose streptozotocin; Diabetes; Insulitis; Thiobarbituric acid reactive substance

## 1. Introduction

Type 1 diabetes is an autoimmune disease characterized by mononuclear cell infiltration in the pancreatic islets of Langerhans (insulitis) followed by β-cell destruction. Noniso-form-specific inhibitors of nitric oxide synthase have been demonstrated to counteract diabetes in genetic animal models such as the nonobese diabetic mouse (Andersson et al., 2001) and the BioBreeding rat (Lindsay et al., 1995; Wu, 1995). In addition, the inducible nitric oxide synthase gene-deficient mouse has been shown to be less susceptible to multiple low-dose streptozocin-induced diabetes, a pharmacological animal model of type 1 diabetes (Flodström et al., 1999), although there are conflicting reports concerning the effects of nitric oxide synthase inhibitors in this model (Holstad and Sandler, 1993; Papaccio et al., 1995b; Rydgren and Sandler, 2002; Sternesjo et al., 1997). Previous studies have also shown that the over-expression of antioxidants such as Cu/Zn superoxide dismutase (Kubisch et al., 1997), catalase (Xu et al., 1999), thioredoxin (Hotta et al., 1998), and metallothionein (Chen et al., 2001) targeted at β-cells counteracts the development of type 1 diabetes. Since the pancreas has been shown to have low levels of the antioxidant enzymes superoxide dismutase and catalase (Lenzen et al., 1996), these findings suggest that oxidative stress plays a key role in the pathogenesis of diabetes (Oberley, 1988). Indeed, nitric oxide and reactive oxygen species induced by inflammatory cytokines such as interleukin-1β, tumor necrosis factor-α, and interferon-γ are considered to be crucial mediators of β-cell death (Corbett and McDaniel, 1992; Eizirik and Pavlovic, 1997; Lakey et al., 2001; Rabinovitch and Suarez-

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Pinzon, 1998; Southern et al., 1990). This is supported by several studies showing that pretreatment with antioxidants before the injection of streptozotocin attenuates the development of hyperglycemia and insulitis in the multiple low-dose streptozotocin model (Heineke et al., 1993; Mabley et al., 2004; Papaccio et al., 1995a; Sandler and Andersson, 1982; Song et al., 2003; Szabó et al., 2002). However, the effects of antioxidants on multiple low-dose streptozotocin-induced diabetes given after streptozotocin injection have not been reported.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent scavenger of hydroxyl and peroxyl radicals (Kawai et al., 1997; Watanabe et al., 1994) and nitric oxide (Satoh et al., 2002), and it has been demonstrated to be beneficial for patients following acute ischemic stroke (Houkin et al., 1998; Yoneda et al., 2003). We have also shown that edaravone protects against methamphetamine-induced and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurotoxicity in mice (Kawasaki et al., 2006, 2007a). These findings suggest that edaravone may also be effective in attenuating type 1 diabetes. The present study examines the effect of edaravone on type 1 diabetes in a multiple low-dose streptozotocin model. In particular, we demonstrate that treatment with edaravone after the injection of streptozocin attenuates the severity of hyperglycemia and insulitis.

## 2. Materials and methods

### 2.1. Experimental animals and drugs

Male C57BL/6J mice (7 weeks old) were housed in groups of 5–6/cage (24×17×12 cm) under controlled environmental conditions (22±1 °C; 12–12 h light–dark cycle, lights on at 08:00 h; food and water *ad libitum*) for at least 1 week before being used in the experiment. The care and handling of the animals were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society. The following drugs were used: edaravone (Mitsubishi Pharma Co., Osaka, Japan), streptozotocin (Sigma-Aldrich Inc., St. Louis, MO, USA), Sensitive Rat Insulin RIA kit (Linco Research, St. Charles, MO, USA), glucose C2 test, sodium citrate, Mayer's hematoxylin solution, butylated hydroxytoluene and thiobarbituric acid (Wako Chemical Industries, Ltd, Osaka, Japan), and paraformaldehyde (Nacalai Tesque, Inc., Kyoto, Japan). Edaravone was dissolved in 1 M NaOH, adjusted to pH 7.4 with 1 M HCl. Streptozotocin was dissolved in 50 mM sodium citrate buffer (pH 4.5). The drugs were injected intraperitoneally at 10 ml/kg.

### 2.2. Induction of diabetes

Mice were treated daily (09:00–10:00 h) with streptozotocin (40 mg/kg) or vehicle (citrate buffer) for 5 consecutive days (day 1–5). Edaravone at 1 and 3 mg/kg was injected twice daily (09:00–10:00 h and 21:00–22:00 h) starting at day 1 or day 6 until day 21. Edaravone was treated 30 min after streptozotocin when edaravone was injected starting at day 1. Blood glucose was measured on days 1, 4, 7, 10, 13, 16, 19 and 22 from blood obtained from the tail vein. Mice were considered to be diabetic after two consecutive blood glucose measurements of ≥11.1 mM. The cumulative incidence of diabetes was calculated as a percentage of hyperglycemic mice per treatment group at each time point. Biopsy specimens of the pancreas were removed on day 10 for histological analysis and day 22 for biochemical and histological analyses.

### 2.3. Measurement of lipid peroxidation

Lipid peroxidation in pancreas was assessed by determining the concentrations of thiobarbituric acid reactive substances (Mabley et al., 2004). Briefly, 50 µl of tissue homogenate in 154 mM KCl was added to a reaction mixture consisting of 375 µl 55.5 mM thiobarbituric acid, 50 µl 281 mM sodium

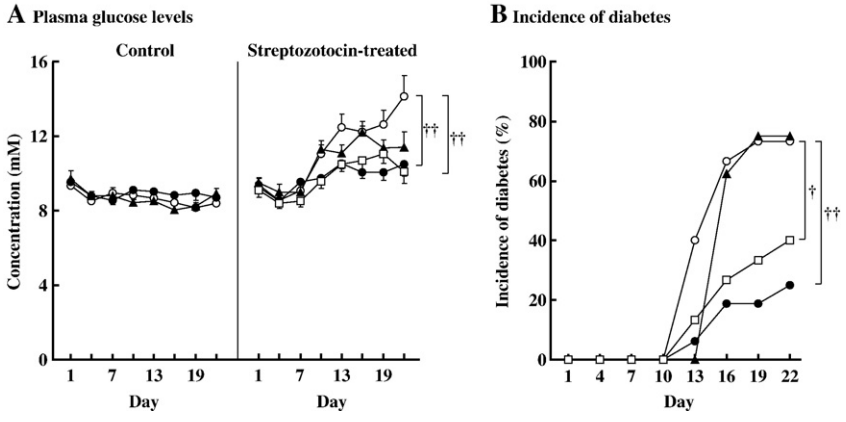


Fig. 1. Effect of edaravone on multiple low-dose streptozotocin-induced hyperglycemia in mice. Mice were treated daily with vehicle or streptozotocin at 40 mg/kg for 5 consecutive days (days 1–5). Vehicle (open circles), edaravone at 1 (closed triangles) and 3 (closed circles) mg/kg were injected twice daily starting on day 1 (days 1–21). Edaravone at 3 mg/kg was also injected twice daily after streptozotocin treatment starting on day 6 (days 6–21) (open squares). Plasma glucose concentrations were determined on days 1, 4, 7, 10, 13, 16, 19 and 22 in control and streptozotocin-treated mice (A). The incidence of diabetes in streptozotocin-treated mice was expressed as the cumulative percentage of mice with blood glucose greater than 11.1 mM for 2 consecutive measurements in the fed state (B). Values are expressed as the mean±S.E.M. of 8 to 16 mice. †$P<0.05$, ††$P<0.01$, compared with the vehicle treatment group of the streptozotocin-treated mice.

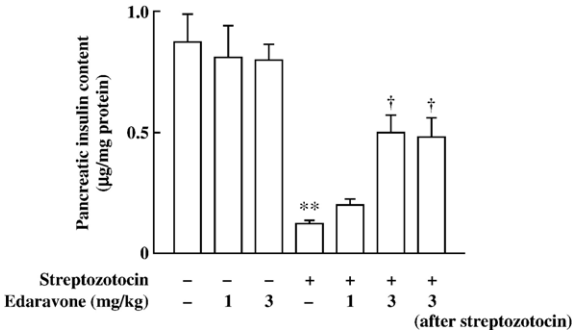


Fig. 2. Effect of edaravone on multiple low-dose streptozotocin-induced decrease in pancreatic insulin contents in mice. Mice were treated daily with vehicle or streptozotocin at 40 mg/kg for 5 consecutive days (days 1–5). Vehicle, edaravone at 1 and 3 mg/kg were injected twice daily starting on day 1 (days 1–21). Edaravone at 3 mg/kg was also injected twice daily after streptozotocin treatment starting on day 6 (days 6–21). Pancreatic insulin contents were measured on day 22. Values are expressed as the mean ± S.E.M. of 5 to 7 mice. $^{**}P<0.01$, compared with the vehicle treatment group of control mice; †$P<0.05$, compared with the vehicle treatment group of the streptozotocin-treated mice.

dodecyl sulfate, 375 μl 4.25 M acetic acid (pH 3.5), 15 μl 36.3 mM butylated hydroxytoluene (dissolved in acetic acid), and 135 μl distilled $H_2O$. The mixture was incubated at 95 °C for 1 h. After cooling at room temperature, 500 μl of *n*-butanol was added and the mixture shaken vigorously. After centrifugation at 4000 ×*g* for 10 min, absorbance of the supernatant (organic layer) was measured at 532 nm using a Shimadzu UV-1650PC spectrophotometer (Kyoto, Japan).

### 2.4. Histological examination

Severity of insulitis was evaluated histologically on day 10 and 22 for each group. Pancreata were fixed with 4% paraformaldehyde and embedded in paraffin. Five-μm-thick sections were cut and stained with hematoxylin and eosin for histological examinations. A minimum of 20 islets was scored for each animal. Scoring of insulitis was carried out in a blinded fashion. Islet inflammation (insulitis) was ranked according to an arbitrary scale (0–3) (Verdaguer et al., 1996; Takamura et al., 1999) as follows: rank 0 denotes no visible infiltration, rank 1 denotes peripheral or peri-islet infiltration (peri-insulitis), rank 2 denotes intra-islet infiltration (intra-insulitis), and rank 3 denotes massive lymphocytic infiltration with islet destruction (severe insulitis). For each animal, a mean score was calculated, which equaled the total score for all islets examined divided by the total number of islets examined.

### 2.5. Pancreatic insulin content

Each pancreatic specimen was homogenized in 1.8 ml of acid ethanol (75% ethanol, 23.5% water, and 1.5% 12 M HCl). The homogenates were stored at 4 °C for 24 h and then centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatants were diluted (1:20,000) and insulin levels were determined using a commercially available radioimmunoassay kit (Linco Research, St. Charles, MO, USA). Pancreatic insulin content was expressed as μg insulin/mg protein.

### 2.6. Statistics

Blood glucose levels were analyzed using two-way analysis of variance (ANOVA) for treatment as the intersubject factor and repeated measures with time as the intrasubject factor. Incidence of diabetes was analyzed using the $\chi^2$ test. Pancreatic insulin content, lipid peroxidation scores, and inflammatory scores were analyzed using one- and two-way ANOVA followed by the Tukey–Kramer test, respectively. Statistical analyses were made using the software package Statview 5.0J for Apple Macintosh (SAS Institute Inc., Cary, NC, USA). A value of $P<0.05$ was considered statistically significant.

## 3. Results

Fig. 1 shows the effect of edaravone on the multiple low-dose streptozotocin-induced increase in blood glucose levels in mice. Edaravone alone did not affect blood glucose levels [$F(7, 196)=1.247$, not significant for 1 mg/kg; $F(7, 189)=1.070$, not significant for 3 mg/kg] (Fig. 1A, left). Treatment with streptozotocin (40 mg/kg) for five consecutive days induced a progressive hyperglycemia [$F(7, 203)=17.257$, $P<0.0001$], and daily treatment with edaravone at 3 mg/kg, but not at 1 mg/kg, starting at day 1, significantly counteracted multiple low-dose

Table 1
Effect of edaravone on insulitis indices in multiple low-dose streptozotocin-treated mice

| | Edaravone | Infiltration (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | No infiltration | | Peri-insulitis | | Intra-insulitis | | Severe insulitis | |
| | | Day 10 | Day 22 | Day 10 | Day 22 | Day 10 | Day 22 | Day 10 | Day 22 |
| Control | Vehicle | 96.3 | 96.0 | 3.8 | 4.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 1 mg/kg | – | 97.5 | – | 2.5 | – | 0.0 | – | 0.0 |
| | 3 mg/kg | – | 96.4 | – | 3.6 | – | 0.0 | – | 0.0 |
| Streptozotocin-treated | Vehicle | 41.9 | 27.5 | 26.9 | 35.0 | 17.5 | 26.0 | 13.8 | 11.5 |
| | 1 mg/kg | – | 31.9 | – | 36.9 | – | 23.8 | – | 7.5 |
| | 3 mg/kg | 63.1 | 66.5 | 25.0 | 24.5 | 9.4 | 6.0 | 2.5 | 3.0 |
| | 3 mg/kg after streptozotocin | 55.6 | 57.5 | 29.4 | 25.0 | 11.3 | 13.1 | 3.8 | 4.4 |

Data are percentage of islets in each group ($n=7$–10 mice) among the total number of islets evaluated. Histological examination was performed as described in Materials and methods.

streptozotocin-induced hyperglycemia [$F$ (7, 147)=1.951, not significant for 1 mg/kg; $F$ (7, 203)=5.361, $P<0.0001$ for 3 mg/kg] (Fig. 1A, right). Furthermore, treatment with edaravone (3 mg/kg), even after the injection of streptozocin starting at day 6, also attenuated the severity of hyperglycemia relative to the controls [$F$ (7, 196)=4.064, $P=0.0003$] (Fig. 1A, right). Fig. 1B shows the cumulative incidence of diabetes in mice. On day 22 after the injection of streptozocin, 73.3% of multiple low-dose streptozotocin-treated mice were hyperglycemic. This effect was significantly attenuated by daily injection of edaravone at 3 mg/kg, but not at 1 mg/kg, with streptozotocin. Post treatment with edaravone (3 mg/kg) was still effective in protecting the treated mice from multiple low-dose streptozotocin-induced diabetes.

Fig. 2 shows the effect of edaravone on the multiple low-dose streptozotocin-induced decrease in pancreatic insulin in mice. One-way ANOVA revealed that the main effect of treatment was significant [$F$ (6, 36)=13.721, $P<0.0001$]. Multiple low-dose streptozotocin treatment markedly decreased pancreatic insulin and this effect was reduced by edaravone at 3 mg/kg [$P<0.05$], but not at 1 mg/kg. Treatment with edaravone (3 mg/kg) after the injection of streptozotocin also counteracted the multiple low-dose streptozotocin-induced reduction in pancreatic insulin [$P<0.05$]. Table 1 shows the effect of edaravone on multiple low-dose streptozotocin-induced mononuclear cell infiltration in the pancreatic islets of mice on day 10 and 22. To assess insulitis quantitatively, we used a previously described scoring system (Verdaguer et al., 1996; Takamura et al., 1999) (Fig. 3): a score of 0 to 3 represents no visible infiltration, peripheral or peri-islet infiltration, intra-islet infiltration, and massive lymphocytic infiltration with islet destruction, respectively. The inflammatory scores were higher in the pancreatic islets of multiple low-dose streptozotocin-treated mice than those of the control on days 10 and 22 [$F$ (2, 1114)=115.436, $P<0.0001$]. Insulitis indices in the edaravone (3 mg/kg)-treated group were significantly lower

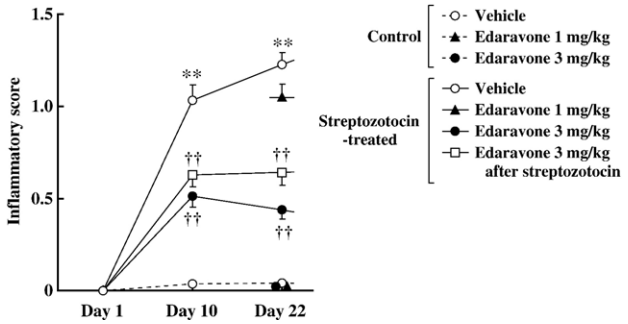


Fig. 3. Inflammatory score in pancreatic islets of multiple low-dose streptozotocin-treated mice. Inflammatory score was based on the histological evaluation of multiple low-dose streptozotocin-treated mice (Table 1). A score of 0 to 3 represents no visible infiltration, peripheral or peri-islet infiltration, intra-islet infiltration, and massive lymphocytic infiltration with islet destruction, respectively. For each animal the mean score (total score for all islets examined/total number of islets examined) was calculated. Values are expressed as the mean±S.E.M. of 7 to 10 mice. **$P<0.01$, compared with the vehicle treatment group of control mice; ††$P<0.01$, compared with the vehicle treatment group of the streptozotocin-treated mice.

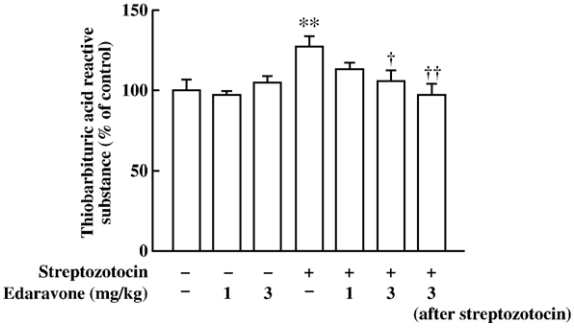


Fig. 4. Effect of edaravone on multiple low-dose streptozotocin-induced lipid peroxidation in pancreatic tissues of mice. Mice were treated daily with vehicle or streptozotocin at 40 mg/kg for 5 consecutive days (days 1–5). Vehicle, edaravone at 1 and 3 mg/kg were injected twice daily starting at day 1 (days 1–21). Edaravone at 3 mg/kg was also injected twice daily after streptozotocin treatment starting at day 6 (days 6–21). Thiobarbituric acid reactive substance levels in pancreatic tissues were measured on day 22. Values are expressed as the mean±S.E.M. of 7 to 12 mice. **$P<0.01$, compared with the vehicle treatment group of control mice; †$P<0.05$, ††$P<0.01$, compared with the vehicle treatment group of the streptozotocin-treated mice.

than those in the vehicle treatment group on day 10 [$F$ (3, 636)=43.476, $P<0.0001$] and 22 [$F$ (6, 1213)=96.245, $P<0.0001$].

Fig. 4 shows the effect of edaravone on multiple low-dose streptozotocin-induced lipid peroxidation in pancreatic tissues of mice on day 22. The level of thiobarbituric acid reactive substance is an index of lipid peroxidation. One-way ANOVA revealed that multiple low-dose streptozotocin treatment induced a significant increase in pancreatic levels of thiobarbituric acid reactive substance [$F$ (6, 56)=3.649, $P=0.0040$]. Edaravone (3 mg/kg), given simultaneously with streptozotocin [$P<0.05$] and after streptozotocin [$P<0.01$], inhibited thiobarbituric acid reactive substance production.

## 4. Discussion

The present study demonstrates that edaravone, a potent scavenger of hydroxyl and peroxyl radicals, attenuates the development of multiple low-dose streptozotocin-induced hyperglycemia and reduction in pancreatic insulin. The results support the previous observation that oxidative stress plays a key role in the pathogenesis of diabetes. Previous studies have shown that desferrioxamine, an iron chelator that inhibits the formation of hydroxyl radicals from hydrogen peroxide via the Fenton reaction, and dimethyl urea, a scavenger of hydroxyl radicals, protect against multiple low-dose streptozotocin-induced diabetes (Mendola et al., 1989; Sandler and Andersson, 1982). These reports suggest that hydroxyl radicals are involved in multiple low-dose streptozotocin-induced diabetes. However, it was not known whether these drugs, when given after streptozotocin injection, are effective in attenuating the multiple low-dose streptozotocin-induced diabetes. The present study clearly demonstrates a significant effect of edaravone even when given after streptozotocin injection. Therefore, edaravone may have therapeutic value for counteracting or delaying the progression of type 1 diabetes when it is applied in the early

stage of insulitis and diabetes. This is in addition to its clinical use for Parkinson's disease (Kawasaki et al., 2006, 2007a) and acute pancreatitis (Araki et al., 2003).

There are conflicting reports on the effects of nitric oxide synthase inhibitors on type 1 diabetes in animal models (Lukic et al., 1991; Kolb et al., 1991; Holstad and Sandler, 1993; Papaccio et al., 1995b). Papaccio et al. (2000) reported that multiple low-dose streptozotocin treatment did not stimulate nitric oxide production at the islet level, although superoxide dismutase activity was decreased. These data suggest that nitric oxide itself may have no major role in multiple low-dose streptozotocin-induced diabetes. However, Szabó et al. (2002) and Mabley et al. (2004) have demonstrated that peroxynitrite plays a role in the pathogenesis of islet cell dysfunction and the destruction associated with the multiple low-dose streptozotocin model of type 1 diabetes. We have recently shown that edaravone protects against nitric oxide-induced cytotoxicity in cultured astrocytes, although edaravone does not affect the release of nitric oxide or its metabolism (Kawasaki et al., 2007b). In addition, we have found in a preliminary experiment that edaravone protects against *S*-nitroso-*N*-acetyl-DL-penicillamine, a nitric oxide donor that induces cytotoxicity in the rat pancreatic β cell line INS-1. These findings suggest that nitric oxide plays a role in the pathogenesis of diabetes and that edaravone may attenuate multiple low-dose streptozotocin-induced diabetes by inhibiting a nitric oxide-mediated mechanism.

The present study shows that the inflammatory score reaches nearly the maximal level on day 10, whereas blood glucose level is higher on day 22 than on day 10. This suggests that insulitis is followed by the development of hyperglycemia in the multiple low-dose streptozotocin model, in agreement with previous observations (Karabatas et al., 2005; Li et al., 2000). Edaravone, given simultaneously with streptozotocin or after streptozotocin, attenuates the multiple low-dose streptozotocin-induced insulitis. Furthermore, we observed that edaravone completely inhibits the multiple low-dose streptozotocin-induced production of thiobarbituric acid reactive substance, an index of lipid peroxidation, in pancreatic tissues of mice 22 days after the injection of streptozotocin. Thus, it is likely that the protective effect of edaravone is mediated by inhibiting oxidative stress in islet β cells of multiple low-dose streptozotocin-treated mice. It has been reported that oxidative stress is involved not only in type 1 diabetes but also in type 2 diabetes (Ihara et al., 2000; Kajimoto and Kaneto, 2004). Thus, it is possible that edaravone may also be useful for the treatment of type 2 diabetes, although Hayashi et al. (2003) reported that edaravone did not affect plasma glucose levels in Otsuka Long-Evans Tokushima Fatty rats, a model of spontaneous development of type 2 diabetes mellitus. Further studies are required to test this possibility.

In conclusion, the present study demonstrates that treatment with the radical scavenger edaravone attenuates the severity of hyperglycemia and insulitis in the multiple low-dose streptozotocin model of type 1 diabetes. The protective effect was observed even after the injection of streptozotocin. The effect of edaravone might be due to an inhibition of oxidative stress in islet β cells of multiple low-dose streptozotocin-treated mice. It is possible that edaravone counteracts multiple low-dose streptozotocin-induced diabetes by protecting pancreatic islets from the activation of inflammatory reaction and immune destruction. These findings suggest that edaravone may be useful for counteracting or delaying the progression of type 1 diabetes.

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