Original Article

TIPS Trends in Pharmaceutical Sciences 2022: 8(2): 85-94.

Renoprotective Effects of Edaravone in a Model of Acute kidney Injury Induced by Rhabdomyolysis in Rats, the Involvement of Nitric oxide

Leila Moezi[1,2], Mohammad Reza Panjehshahin[1], Simin Torabinezhad[3], Eskandar Kamali-Sarvestani[4], Shirin Farjadian[5], Fatema Pirsalami[1], Azin Ebrahimpour[5], Somayeh Oftadehgan[1], Azar Purkhosrow[1], Maryam Mojahedtaghi[1], Elahe Sattarinezhad[1*]

[1]Department of Pharmacology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.
[2]Nanomedicine and Nanobiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[3]Nephrology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[4]Autoimmune Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[5]Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran.

Abstract

Edaravone is a free radical scavenger which is used as a drug for the treatment of cerebral infarction and amyotrophic lateral sclerosis. Edaravone is distributed widely in the body and its effects are not limited to the neural tissue. Many studies indicate that edaravone has some nitric oxide synthase (NOS) modulating properties. In this research we evaluated the effects of edaravone (1, 5 and 10 mg/kg) alone or in concombination with diphenyliodonium chloride (a specific endothelial NOS inhibitor) or aminoguanidine (a specific inducible NOS inhibitor) on oxidative stress, and renal tissue and function in a model of acute kidney injury induced by a single intramuscular injection of hypertonic glycerol solution. Effects of edaravone on gene expressions of eNOS and iNOS (by RT-PCR) were also investigated. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. At the end of this study, edaravone attenuated oxidative stress and improved renal tissue damage and dysfunction. Aminoguanidine enhanced the renoprotective effects of edaravone. Edaravone showed no remarkable effect on the expression of eNOS gene but it reduced the induction of iNOS gene significantly. The results of this study showed that edaravone could protect against rhabdomyolysis-induced acute kidney injury using its antioxidant activity and inhibiting effect on iNOS gene expression.

Keywords: Edaravone, Oxidative Stress, Rhabdomyolysis, Acute kidney injury, Nitric oxide

Please cite this article as: Leila Moezi, Mohammad Reza Panjehshahin, Simin Torabinezhad, Eskandar Kamali-Sarvestani, Shirin Farjadian, Fatema Pirsalami, et al. Renoprotective Effects of Edaravone in a Model of Acute kidney Injury Induced by Rhabdomyolysis in Rats, the Involvement of Nitric oxide. Trends in Pharmaceutical Sciences. 2022;8(2):85-94. doi: 10.30476/TIPS.2022.95089.1141

1. Introduction

Acute kidney injury (AKI), is a sudden impairment of renal function generally characterized by a rapid fall in glomerular filtration rate (GFR), fluid and electrolyte imbalance, and retention of nitrogenous waste products over a period of hours or days (1). The incidence of AKI has increased greatly over time (2). One of the important types of AKI is rhabdomyolysis-induced AKI. Rhabdomyolysis is a syndrome characterized by the lysis of skeletal muscles with massive release of myoglobin into the extracellular fluid and circulation leading to infiltration of myoglobin into

Corresponding Author: Elahe Sattarinezhad, Department of Pharmacology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
Email: elahesat@yahoo.com

Received: 12/12/2021; Accepted: 01/06/2022

renal tubules (3). Myoglobin has a main role in the pathophysiology of rhabdomyolysis-induced AKI and acts as a toxin causing renal dysfunction (4). Nitric oxide is an important mediator in maintaining vascular tone in renal vessels. Nitric oxide is synthesized from its precursor L-arginine by the action of nitric oxide synthase (NOS) enzyme which has three isoforms: neuronal NOS (nNOS), mainly expressed in brain; inducible NOS (iNOS) (5) that is expressed in macrophages after sufficient inflammatory stimuli; and endothelial NOS (eNOS) mainly expressed in endothelial cells (6). All of these isoforms are present in the kidney. Nitric oxide produced by eNOS has a main role in regulating the tone of renal vessels through vasodilation of glomeruli (7). During inflammation, iNOS induces the synthesis of high amounts of nitric oxide (8) that is cytotoxic and mediates the inflammatory damage of kidney tissue (6).

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent free radical scavenger drug which has been used successfully for the treatment of cerebral infarction and amyotrophic lateral sclerosis (ALS). Edaravone ameliorates oxidative stress, protects neurons, and prevents cerebral infarction following brain ischemia (9). Currently, it has received great attention for its potential application in protecting against other diseases associated with oxidative stress (10). Edaravone has been shown to inhibit iNOS gene expression in a model of inflammation in rat abdominal aorta (11). Edaravone pretreatment decreased the level of iNOS mRNA in a model of tourniquet-induced ischemia-reperfusion injury in rats. In a model of liver injury induced by endotoxins, edaravone inhibited the iNOS promoter activity and mRNA stabilization (12). Edaravone also has enhanced endothelial nitric oxide synthase gene expression in ischemic spinal cord injury (10, 13, 14). It has also been demonstrated that edaravone restores the reduction in eNOS gene expression induced by oxidized low-density lipoprotein in endothelial cells (14) and in the rabbit artery after irradiation (15). Our team also showed the inhibiting effects of edaravone on iNOS gene expression in a model of cyclosporine-induced chronic nephropathy, previously (16). To our knowledge, the renoprotective effects of edaravone in rhabdomyolysis have not been studied. Therefore, the main purpose of this research was to evaluate the protective effects of edaravone in a model of rhabdomyolysis-induced acute kidney injury in rats, and also to investigate the possible interaction of edaravone with gene expressions of endothelial and inducible nitric oxide synthase enzymes in kidney tissue. The results of this study may offer edaravone as an adjunctive drug for amelioration of acute kidney injury induced by rhabdomyolysis.

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats (200-250 g), obtained from Laboratory Animal Breeding Center, Shiraz University of Medical Science, Shiraz, Iran, were used in this study. The animals were housed in standard cages and kept in the controlled environment of a vivarium with a 12 h light/dark cycle, temperature 20-25 °C, and humidity 25-35%, and access to food and water ad libitum. All animal procedures were approved by the Institutional Ethical Committee for Care and Use of Animals in Shiraz University of Medical Sciences.

2.2. Materials

Glycerol, edaravone, diphenylidonium chloride (DPI) and aminoguanidine (AG) were purchased from Sigma Aldrich Company (USA). All materials were dissolved in 0.9% sodium chloride (normal saline). The solutions were freshly prepared and used on the same day of the experiment.

2.3. Experimental design

Animals were randomly distributed into nine groups of 7 to 10 animals and treated as follows.

Day 1: The animals were deprived of drinking water for 24 h.

Day 2: To induce AKI, animals were treated with an intramuscular injection of 8 ml/kg of hypertonic glycerol solution as equally divided doses into the hind limbs of rats (except vehicle group).

Days 3 to 6: Animals were treated as follow: Vehicle group: normal saline, Control group: normal saline, Edaravone groups: intraperitoneal (ip) dos-

es of edaravone (1, 5, and 10 mg/kg).
DPI group: DPI (4 mg/kg, ip).
AG group: AG (100 mg/kg, ip).
DPI + edaravone group: DPI (4 mg/kg, ip) + edaravone (10 mg/kg, ip).
AG + edaravone group: AG (100 mg/kg, ip) + edaravone (10 mg/kg, ip).
Day 7: The rats were anesthetized with ketamine (40 mg/kg, ip) and xylazine (10 mg/kg, ip). Blood samples were collected from abdominal aorta. After centrifuging, the plasma was isolated from the whole blood and kept at -80 °C until measuring blood urea nitrogen (BUN) and serum creatinine level. Then, the rats were sacrificed, the left kidneys were removed and transferred into RNase-free sample tubes and immediately placed in liquid nitrogen and transferred to a freezer to maintain at -80 °C for the measurement of renal antioxidant enzyme activities, malondialdehyde (MDA) content of the kidney, and gene expression analyses by real time PCR. The right kidneys were also removed and placed in 10% formalin solution for histopathological assessments.

2.4. Methods

2.4.1. Determination of blood urea nitrogen (BUN) and serum creatinine levels

Plasma concentrations of blood urea nitrogen (BUN) and serum creatinine were measured using standard kits (Pars Azemoon, Shiraz, Iran).

Determination of renal malondialdehyde (MDA) content, superoxide dismutase (SOD), and glutathione reductase (GRH) enzyme activities.

Some pieces of the left kidney was homogenized in cold normal saline and centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatant was used to assay renal malondialdehyde (MDA) content and antioxidant enzyme activities using standard kits (Biorexfars, Shiraz, Iran).

2.5. Measurement of eNOS and iNOS mRNA levels by RT-PCR

In the first step, the appropriate primers for eNOS and iNOS enzymes were designed. The sequences of the forward and reverse primers used for amplification of β-actin, eNOS & iNOS genes are showed in Table 1. Total RNA was extracted from kidney tissue using Tri-Pure extraction kit (Roche Applied Science, Germany). The purity of the extracted RNA was determined by OD ratio (the ratio of optical density measured at 260 and 280 nm) using a Nanodrop instrument. The OD ratio of 1.8 to 2 and the RNA concentration greater than100 ng/µl considered as desirable for cDNA synthesis. To determine the integrity of the extracted total RNA, gel electrophoresis was performed. Appropriate RNA was described to have two sharp bands in 18s and 28s without smearing. The cDNA was synthesized from 1 µg of total RNA using H minus first strand cDNA synthesis kit (Thermoscientific, USA), according to the manufacturer's instructions. All the reagents were gently mixed, centrifuged, and transferred into thermal cycler. The thermal program was as follows: 25 °C for 5 min followed by 42 °C for 60 min. The reaction was terminated by heating at 70 °C for 5 min. To determine the relative levels of eNOS and iNOS genes, SYBR Premix Ex Taq II kit (Takara, Japan) was used. The procedures were carried out according to the manufacturer′s instructions. β-Actin gene was used as an internal control (housekeeping gene). In each set of samples, one negative control was also included with no cDNA addition. An ABI 7500 real-time system (Applied Biosystems, USA) was used for thermal cycling and fluorescence detection. Relative changes in gene expressions of eNOS and iNOS enzymes were calculated using the comparative CT method and normalized with the mean Ct of the housekeeping gene, β-Actin. Table 2 shows the conditions of real-time PCR for

Table 1. The sequences of the forward and reverse primers used for amplification of β-Actin, eNOS & iNOS genes.

Gene	Forward primer	Reverse primer
β-actin	5'-GCA AAT GCT TCT AGG CGG AG-3'	5'-AAG GGG TGT AAA AAA ACG CAG C-3'
eNOS	5'-ATT GGC ATG AGG GAC CTG TG-3'	5'-CCG GGT GTC TAG ATC CAT GC-3'
iNOS	5'-AGC TAC GCC TTC AAC ACC AA-3'	5'-CCC AGG CCA AAT ACC GCA TA-3'

Table 2. The conditions of real time PCR for amplification of eNOS and iNOS genes.

Steps of PCR	Temperature (°C)	Time (min)
Denaturation	95	15
Annealing	60	30
Extension	60	30

amplification of eNOS and iNOS genes.

2.6. Histopathological methods

The right kidneys of the animals were immediately washed with cold normal saline and the following processes were performed.The kidney tissue was embedded in formaldehyde solution (10% v/v) for 24 h. The tissue specimens were placed in serial dilutions of ethanol including 70%, 80%, 96% (v/v), and three dishes of absolute alcohol. The maintenance time necessary for each concentration was 1 h. The tissue specimens were placed in xylol solution for 1 h. The tissue specimens were put in the melted paraffin, embedded and then transferred into refrigerator to become firm. Each kidney was cut into 2-micrometer thick sections by a Microtom instrument. The tissue sections were placed in ethanol (20% v/v) solution then put on the transparent slides and transferred into oven to be dehydrated and deparaffinized at 80 °C for 45 minutes.The prepared slides of the kidney tissue were stained with hematoxylin and eosin (H&E), Periodic acid-Schiff (PAS), Jones', and Masson's trichrome staining (17). The histopathological evaluation for all of the specimens was performed by an expert pathologist in a blind manner for finding edema, tubular casts, sloughing, and necrosis, and loss of brush borders as the hallmarks of rhabdomyolysis-induced AKI. At least, 10 fields for each kidney slide were evaluated and the severity of kidney tissue damage was scored.

2.7. Statistical analysis

The data were expressed as mean ±SEM and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey′s multiple comparison test. A value of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Effects of edaravone, diphenyliodonium chloride, and aminoguanidine treatments on glycerol-induced acute kidney injury

Figure 1. illustrates the effects of edaravone, diphenyliodonium chloride, and aminoguanidine on renal impairment induced by glycerol injection. Administration of glycerol increased BUN and serum creatinine level significantly compared to the vehicle group ($P<0.001$). Administration of

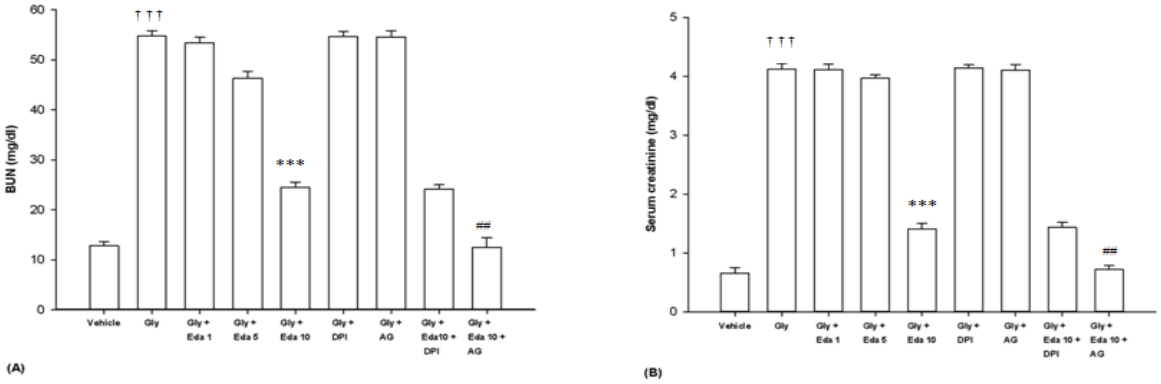

Figure 1. Effects of edaravone (1, 5, 10 mg/kg), diphenyliodonium chloride (4 mg/kg), and aminoguanidine (100 mg/kg) on BUN (A), and serum creatinine (B) levels in rats treated with vehicle or hypertonic glycerol solution. All values expressed as mean ± SEM. † † † $p<0.001$ as compared to vehicle group. *** $p<0.001$ as compared to glycerol group. ## $p<0.01$ as compared to glycerol + edaravone group. One-way ANOVA followed by Tukey's test. Gly: glycerol; Eda: edaravone; DPI: diphenyliodonium chloride; AG: aminoguanidine.

Table 3. Effect of edaravone (1, 5, 10 mg/kg) treatment on renal MDA content, and SOD, and GR enzyme activities in acute glycerol (8 ml/kg) treated rats.

Groups	MDA (nM/g of tissue)	SOD (units/mg protein)	GR (Units/L)
Vehicle	62.20±1.20	1.55±1.20	1397.50±2.80
Glycerol (8 ml/kg)	119.55±1.64†††	0.58±1.32†††	310.88±2.84†††
Glycerol + Eda (1 mg/kg)	119.75±1.85	0.62±1.10	318.12±2.08
Glycerol + Eda (5 mg/kg)	104.00±1.28**	0.69±1.42	367.12±2.56**
Glycerol + Eda (10 mg/kg)	70.00±1.63***	1.43±1.50***	994.10±2.83***

All values expressed as mean±SEM. $^{\dagger\dagger\dagger}P<0.001$ as compared to vehicle group. $^{**}P<0.01$ as compared to glycerol group. $^{***}P<0.001$ as compared to glycerol group (one-way ANOVA followed by Tukey's test). Eda, edaravone; MDA, malondialdehyde; SOD, superoxide dismutase; GR, glutathione reductase.

edaravone (10 mg/kg) significantly attenuated the rise in BUN and serum creatinine level produced by glycerol ($P<0.001$). Administration of diphenyliodonium chloride (4 mg/kg) or aminoguanidine (100 mg/kg) to animals had no significant effect on the elevated BUN and serum creatinine levels induced by glycerol. Addition of aminoguanidine (100 mg/kg) to edaravone (10 mg/kg) reduced BUN and serum creatinine level ($P<0.01$). Addition of diphenyliodonium chloride (4 mg/kg) to edaravone produced no significant change in the effect of edaravone on the level of BUN and serum creatinine (Figure 1A and B).

3.2. Effect of edaravone on glycerol-induced lipid peroxidation and alteration in renal antioxidant enzymes

Administration of glycerol induced a significant increase in MDA level as compared to the vehicle group ($P<0.001$). Treatment with edaravone (10 mg/kg) significantly lowered the elevated MDA level induced by glycerol ($P<0.001$). Treatment with glycerol led to a significant decrease in superoxide dismutase and glutathione reductase enzyme activities in kidney homogenates ($P<0.001$), whereas, administration of edaravone (10 mg/kg) preserved the activities of these enzymes ($P<0.001$) (Table 3).

3.3. Effect of edaravone, diphenyliodonium chloride, and aminoguanidine treatments on glycerol-induced changes in renal histology

The histopathological changes and total histopathological scores were summarized in Tables 4 & 5 respectively. The vehicle group did not show any significant histological changes. By contrast, the kidneys of rats treated with glycerol showed marked histological changes in the cortex and outer medulla. The renal sections showed severe interstitial edema, hyaline and hemorrhagic

Table 4. Effect of edaravone (1, 5, 10 mg/kg), DPI (4 mg/kg), and AG (100 mg/kg) treatments on histological changes of kidney tissue in rats treated with vehicle or a single intramuscular dose of hypertonic glycerol solution (8 ml/kg).

Groups	**Edema**	**Loss of BB**	**Necrosis**	**Sloughing**	**Cast**
Vehicle	0.00	0.00	0.00	0.00	0.00
Glycerol	0.78±0.15	1.22±0.15	1.11±0.26	1.33±0.17	2.33±0.17
Glycerol + Eda (1 mg/kg)	0.50±0.19	1.25±0.16	0.87±0.29	1.25±0.16	2.00±0.27
Glycerol + Eda (5 mg/kg)	0.00	0.75±0.16	0.00	0.87±0.13	1.25±0.25
Glycerol + Eda (10 mg/kg)	0.00	0.30±0.15	0.00	0.30±0.15	0.70±0.21
Glycerol + DPI	0.75±0.16	1.25±0.16	0.75±0.16	1.25±0.16	2.50±0.19
Glycerol + AG	0.71±0.18	1.28±0.18	0.71±0.18	1.28±0.18	2.43±0.20
Glycerol + Eda (10 mg/kg) + DPI	0.00	0.35±0.16	0.00	0.35±0.16	0.80±0.17
Glycerol + Eda (10 mg/kg) + AG	0.00	0.31±0.18	0.00	0.33±0.16	0.75±0.29

All values expressed as mean±SEM. BB, brush borders; Eda, edaravone; DPI, diphenyliodonium chloride; AG, aminoguanidine.

Table 5. Effects of edaravone (1, 5, 10 mg/kg), DPI (4 mg/kg), and AG (100 mg/kg) treatment on total histopathological scores of kidney tissue injury in rats treated with vehicle or a single intramuscular dose of hypertonic glycerol solution (8 ml/kg).

Groups	Total histopathological score
Vehicle	0.00
Glycerol	6.78±1.20***
Glycerol + Eda (1 mg/kg)	5.88±2.42
Glycerol + Eda (5 mg/kg)	2.88±1.25††
Glycerol + Eda (10 mg/kg)	1.30±1.34†††
Glycerol + DPI	6.50±1.20
Glycerol + AG	6.43±0.79
Glycerol + Eda (10 mg/kg) + DPI	1.30±1.49
Glycerol + Eda (10 mg/kg) + AG	1.30±1.46

All values expressed as mean ± S.E.M. †††P<0.001 as compared to vehicle group; **P<0.01 as compared to glycerol group; ***P<0.001 as compared to glycerol group. One-way ANOVA followed by Tukey's test. Eda, edaravone; DPI, diphenyliodonium chloride; AG, aminoguanidine.

casts, sloughing , tubular necrosis, and loss of tubular brush borders. Treatment with edaravone (1, 5 mg/kg) did not improve the renal histological changes induced by glycerol; however, edaravone (10 mg/kg) showed significant histological protection (Figure 2). On the other hand, administration of diphenyliodonium chloride (4 mg/kg) or aminoguanidine (100 mg/kg) did not show any changes in the histological injuries produced by glycerol injection. Moreover, concomitant administration of diphenyliodonium chloride (4 mg/kg) or aminoguanidine (100 mg/kg) with edaravone (10 mg/kg) did not alter the renoprotective effects of edaravone (10 mg/kg), significantly.

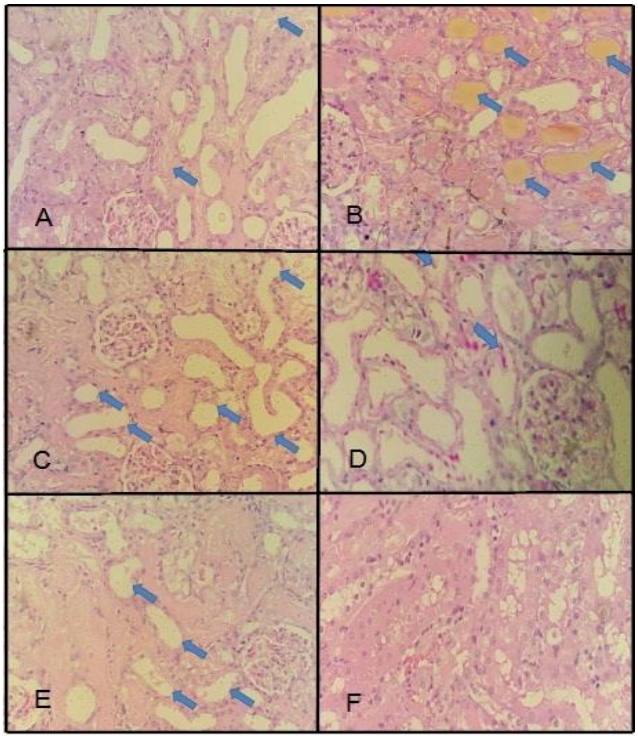

Figure 2. Cross sections of kidney tissue of rats treated with glycerol shows: severe interstitial edema (A), many tubular casts (B), severe necrosis (C), marked sloughing of renal tubular cells (D), loss of brush borders (E). Near normal morphology in kidney tissue of rats treated with glycerol along with edaravone (10 mg/kg) (F). A, B, C, E, F: Hematoxylin & eosin and D: Periodic Acid-shiff staining.

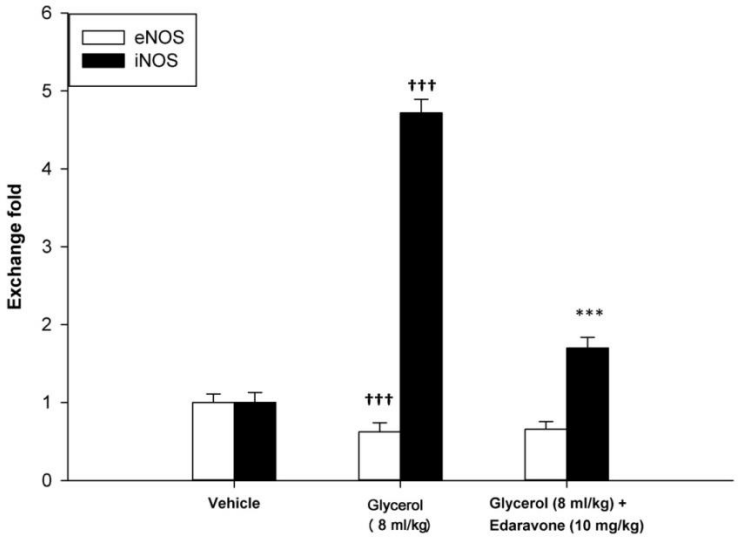

Figure 3. Effects of edaravone (10 mg/kg) on renal eNOS and iNOS mRNA levels in acute glycerol-treated rats. Data expressed as mean±SEM. One-way ANOVA followed by Tukey's test. $^{\dagger\dagger\dagger}P<0.001$ as compared to vehicle group. $^{***}P<0.001$ as compared to glycerol-treated group.

3.4. Effect of edaravone administration on eNOS and iNOS mRNA levels

The results of RT-PCR analysis is depicted in Figure 3. Glycerol administration elevated the mRNA levels of both eNOS and iNOS enzymes significantly ($P<0.001$). Treatment with edaravone (10 mg/kg) significantly reduced the mRNA level of iNOS ($P<0.001$) but it had no significant effect on the level of eNOS mRNA.

4. Discussion

In this study, we showed that injection of a single dose of intramuscular hypertonic glycerol in normal saline solution into hind limbs of rats resulted in severe rhabdomyolysis, oxidative stress, and dramatic high level of BUN and serum creatinine levels. Renal dysfunction was accompanied with a marked increase of lipid peroxidation and a significant reduction of activities of antioxidant enzymes glutathione reductase and superoxide dismutase. The histological pattern of glycerol-treated rat kidneys showed characteristic renal tubular cast deposits, tubular necrosis, interstitial edema, sloughing of renal tubular cells, and loss of tubular brush borders. Dehydration of the animals for 24 h, allowed the acute renal injury to be fully expressed (18, 19). Although the precise mechanisms of myoglobinuric AKI are unclear, some experimental evidence suggests that at least three main pathophysiological mechanisms may be involved: tubular obstruction due to the cast formation, intrarenal vasoconstriction, and direct ischemic tubule injury (20, 21). Many studies indicate to the main role of oxidative stress in the pathogenesis of this type of kidney injury. This destructive effect has been related to the heme moiety in myoglobine molecule. It has been proposed that the catalytic effect of ferric ion in catalyzing the production of massive amounts of hydroxyl radical released in renal tubules during rhabdomyolysis is followed by marked hemodynamic abnormalities and severe renal tubular damage (22, 23). There is also evidence that scavenging the nitric oxide by reactive oxygen species causes a huge production of peroxynitrite that causes further tissue injury (24). More recent studies indicate that myoglobin has peroxidase-like enzyme activity in addition to direct cytotoxic effects that tends to uncontrolled formation of reactive oxygen species and cellular and tissue damage (25). In this research, treatment of rats with edaravone (10 mg/kg) protected against acute kidney injury. Edaravone strongly lowered the elevated levels of BUN and serum creatinine. It also prevented the rise in the renal lipid peroxides produced by rhabdomy-

olysis. Furthermore, treatment with edaravone restored the reduced levels of glutathione reductase and superoxide dismutase in kidney tissue. These findings were further confirmed by the histological evaluations. The animals treated with edaravone showed a significant improvement in renal histology as compared to glycerol-treated rats. Based on our results and regarding the crucial role of oxidative stress in the pathogenesis of rhabdomyolysis-induced acute kidney injury, it seems that the antioxidant property of edaravone may have a key role in producing its potent renoprotective effects.

It has also been evidenced that edaravone in addition to having potent free radical scavenging effect, modulates nitric oxide production (26). Nitric oxide is one of the most important mediators in renal vascular homeostasis (27). Nitric oxide is synthesized from its precursor L-arginine by three NOS isoforms which are all present in the kidney. Two isoforms are constitutively expressed (eNOS and nNOS), and one isoform is inducible (28). During inflammation, iNOS catalyses the production of large amounts of nitric oxide that easily reacts with other reactive oxygen species mainly superoxide to form a highly reactive free radical peroxynitrite which mediates cytotoxic effects (29). Therefore, nitric oxide produced by iNOS is involved in the inflammatory damage of cells and tissues (6). On the other hand, it has been shown that administration of edaravone in various animal models of ROS-mediated tissue injury causes the inhibition of iNOS gene expression (30). The results of these studies are in line with ours. Addition of aminoguanidine (a specific iNOS inhibitor) to edaravone enhanced the protective effect of edaravone on renal function suggesting that edaravone may have inhibiting effect on iNOS gene expression. This idea was supported by real-time PCR analysis. In this study we showed that rhabdomyolysis elevated the mRNA level of iNOS in the kidney tissue and edaravone lowered it significantly. Therefore, it seems that edaravone prevents iNOS expression and inhibits the subsequent vicious cycle of more inflammation. Furthermore, it was observed in our study that the renal protection afforded by edaravone was not altered with addition of diphenyliodonium chloride (a specific eNOS inhibitor) indicating that edaravone may have no significant effect on eNOS gene expression. This idea was confirmed by real-time PCR analysis. In the present study, acute glycerol injection increased eNOS gene expression significantly compared to the vehicle whereas edaravone could not make any significant change in the elevated eNOS gene expression induced by glycerol. Moreover, it has been shown that edaravone has improved endothelial vasodilation through restoration of the decreased eNOS gene expression in some animal models of ROS-mediated tissue injury (31). It has been suggested that free radical scavenging by edaravone prevents the decrease of eNOS activity rather than up regulation of eNOS (11), but this point needs further examination.

The same results for edaravone have been reported in several studies using other animal models of kidney injury caused by ROS (32, 33). To our knowledge, the renoprotective effect of edaravone has not been examined in rhabdomyolysis-induced acute kidney injury and our study is the first in this topic. However, this study has one limitation. This is a study which examined the renoprotective effects of edaravone and its effects on oxidative stress and mRNA levels of eNOS and iNOS in the kidney. This study lacks the measurement of protein levels of these enzymes to confirm the role of nitric oxide modulating effect of edaravone in producing renoprotective properties of this drug.

5. Conclusion

The results of the present study indicate that the novel free radical scavenger edaravone may act as a renoprotective agent against glycerol-induced AKI by its antioxidant property and partially through inhibiting iNOS gene expression.

Acknowledgment

The present article was extracted from the thesis written by Elahe Sattarinezhad and was financially supported by Shiraz University of Medical Sciences grants No 92-03-04- 6547.

Conflict of Interest

None declared

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