

➤ **Original Article** ◀

Pretreatment with the Free Radical Scavenger Edaravone Mitigates Kidney Glycogen Depletion and Neutrophil Infiltration after Leg Ischemia in a Rat Model: A Pilot Study

Mitsuhiro Yamamura, MD, FICA, Yuji Miyamoto, MD, Masataka Mitsuno, MD, Hiroe Tanaka, MD, and Masaaki Ryomoto, MD

Objective: We have previously shown that pretreatment with the free radical scavenger edaravone (Radicut[®], Mitsubishi Tanabe Pharma Co., Japan) mitigated skeletal muscle damage due to ischemia reperfusion. In this study, we sought to validate its use in an experimental model of myoneuropathic-metabolic syndrome (MNMS).

Methods: Either edaravone (3.0 mg/kg; edaravone group; n=4) or saline (saline group; n=6) was intraperitoneally injected into male Lewis rats (508±31 g). Normal kidneys were harvested as control (n=3). MNMS was induced by bilaterally clamping the common femoral arteries for 5 h and declamping 5 h later. Kidney damage was evaluated by quantifying Periodic Acid Schiff (PAS)-positive area (glycogen storage) and esterase-positive cells (neutrophil infiltration).

Results: The PAS-positive area in the saline group was significantly lower than that in the normal group (36.9±2.6 vs. 66.9±1.2%, P<0.01); the PAS-positive area in the edaravone group remained comparable to that in the normal group (52.9±0.9%, P<0.01). Esterase-positive cells in the saline group were significantly higher than in normal kidneys (62.4±5.6 vs. 17.5±2.4 cells/mm², P<0.01), while they were significantly reduced in the edaravone group (32.8±5.7 cells/mm², P<0.01).

Conclusion: Edaravone pretreatment mitigates MNMS-induced kidney damage by reducing both glycogen depletion and neutrophil infiltration.

Keywords: free radical, edaravone (Radicut[®]), myoneuropathic-metabolic syndrome (MNMS), reperfusion injury

Department of Cardiovascular Surgery, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

Received: February 28, 2017; Accepted: September 7, 2017
Corresponding author: Mitsuhiro Yamamura, MD, FICA. Department of Cardiovascular Surgery, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan
Tel: +81-798-45-6851, Fax: +81-798-45-6853
E-mail: yamachan@hcm-mail.hyo-med.ac.jp

Introduction

In 1960, Haimovici was the first to report two cases of arterial embolism with acute massive ischemic myopathy and myoglobinuria; this syndrome is now termed the myoneuropathic-metabolic syndrome (MNMS).¹⁾ Since this case report, it has become clear that free radicals cause MNMS and that it affects the muscles and kidneys.²⁾ However, currently, there are no commercially available free radical scavengers that have been proven to be clinically effective for preventing MNMS.

Edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one; C₁₀H₁₀N₂; molecular weight = 174.2; Radicut[®], Mitsubishi Tanabe Pharma Co., Tokyo, Japan) is the first commercially available free radical scavenger.^{3,4)} In Japan, edaravone has been shown to be effective in treating patients with acute cerebral infarction since 2001.⁵⁾ Initially, we hypothesized that free radical scavengers can also mitigate muscle reperfusion injury following leg ischemia⁶⁾ and reported that edaravone can diminish muscle damage following leg ischemia in a rat model.⁶⁻⁹⁾

Therefore, we sought to validate an experimental model of MNMS and used this model to evaluate whether edaravone prevented MNMS-induced kidney damage following leg ischemia by quantifying changes in glycogen storage and activated neutrophil infiltration in the rat kidney.

Materials and Methods

MNMS-induced kidney damage model

MNMS-induced kidney damage in rats was established by microscopic analyses (OME-J&N J73507R, Olympus, Tokyo, Japan), as previously reported.⁶⁻⁹⁾ Lewis male rats (508±31 g, n=10) were intraperitoneally injected with either 3.0 mg/kg of edaravone (edaravone group; n=4) or same dose of saline (saline group; n=6) prior to inducing leg ischemia and MNMS was established as follows. Normal kidneys were harvested as control (n=3). General



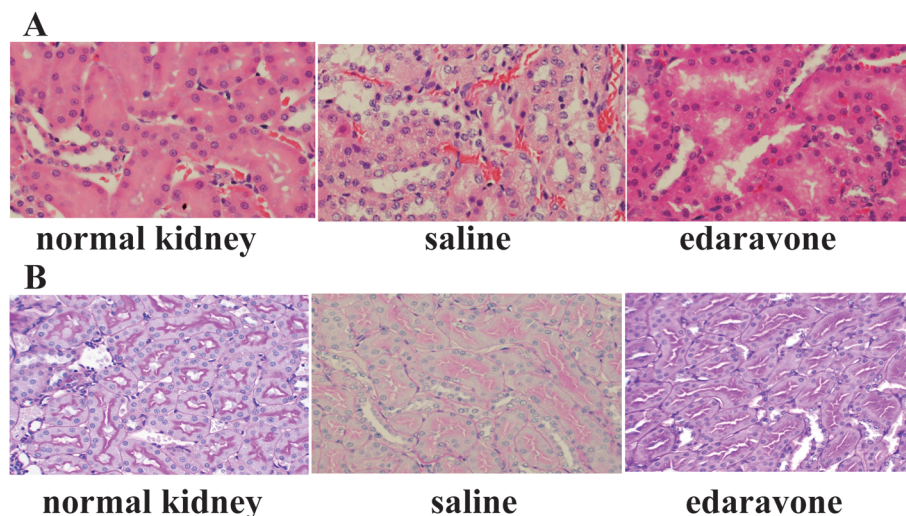


Fig. 1 Tubular cell sections (original magnification 400 \times). **A:** H&E stain. There is a very little loss of tubular cells and there are fewer myoglobin casts in the edaravone-treated group. **B:** PAS stain shows very little loss of glycogen storage in the edaravone-treated group.

anesthesia in both groups of animals was induced with isoflurane (Forane[®] inhalant liquid, Abbott Co., Tokyo, Japan). Both the deep femoral arteries and epigastric arteries were ligated to completely block their collateral supply. A model of MNMS-induced kidney damage was established by bilaterally clamping the common femoral arteries for 5 h.

After a further 5 h, the animals were sacrificed by an intraperitoneal overdose of sodium pentobarbital (>100 mg/kg, Nembutal[®], Abbot Co., IL, USA). Kidneys from both experimental groups and the normal group were harvested for histological analysis. Serum creatinine levels were also measured in all animals (SRL Co., Tokyo, Japan).

All animal procedures complied with the Ethics Criteria of the Animal Research Committee of the Hyogo College of Medicine (Nos. B-11-202 & 14-004).

Histological study of kidney damage

Using a cryostat microtome (Model CM3050S, Leica, Wetzlar, Germany), 5- μ m sections of kidneys from the experimental animals were mounted onto microslides (Silanized Slides, Dako Japan, Kyoto, Japan) and stained with Hematoxylin–Eosin (H&E) and Periodic Acid Schiff (PAS) to assess glycogen storage and with naphthol AS-D chloroacetate esterase to detect activated neutrophils (Mitsubishi Chemical Medience Corporation, Kyodo Byori, Inc., Kobe, Japan). Kidney sections from normal animals were also similarly stained and used as positive controls for PAS staining and negative controls for naphthol AS-D chloroacetate esterase staining.

Glycogen storage in tubular cells

Kidney damage was evaluated by quantifying glycogen storage in tubular cells, which was expressed as percentage PAS-positive area in tubular cells (%). The PAS-positive areas were measured thrice in different fields using computerized densitometry (National Institutes of Health Image J Program, Ver. 1.37; <http://rsb.info.nih.gov/ij/Java>). From these densitometry images, approximately 125–250 dots were used to define the PAS-positive area, and this area was used to calculate percentage PAS-positive area relative to total area using the following formula:

$$\begin{aligned} \text{Percentage PAS-positive area} \\ = (\text{PAS-positive area} / \text{total area}) \times 100 \end{aligned}$$

Neutrophil infiltration around the glomeruli

Kidney damage was also evaluated by measuring the density of naphthol AS-D chloroacetate esterase-positive cells (cells/mm²) as an indicator of neutrophil infiltration around the glomeruli. The numbers of naphthol AS-D chloroacetate esterase-positive cells were manually counted in three different fields of view at an original magnification of 400 \times .

Statistical analysis

Data are presented as mean \pm standard error of mean (SEM) and were analyzed using unpaired t tests (Stat View J-4.5[®] for Macintosh, Abacus Concept, Berkeley, CA, USA). $P < 0.05$ was considered statistically significant.

Results

There were no operative deaths in the saline and edaravone groups.

Serum creatinine levels

No significant difference in serum creatinine levels was observed between the saline group and the edaravone group (0.31 ± 0.07 vs. 0.35 ± 0.03 mg/dl, $P = 0.15$).

Histological study of tubular cells

Representative tubular cell sections stained with H&E are shown in Fig. 1A. There was a severe loss of tubular cells, and some red myoglobin casts in the saline group. Conversely, in the edaravone group, there was a minimal loss of tubular cells and fewer myoglobin casts were observed.

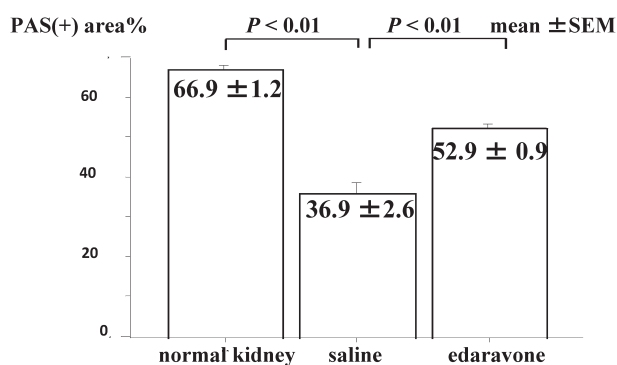


Fig. 2 Comparison of percentage PAS-positive area. The PAS-positive area in the edaravone group remained comparable to that of the normal group ($P < 0.01$).

Representative tubular cell sections stained with PAS are shown in Fig. 1B; purple PAS staining indicates glycogen storage. There was a severe loss of glycogen storage in the saline group, whereas there was very little loss in the edaravone group.

A statistical comparison of PAS-positive area (%) among the groups is shown in Fig. 2. The PAS-positive area in the saline group was significantly lower than that in the normal group (36.9 ± 2.6 vs. 66.9 ± 1.2 %, $P < 0.01$), whereas the PAS-positive area in the kidneys of the edaravone group was comparable to that in the kidneys of the normal group (52.9 ± 0.9 %, $P < 0.01$). Thus, edaravone pretreatment not only reduced the MNMS-induced loss of tubular cells but also glycogen depletion.

Histological study of glomeruli

Representative glomerular sections stained with H&E are shown in Fig. 3A. There was a severe glomerular infiltration with neutrophils in the saline group. However, in the edaravone group, the glomerular cells appeared to be similar to those in the normal group.

Representative glomerular sections stained with naphthol AS-D chloroacetate esterase stain are shown in Fig. 3B. The saline group showed significantly greater activated neutrophil infiltration (red arrows, Fig. 3B) than the normal group. However, the neutrophil infiltration in the edaravone group appeared to be similar to that seen in the normal group.

The density of naphthol AS-D chloroacetate esterase-positive cells (cells/mm²) was compared among the three groups and is shown in Fig. 4. The density of esterase-pos-

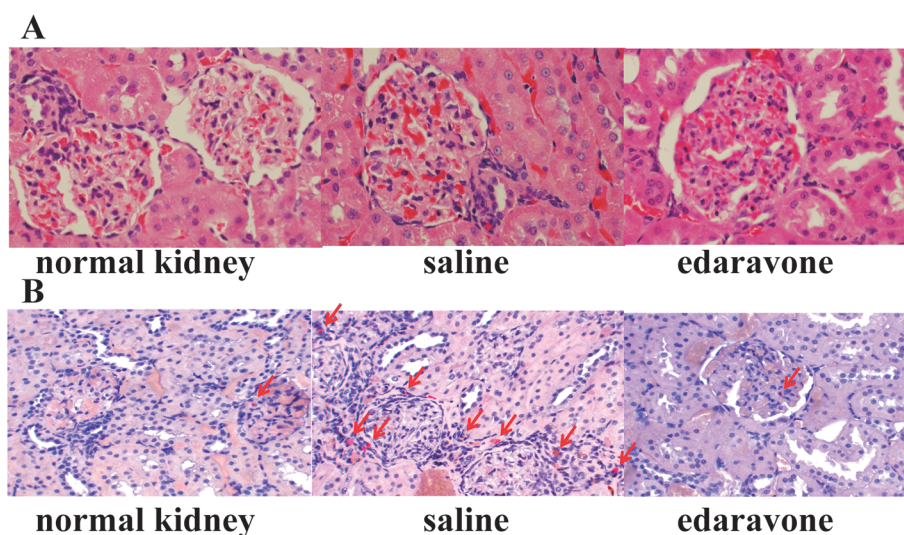


Fig. 3 Glomerular sections (original magnification 400 \times). **A:** H&E stain. Glomerular cells in the edaravone group appear to be similar to those in the normal group. **B:** AS-D chloroacetate esterase stain. Activated neutrophil infiltration in the edaravone group appears similar to that in the normal group.

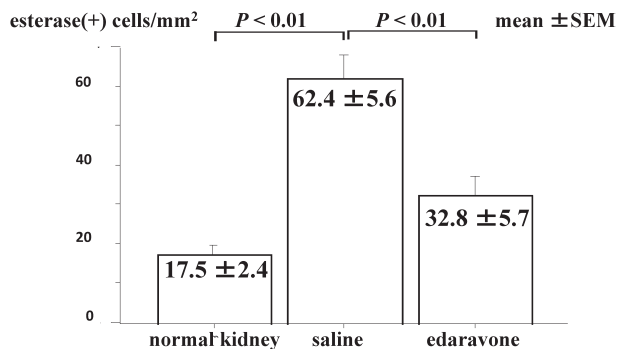


Fig. 4 Comparison of AS-D chloroacetate esterase-positive cell density. The density of naphthol AS-D chloroacetate esterase-positive cells was significantly lower in the edaravone group than in the normal group ($P < 0.01$).

itive cells was significantly higher in the kidney sections from the saline group than those from the normal group (62.4 ± 5.6 vs. 17.5 ± 2.4 cells/mm², $P < 0.01$). Interestingly, the density of esterase-positive cells was significantly lower in the edaravone group than in the normal group (32.8 ± 5.7 cells/mm², $P < 0.01$). Thus, edaravone reduced MNMS-induced infiltration of activated neutrophils around the glomeruli.

Discussion

What is edaravone?

Previous experimental studies have reported the successful use of free radical scavengers. However, there are currently no commercially available free radical scavengers for the prevention of MNMS. Edaravone is the first commercially available free radical scavenger and has been available in Japan since 2001.^{3,4)} Previous experimental studies have shown that edaravone can reduce postoperative spinal cord infarction in rabbits,¹⁰⁾ and cerebral damage during aortic arch surgery in dogs.¹¹⁾ Edaravone has also been used in experimental studies on cardiac surgery.^{12,13)} Moreover, some experimental studies in vascular surgery have shown that edaravone suppressed abdominal aortic aneurysm (AAA) formation in elastase and calcium chloride-induced rat models¹⁴⁾ and angiotensin II-induced hypertension rat models.¹⁵⁾

Recently, edaravone has been approved for the treatment of amyotrophic lateral sclerosis in Japan as it may help suppress superoxide-induced neuronal damage. Double-blind, phase 3 studies of edaravone (MCI-186) have been completed in the United States (NIH Clinical Trials 00415519, 00424463, 01492686), and FDA approval is expected very soon.¹⁶⁾

MNMS-induced kidney damage

It is well known that Haimovici was the first to report

two cases of MNMS with acute massive ischemic myopathy and myoglobinuria.¹⁾ According to his theory, as described in his textbook, myoglobin casts cause a severe loss in tubular concentration and function, leading to myoglobinuria.^{2,17)} In accordance with this, it is accepted that myoglobinuria is caused by myoglobin released from damaged muscles and that the released myoglobin has a direct and toxic effect on tubular cells.²⁾ Thus, we confirmed MNMS-induced kidney damage by quantifying the loss of glycogen storage in tubular cells.

Next, we hypothesized that activated neutrophil infiltration may also cause kidney damage around the glomeruli and lead to severe kidney dysfunction and anuria. In support of this hypothesis, we show that MNMS induction leads to activated neutrophil infiltration around the glomeruli. Such trapping of the activated neutrophils around the glomeruli may also damage glomerular function.

Based on the abovementioned and the observed beneficial effects of edaravone against kidney damage, we also hypothesized that the protective mechanisms would involve two putative pathways, namely, I) reduction in myoglobin and II) reduction in activated neutrophils.

We have previously demonstrated that edaravone can suppress muscle damage following leg ischemia.⁶⁻⁹⁾ As it is accepted that myoglobin is released from damaged muscles, we think that edaravone can help reduce myoglobin release from the damaged muscles in the MNMS-induced kidney damage model.

Another probable beneficial mechanism is the free radical scavenging effect of edaravone. We have recently confirmed that edaravone can reduce activated neutrophil infiltration in MNMS-induced lung damage, as quantified by staining with naphthol AS-D chloroacetate esterase, and that this effect is due to the free radical scavenging activity of edaravone.¹⁸⁾ Similarly, we propose that the observed reduction in activated neutrophil infiltration may be due to the free radical scavenging effect of edaravone. However, it is important to note that this hypothesis is only speculative, as we could not prove a direct reduction in free radical levels due to their instability during measurement.

Study limitations

This study has certain limitations.

Is the saline group equivalent to the control group?

According to the Japanese Pharmaceutical Reference Guidelines for edaravone (Radicut[®]), Radicut[®] contains not only edaravone but also other chemical products (sodium chloride, sodium bisulfite, sodium hydroxide, and other excipients) as solvents. We used a saline-administered group because Radicut[®] is now commercially available; it would have been better to use the abovementioned

solvent(s) as a control group if this was a preclinical study of edaravone.

When would edaravone be administered?

This is an off-label application of edaravone in an experimental model of NMNS, and the Japanese Pharmaceutical Reference Guidelines for edaravone (Radicut®) do not allow its use for severe kidney dysfunction. Experimentally, we administered edaravone before inducing limb ischemia, and we recognize that this might not be a feasible clinical situation. Further, our protocol considered that edaravone administered after the onset of limb ischemia might further deteriorate kidney function to critical levels in this animal model of NMNS-induced kidney dysfunction. However, we found no significant differences in serum creatinine levels when edaravone was administered before inducing ischemia. Nonetheless, our results warrant further exploration of the use of edaravone as a therapeutic option for NMNS in scenarios that closely resemble real clinical situations. Thus, we expect to be able to study the effects of administering edaravone during limb ischemia, as in our previous study,¹⁸⁾ and further studies are needed to determine the effects of edaravone before clinical use.

Conclusion

In conclusion, we report that pretreatment with the free radical scavenger edaravone can mitigate kidney glycogen depletion and MNMS-induced neutrophil infiltration. We hope that suppression of MNMS with edaravone can help revise surgical indications for occlusive arterial diseases in the near future.

Acknowledgments

We wish to thank Chie Sakashita, Ritsuko Fujimoto, Nobuyuki Adachi (Joint-Use Research Facilities, Hyogo College of Medicine) and Tomokazu Kawakami (The Institute of Experimental Sciences, Hyogo College of Medicine) for their technical support. We also thank Mitsubishi Tanabe Pharma Co., Japan for kindly donating edaravone (Radicut®).

Disclosure Statements

MY received a research grant from a Grant-in Aid for Researchers, Hyogo College of Medicine 2012–2013 & 2016–2017.

Additional Note

This study was partially presented at The Arteriosclerosis,

Thrombosis, and Vascular Biology 2014 & 2015 (Toronto & San Francisco) and Vascular Annual Meeting of the Society for Vascular Surgery 2014 & 2015 (Boston & Chicago).

Author Contributions

Conception and design: MY
 Analysis and interpretation: MY
 Data collection: MY
 Writing the article: MY, YM
 Critical revision of the article: all authors
 Statistical analysis: MY
 Obtaining funding: MY
 Final approval of the article: MY, YM
 Overall responsibility: MY

References

- 1) Haimovici H. Arterial embolism with acute massive ischemic myopathy and myoglobinuria: evaluation of a hitherto unreported syndrome with report of two cases. *Surgery* 1960; **47**: 739-47.
- 2) Haimovici H. Metabolic complications of acute arterial occlusions and skeletal muscle ischemia: myoneuropathic-metabolic syndrome. In: Haimovici H ed. *Haimovici's Vascular Surgery*, 4th edition. Cambridge, Massachusetts: Blackwell Science Inc., 1996: 509-30.
- 3) Yamamoto Y, Kuwahara T, Watanabe K, et al. Antioxidant activity of 3-methyl-1-phenyl-2-pyrazoline-5-one. *Redox Rep* 1996; **2**: 333-8.
- 4) Watanabe K, Watanabe K, Hayase T. Radical scavenging mechanism of MCI-186. *Jpn Pharmacol Ther* 1997; **25 Suppl**: S1699-707.
- 5) Houkin K, Nakayama N, Kamada K, et al. Neuroprotective effect of the radical scavenger MCI-186 in patients with cerebral infarction: clinical evaluation using magnetic resonance imaging and spectroscopy. *J Stroke Cerebrovasc Dis* 1998; **7**: 315-22.
- 6) Yamamura M, Miyamoto Y, Mitsuno M, et al. Suppression of rat lower extremity postoperative reperfusion injury with edaravone. *Int J Angiol* 2006; **15**: 34-6.
- 7) Yamamura M, Miyamoto Y, Mitsuno M, et al. Edaravone suppresses postoperative reperfusion injury in the rat lower extremity: an immunohistological study. *Int J Angiol* 2007; **16**: 17-9.
- 8) Yamamura M, Miyamoto Y, Mitsuno M, et al. Edaravone reduces mitochondrial damage due to reperfusion injury following leg ischemia in rats. *Int J Angiol* 2010; **19**: e129-31.
- 9) Yamamura M, Miyamoto Y, Mitsuno M, et al. Edaravone suppresses reperfusion injury following leg ischemia in rats: a transmission electron microscopic study. *Int J Angiol* 2013; **22**: 267-70.
- 10) Chiba K, Makuuchi H, Murakami H, et al. Effects of edaravone on prevention of paraplegia caused by ischemic spinal cord. *Jpn J Cardiovasc Surg* 2008; **37**: 82-90. (Abst in English)

- 11) Kitanaka Y, Makuuchi H, Murakami H, et al. Effects of edaravone on cerebral protection during aortic arch surgery. *Jpn J Cardiovasc Surg* 2011; **40**: 48-53. (Abst in English)
- 12) Minhaz U, Tanaka M, Tsukamoto H, et al. Effect of MCI-186 on postischemic reperfusion injury in isolated rat heart. *Free Radic Res* 1996; **24**: 361-7.
- 13) Wu T-W, Zeng L-H, Wu J, et al. Myocardial protection of MCI-186 in rabbit ischemia-reperfusion. *Life Sci* 2002; **71**: 2249-55.
- 14) Morimoto K, Hasegawa T, Tanaka A, et al. Free-radical scavenger edaravone inhibits both formation and development of abdominal aortic aneurysms in rats. *J Vasc Surg* 2012; **55**: 1749-58.
- 15) Uchida HA, Takatsuka T, Shikata K, et al. Edaravone attenuate angiotensin II-induced abdominal aortic aneurysms in apolipoprotein E-deficient mice. *Arteriosclerosis Thrombosis and Vascular Biology* 2011 Scientific Sessions Final Program and Abstracts 2011; 58.
- 16) Abe K, Aoki M, Tsuji S, et al.; Writing Group; Edaravone (MCI-186) ALS 19 Study Group. Safety and efficacy of edaravone in well-defined patients with amyotrophic lateral sclerosis: a randomized double-blind placebo-controlled trial. *Lancet Neurol* 2017; **16**: 505-12.
- 17) Haimovici H. Arterial embolism myoglobinuria and renal tubular necrosis. *Arch Surg* 1970; **100**: 639-45.
- 18) Yamamura M, Miyamoto Y, Mitsuno M, et al. Edaravone injected at the start of reperfusion suppresses myonephropathic metabolic syndrome in rat. *Int J Angiol* 2014; **23**: 193-6.