Edaravone Injected at the Start of Reperfusion Suppresses Myonephropathic Metabolic Syndrome in Rats

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Abstract

The purpose of this study was to evaluate whether edaravone (Radicut®, Mitsubishi Tanabe Pharma Co., Osaka, Japan) injected at the start of reperfusion can suppress myonephropathic-metabolic syndrome (MNMS). MNMS models were made by clamping the bilateral common femoral arteries for 5 hours. At de-clamping (at the start of reperfusion), they were intra-peritoneal injected with 9.0 mg/kg of edaravone (the edaravone group, $n = 5$) or an equal volume of saline (the control group, $n = 5$). At five hours after de-clamping, the lower extremity muscles were stained with hematoxylin & eosin (H&E) to count the viable cells, and periodic acid- Schiff (PAS) to assess the glycogen storage. The lungs were also stained with H&E to express the alveolar wall thickness, and naphthol AS-D chloroacetate esterase to label infiltrating active neutrophils.

The viable muscle cells in the edaravone group was significantly greater than that of the control group (593 ± 60 vs. 258 ± 31 cells/mm², $p < 0.01$). The PAS-positive area in the edaravone group was also significantly higher than that in the control group (30.1 ± 6.9 vs. $7.3 \pm 2.1\%$, $p < 0.001$). The alveolar wall thickness in the edaravone group was significantly lower than that in the control group (63.6 ± 5.6 vs. $17.2 \pm 5.2\%$, $p < 0.001$). The active neutrophil infiltration in the edaravone group was also significantly lower than that in the control group (249 ± 59 vs. 68 ± 8 cells/mm², $p < 0.001$).

We conclude that edaravone injected at the start of reperfusion can suppress not only muscle reperfusion injury but also lung damage.

Keywords

- ▶ myonephropathic metabolic syndrome
- ▶ reperfusion injury
- ▶ free radical
- ▶ edaravone (Radicut)

In 1960, Haimovici reported two postoperative cases of myonephropathic metabolic syndrome (MNMS).¹ It has since become widely acknowledged that free radicals cause MNMS following leg reperfusion injury. However, there were no commercially available free radical scavengers to prevent MNMS. Edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one, C₁₀H₁₀N₂O, molecular weight = 174.2 kDa, Radicut, Mitsubishi Tanabe Pharma Co., Osaka, Japan) is the first free radical scavenger to be introduced for the treatment of patients with acute cerebral infarction.² We previously reported that edaravone injected before declamping could prevent reperfusion injury in rats.³ In

addition, we also reported that edaravone injected before reperfusion could prevent MNMS following leg ischemia.⁴

The purpose of this study is to evaluate whether edaravone injected at the start of reperfusion can suppress muscle injury and lung damage following leg ischemia.

Materials and Methods

Myonephropathic Metabolic Syndrome Rat Model

MNMS rat model was established under a microscope (OME-J&N J73507R, Olympus, Tokyo, Japan), as previously

described.^{3,4} Complete general anesthesia was induced by intraperitoneal administration of 30 mg/kg of sodium pentobarbital (Nembutal, Abbot Co., IL). Male Lewis rats were subjected to MNMS models by clamping the bilateral common femoral arteries for 5 hours. At declamping (at the start of reperfusion), Lewis male rats (525 ± 78 g) were divided into the two groups: the edaravone group ($n = 5$), we administered edaravone (9.0 mg/kg) into peritoneal cavity at the start of reperfusion; and the control group ($n = 5$), we also administered an equal volume of saline. At 5 hours after declamping, the lower extremity muscles and both lungs were harvested for histological studies.

All animal procedures complied with the criteria of Hyogo College of Medicine Animal Research Committee criteria (No. B-11-202).

Muscle Histological Studies

The muscles sections were stained with hematoxylin and eosin (H&E) to count the viable muscle cells. The numbers of viable muscle cells were counted manually in three fields of view at an original magnifications $\times 200$. The density of viable muscle cells was calculated and expressed per area (cells/mm^2). The sections were also stained with periodic acid Schiff (PAS), to assess glycogen storage in muscle cells. The percentages of PAS-positive area were measured in three fields of view at an original magnifications $\times 200$, using computerized densitometry (National Institutes of Health Image Program J, Ver. 1.37, <http://rsb.info.nih.gov/ij/>, Java 1.50 19; National Institutes of Health, MD).

Lung Histological Studies

The lung sections were stained with H&E and naphthol AS-D chloroacetate esterase, to label active neutrophils (Mitsubishi Chemical Medience Corporation, Kyodo Byori, Inc., Kobe, Japan). Lung damage was expressed as the percentage area of the alveolar wall thickness stained with H&E, using computerized densitometry (NIH Image Program J, Ver. 1.37) on three times in different fields (original magnification $\times 200$). The infiltration of active neutrophils was expressed as the density of naphthol AS-D chloroacetate esterase-positive cells per area (cells/mm^2). The numbers of naphthol AS-D chloroacetate esterase-positive cells were counted manually in three fields of view at an original magnifications $\times 400$.

Statistical Analysis

All results are expressed as the mean $\pm$ standard error of mean. Unpaired *t*-tests were used to compare data between the groups were (Stat View J-4.5 for Macintosh, Abacus Concept, Berkeley, CA). *p*-Values < 0.01 were considered to indicate statistical significance.

Results

Histological Results of Muscle

Representative muscle sections of the lower extremity stained with H&E were shown in **Fig. 1**. In the control group, there were significant swelling muscles and occlusion

Fig. 1 Representative muscle section H&E $\times 200$. H&E, hematoxylin and eosin.

Fig. 2 Comparison of viable muscle cell. SEM, standard error of mean.

of capillary vessels (**Fig. 1**, left). However, in the edaravone group, there are more live muscles cells whose cell nuclei stained in blue (**Fig. 1**, right). Statistical comparison for the viable muscle cells was shown in **Fig. 2**. The density of viable muscle cells in the edaravone group was significantly greater than that of the control group (593 ± 60 cells/mm^2 vs. 258 ± 31 cells/mm^2 , $p < 0.01$).

Representative muscle sections stained with PAS were shown in **Fig. 3**. Purple staining indicated positive PAS staining, which means rich of glycogen storage. The PAS muscle staining in the edaravone group (**Fig. 3**, left) was much stronger than that in the control group (**Fig. 3**, right).

Fig. 3 Representative muscle section PAS $\times 200$. PAS, periodic acid Schiff.

Fig. 4 Comparison of glycogen storage. SEM, standard error of mean.

Fig. 5 Representative lung section H&E ×200. H&E, hematoxylin and eosin.

Statistical comparison for PAS-positive area was shown in ▶Fig. 4. The mean percentage of PAS-positive area in the edaravone group was also significantly higher than that in the control group ($30.1 \pm 6.9\%$ vs. $7.3 \pm 2.1\%$, $p < 0.001$).

These results indicate that edaravone, even injected at the start of reperfusion, can also suppress muscle injury following leg ischemia in rats, storing a high level of glycogen in viable muscle cells.

Histological Results of Lung

Representative lung sections stained with H&E are shown in ▶Fig. 5. The alveolar wall was very thick in the control

Fig. 6 Comparison of lung damage. SEM, standard error of mean.

Fig. 7 Representative lung section naphthol AS-D chloroacetate esterase stain ×400.

group (▶Fig. 5, left). However, the alveolar wall in the edaravone group appeared nearly normal with less cell infiltrations (▶Fig. 5, right). Statistical comparison for the percentage area of alveolar wall thickness was shown in ▶Fig. 6. The mean percentage area of alveolar wall thickness in the edaravone group was significantly lower than that in the control group ($63.6 \pm 5.6\%$ vs. $17.2 \pm 5.2\%$, $p < 0.001$).

Representative lung sections stained with naphthol AS-D chloroacetate esterase were shown in ▶Fig. 7. Red staining, corresponding to naphthol AS-D chloroacetate esterase staining, represented active neutrophils. There are many active neutrophils infiltration in the control group. Moreover, these active neutrophils were much strongly stained with naphthol AS-D chloroacetate (▶Fig. 7, left). However, in the edaravone group, there are less active neutrophils infiltrations (▶Fig. 7, right). Statistical comparison for the density of active neutrophil infiltration was shown in ▶Fig. 8. There are significantly less active neutrophils infiltrations in the edaravone group than that in the control group (249 ± 59 cells/mm² vs. 68 ± 8 cells/mm², $p < 0.001$).

These results indicate that edaravone injected at the start of reperfusion can also suppress lung damage following leg ischemia in rats, by reducing of active neutrophils infiltration into the alveolar wall.

Fig. 8 Comparison of active neutrophil infiltration. SEM, standard error of mean.

Discussion

Muscle Reperfusion Injury

It is well known that free radicals cause damage to not only muscles but also lungs. Since 2001 in Japan, edaravone is the first free radical scavenger and now commercially available for the treatment for acute cerebral infarction. Several Japanese experimental studies showed that edaravone reduced cerebral damage during aortic arch surgery⁵ and suppressed postoperative spinal cord infarction.⁶ In addition, edaravone attenuated experimental abdominal aortic aneurysmal formation in apolipoprotein E-deficient mice.⁷ Recently, edaravone inhibited experimental abdominal aortic aneurysmal development in rats.⁸

We previously showed that edaravone injected before declamping could suppress muscle reperfusion injury, by reducing the mitochondrial damage.⁹ However, regarding the timing of administration, edaravone may be given after declamping in the clinical settings. Therefore, in this study, we investigated whether edaravone is effective, even if injected at the start of reperfusion. And we found that edaravone can also suppress muscle injury following leg ischemia.

Lung Damage

Because free radicals cause MNMS, which damages not only muscles but also other organs such as lungs and kidneys, we evaluated whether edaravone can reduce MNMS-induced lung damage. Our histological study of H&E stain previously showed that edaravone injected before declamping could reduce lung damage following leg ischemia in rats.⁴ Here in this study, we found that edaravone even if injected at the start of reperfusion can also suppress lung damage following leg ischemia. In addition, we try to identify what kind of cells is infiltrating into lung and found there are many active neutrophils infiltration into lung, using naphthol AS-D chloroacetate esterase stain.

To explain the beneficial effect of edaravone for lung damage, we hypothesized that edaravone has the two types of mechanisms. At first, we thought that the suppression of muscle reperfusion injury reduces the active neutrophils as mentioned above. And the next, because edaravone has the protecting effect on endothelial cell in vivo¹⁰ and in vitro,¹¹ edaravone can directly reduce active neutrophils infiltrations by protecting injured lung endothelial cells.

In conclusion, we emphasize that the free radical scavenger edaravone injected at the start of reperfusion can suppress not only muscle reperfusion injury but also lung damage in rats. Therefore, we believe that edaravone might be useful in clinical settings for MNMS following leg ischemia.

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