Edaravone suppresses postoperative reperfusion injury in rat lower extremity: An immunohistological study

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PURPOSE: The present study evaluated whether the free radical scavenger edaravone (Radicut [Mitsubishi Pharma Co, Japan]) can suppress lower extremity postoperative reperfusion injury by evaluating muscle cell viability with immunohistological stain (cytochrome c oxidase stain).

METHODS: Eight Lewis male rats (460 g to 510 g) were divided into two groups. In the control group, postoperative reperfusion injury models were created by clamping the bilateral common femoral arteries for 5 h and then releasing. In the other group, 9.0 mg/kg of edaravone was administered before clamping the bilateral common femoral arteries. After 5 h of reperfusion, the bilateral

triceps muscles in both groups were stained with cytochrome c oxidase stain (each $n=4 \times 2$). The positive areas of cytochrome c oxidase stain were measured and compared, using computerized densitometry (National Institutes of Health Image program, version 1.61).

RESULTS: In the control group, the lower triceps muscles were not stained with cytochrome c oxidase. In the edaravone group, the lower triceps muscles were strongly stained with cytochrome c oxidase. The positive areas of cytochrome c oxidase stain were significantly greater in the edaravone group ($133,000 \pm 12,000 \mu^2/\text{mm}^2$, $P<0.01$) compared with the control group ($8000 \pm 1300 \mu^2/\text{mm}^2$).

CONCLUSION: The present study suggests that the preoperative administration of 9.0 mg/kg of edaravone may suppress postoperative reperfusion injury in a rat model.

Key Words: *Cytochrome c oxidase stain; Edaravone (Radicut); Postoperative reperfusion injury*

Free radicals cause lower extremity postoperative reperfusion injury, which has been termed myoneuropathic metabolic syndrome (MNMS) (1). Currently, there is no commercially available free radical scavenger that is proven to be clinically effective for preventing MNMS.

Edaravone (Radicut, Mitsubishi Pharma Co, Japan) (3-methyl-1-phenyl-2-pyrazoline-5-one, $\text{C}_{10}\text{H}_{10}\text{N}_2\text{O}$, molecular weight 174.2) is the first free radical scavenger proven to be effective after acute cerebral infarction (2). We previously suggested that edaravone might suppress postoperative reperfusion injury in a rat model (3).

The present study focuses on the rat muscle cell viability. Cells that stain positive for cytochrome c oxidase have high mitochondrial activity. We assessed whether edaravone can suppress postoperative reperfusion injury by evaluating muscle cell viability with cytochrome c oxidase stain.

MATERIALS AND METHODS

Postoperative reperfusion injury model

All animal care complied with Hyogo College of Medicine Animal Research Committee criteria (No. 180). Using 30 mg/kg of sodium pentobarbital (Nembutal, Abbot Laboratories, USA), general anesthesia was induced in eight male Lewis rats (460 g to 510 g). The rats were divided into two groups of four: the edaravone group, in which edaravone

(9.0 mg/kg) was administered, and the control group, in which the same volume of saline was administered preoperatively.

A postoperative reperfusion model was created by clamping the bilateral common femoral arteries for 5 h then releasing, as previously described (3). Using a microscope (OME-J&N J73507R, Olympus JAPAN) with 30 $\times$ magnification, both common femoral arteries were clamped for 5 h, after ligation of both deep femoral arteries and epigastric arteries. Five hours after declamping of the bilateral common femoral arteries, lower extremity postoperative reperfusion injury was assessed.

Cytochrome c oxidase stain

Triceps muscles of each rat were harvested bilaterally for histological study. Using a cryostat microtome CM3050S (Leica, Germany), 5 μm sections were taken from each lower triceps muscle and placed on silanized microslides (Dako, Japan). They were stained immediately with cytochrome c oxidase stain (each $n=4 \times 2$) (4). Nonreperfused lower triceps muscle was also harvested as positive control of cytochrome c oxidase staining.

The positive areas of cytochrome c oxidase stain were measured three times on different fields, using computerized densitometry (National Institutes of Health [NIH] Image Version 1.61). On NIH Image Version 1.61 densitometry, between 125 and 250 over dots was defined and calculated as

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Figure 1) Comparison of the lower triceps muscles after postoperative reperfusion injury in control (A) and edaravone-treated (B) rats. Strong brown staining of muscle indicates the positive area of cytochrome c oxidase. Original magnification $\times 200$

Figure 2) Positive areas of cytochrome c oxidase stain

the positive areas (<http://rsb.info.nih.gov/ni-image/manual/menus/analyze.html>)

Statistical analysis

All results are expressed as mean $\pm$ SEM. The results were statistically analyzed by unpaired *t* test (Stat View J-4.5 for Macintosh, Abacus Concept, USA). $P < 0.05$ was considered to be significant.

RESULTS

There was no operative deaths in either group. Triceps muscles were harvested bilaterally for histological study. The lower triceps muscles from the saline-treated rats were elastic hard. In contrast, the lower triceps muscles from the edaravone group were as soft as nonreperfused tissue.

Cytochrome c oxidase stain

Cytochrome c oxidase-stained muscles of control and edaravone-treated rats are shown in Figures 1A and 1B. Positive areas of cytochrome c oxidase staining are brown in colour. The lower triceps muscles of the rats treated with edaravone

were distinctly 'marbled' in appearance, indicating a more positive reaction to the stain than the muscles of the saline-treated rats.

Computerized densitometry

The mean positive areas of cytochrome c oxidase stain were shown in Figure 2.

In the rats treated with edaravone, the positive areas of cytochrome c oxidase stain were significantly greater ($133,000 \pm 12,000 \mu^2/\text{mm}^2$, $P < 0.01$) than those of the controls ($8000 \pm 1300 \mu^2/\text{mm}^2$). Therefore, edaravone maintains a high level of muscle mitochondrial activity during postoperative reperfusion injury.

DISCUSSION

Edaravone

In experimental studies, there have been some reports of successful suppression of lower extremity postoperative reperfusion injury by free radical scavengers (5), but no effective clinical application of free radical scavengers for MNMS. Edaravone has been commercially available in Japan as a free radical scavenger for acute cerebral infarction since 2001 (2). In another study (6), we showed that edaravone could suppress the postoperative intimal hyperplasia of the vein graft in a rat model *in vivo*.

Free radicals cause lower extremity postoperative reperfusion injury, which has been termed MNMS (1). In our last study, we suggested that edaravone may suppress postoperative reperfusion injury in a rat model, as assessed by counting the nucleus cell numbers stained with hematoxylin and eosin (3). This time, we focused on rat muscle cell viability. After edaravone was given, the positive areas of cytochrome c oxidase stain were significantly greater than that in muscle without edaravone treatment ($P < 0.01$). This would suggest a clinical preoperative benefit in administering edaravone as a protective measure against the development of postoperative MNMS.

Mechanism of action

The mechanism of action of edaravone for acute cerebral infarction is believed to be the scavenging effect of the peroxy

free radical (7). Nitric oxide is formed by endothelial cells from L-arginine via endothelial nitric oxide synthase (8,9). Nitric oxide reacts with superoxide anion during reperfusion injury and generates peroxynitrite (9,10). We believe that edaravone's mechanism of action for lower extremity postoperative reperfusion injury is the scavenging effect of peroxynitrite. It is very interesting that edaravone has not only the scavenging effect of the peroxy free radical, but also prevents vascular endothelial cell injury (2).

The present study did not demonstrate free radical activity. Our results only show that edaravone maintains a high level of muscle mitochondrial activity during postoperative reperfusion injury. Many details are still unclear. How edaravone suppresses postoperative reperfusion injury still needs to be better defined, to bring edaravone toward clinical use.

Other issues must be addressed before clinical use of edaravone. In the present study, we administered edaravone just before clamping both common femoral arteries. Clinically, edaravone may be administered during clamping both common femoral arteries or at the same time of release.

CONCLUSION

The preoperative administration of 9.0 mg/kg edaravone can suppress rat lower extremity postoperative reperfusion injury.

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