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西島, 卓矢

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Basic Science

Necrostatin-1 Attenuates Delayed Paraplegia after Transient Spinal Cord Ischemia in Rabbits by Inhibiting the Upregulation of Receptor-Interacting Protein Kinase 1 and 3

Takuya Nishijima,¹ Satoshi Fujita,¹ Takeaki Harada,¹ Hikaru Uchiyama,¹ Kensaku Matsuda,¹ Hiroshi Mitsuo,¹ Tomoki Ushijima,¹ Meikun Kan-o,¹ Gen Shinohara,¹ Satoshi Kimura,¹ Yasuhisa Oishi,² Hiromichi Sonoda,¹ and Akira Shiose,¹ Fukuoka, Japan

Background: Delayed-onset paraplegia is a disastrous complication after thoracoabdominal aortic open surgery and thoracic endovascular aortic repair. Studies have revealed that transient spinal cord ischemia caused by temporary occlusion of the aorta induces delayed motor neuron death owing to apoptosis and necroptosis. Recently, necrostatin-1 (Nec-1), a necroptosis inhibitor, has been reported to reduce cerebral and myocardial infarction in rats or pigs. In this study, we investigated the efficacy of Nec-1 in delayed paraplegia after transient spinal cord ischemia in rabbits and assessed the expression of necroptosis- and apoptosis-related proteins in motor neurons.

Methods: This study used rabbit transient spinal cord ischemia models using a balloon catheter. They were divided into a vehicle-treated group ($n = 24$), Nec-1-treated group ($n = 24$), and sham-controls ($n = 6$). In the Nec-1-treated group, 1 mg/kg of Nec-1 was intravascularly administered immediately before ischemia induction. Neurological function was assessed using the modified Tarlov score, and the spinal cord was removed 8 hr and 1, 2, and 7 days after reperfusion. Morphological changes were examined using hematoxylin and eosin staining. The expression levels of necroptosis-related proteins (receptor-interacting protein kinase [RIP] 1 and 3) and apoptosis-related proteins (Bax and caspase-8) were assessed using western blotting and histochemical analysis. We also performed double-fluorescence immunohistochemical studies of RIP1, RIP3, Bax, and caspase-8.

Results: Neurological function significantly improved in the Nec-1-treated group compared with that in the vehicle-treated group 7 days after reperfusion (median 3 and 0, $P = 0.025$). Motor neurons observed 7 days after reperfusion were significantly decreased in both groups compared with the sham group (vehicle-treated, $P < 0.001$; Nec-1-treated, $P < 0.001$). However, significantly more motor neurons survived in the Nec-1-treated group than in the vehicle-treated group ($P < 0.001$). Western blot analysis revealed RIP1, RIP3, Bax, and caspase-8 upregulation 8 hr after reperfusion in the vehicle-treated group (RIP1, $P = 0.001$; RIP3, $P = 0.045$; Bax, $P = 0.042$; caspase-8, $P = 0.047$). In the Nec-1-treated group, the upregulation of RIP1 and RIP3 was not observed at any time point, whereas that of Bax and caspase-8 was observed 8 hr after reperfusion (Bax, $P = 0.029$; caspase-8, $P = 0.021$). Immunohistochemical

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¹Department of Cardiovascular Surgery, Kyushu University Hospital, Fukuoka, Japan.

²Advanced Aortic Therapeutics, Faculty of Medicine, Kyushu University Graduate School of Medicine, Fukuoka, Japan.

Correspondence to: Akira Shiose, Department of Cardiovascular Surgery, Kyushu University Hospital, 3-1-1 Maidashi, Higashiku, Fukuoka 812-8582, Japan; E-mail: shiose.akira.799@m.kyushu-u.ac.jp

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study revealed the immunoreactivity of these proteins in motor neurons. Double-fluorescence immunohistochemistry revealed the induction of RIP1 and RIP3, and that of Bax and caspase-8, in the same motor neurons.

Conclusions: These data suggest that Nec-1 reduces delayed motor neuron death and attenuates delayed paraplegia after transient spinal cord ischemia in rabbits by selectively inhibiting necroptosis of motor neurons with minimal effect on their apoptosis.

INTRODUCTION

Spinal cord injury (SCI) after successful thoracoabdominal aortic surgery is a disastrous complication. Hypothermia and cerebrospinal fluid drainage decrease the risk; however, paraplegia incidence after thoracoabdominal aortic surgery remains high (11.4–13.2%).^{1,2} Recently, thoracic endovascular repair (TEVAR) has been proposed as an alternative to open repair and contributes to reduced hospital mortality or postoperative morbidity³; however, the incidence of SCI after TEVAR is also high (3–12%).^{3–5} Naloxone and steroids can reduce this complication^{6,7}; nevertheless, their exact efficacy remains uncertain. Therefore, identifying new effective drug therapies for this complication is essential.

Acute spinal cord dysfunction is caused by ischemic damage during cross-clamping. However, after thoracoabdominal aortic repair, patients sometimes develop delayed-onset paraplegia even when they wake without any neurological deficits immediately after surgery.⁸ Previous studies have revealed that neuronal survival after spinal cord ischemia is affected by apoptosis or autophagy under conditions of nonlethal stress.^{9–12} Moreover, our recent study demonstrated the involvement of necroptosis, a novel type of cell death that is similar to necrosis but regulated like apoptosis.¹³

Necroptosis relies on the formation of a necrosome, which is mainly composed of receptor-interacting protein-1 (RIP1), receptor-interacting protein-3 (RIP3), and mixed lineage kinase domain-like protein, and it is activated by phosphorylation of RIP1^{14–17} (Fig. 1). Studies have revealed that necroptosis is crucial to ischemia-reperfusion injuries, and blocking this pathway attenuates diseases such as cerebral infarction in rats or myocardial infarction in pigs, especially when using a specific RIP1 inhibitor, necrostatin-1 (Nec-1).^{18–20} It inhibits RIP1 autophosphorylation and blocks RIP1-RIP3-mixed lineage kinase domain-like protein signal transduction.¹⁷ Studies have indicated that Nec-1 affects not only necroptosis but also apoptosis in some models because they have several common pathways.^{21,22} However, apoptosis regulation by Nec-1 differs between diseases and models,¹⁷

and the exact crosstalk between apoptosis and necroptosis is not fully understood.

A previous study demonstrated that Nec-1 improves neurological dysfunction in the early phase after spinal cord ischemia and exhibits a protective effect against acute spinal cord ischemia injury.²³ However, its efficacy in delayed paraplegia and delayed motor neuron death after transient ischemia remains unknown. Moreover, how Nec-1 affects motor neuron apoptosis after transient ischemia remains unknown. Therefore, in this study, we investigated its efficacy in delayed paraplegia and delayed motor neuron death after transient spinal cord ischemia in rabbits. In addition, the expression levels of necroptosis-related proteins (RIP1 and RIP3) and apoptosis-related proteins (Bax and caspase-8) were assessed to reveal the influence of Nec-1 on necroptosis and apoptosis in motor neurons of the spinal cord.

MATERIAL AND METHODS

Animal Models

Animals were treated in accordance with the guiding principles for the care and use of animals during the experiments. The animal care committee of the Kyushu University School of Medicine approved the experimental and animal care protocols (approval number: A22-389-0).

In total, 54 Japanese domesticated rabbits weighing 2.5–3.0 kg were divided into 3 groups: the vehicle-treated control group (group V; $n = 24$), the Nec-1-treated group (group N; $n = 24$), and the sham control group (group S; $n = 6$). The N group was administered Nec-1 dissolved with dimethyl sulfoxide (DMSO) and underwent transient spinal cord ischemia surgery. The V group was administered the same amount of DMSO as the N group to ensure precise comparisons and also underwent transient spinal cord ischemia surgery. The sham control group underwent sham surgery. Anesthesia was induced by intramuscular administration of 50 mg/kg ketamine and maintained with 2% halothane inhalation and 100% oxygen. A 5-French pediatric 8-mm balloon catheter (CI-300/BA5F50; Gadellius Medical Products, Inc.,

excised immediately after death using a 1-mL syringe plunger. Tissue samples for western blot analysis and immunohistochemical studies were frozen and stored at -80°C . Histology samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and stored at 4°C for 1 week. They were then transversely cut at L2 or L3, approximately, and embedded in paraffin.

Neurological Assessment

Neurological function was evaluated before the rabbits were euthanized at each time point. They were classified according to a modified Tarlov score²⁷ as follows: 0, no movement; 1, slight movement; 2, sitting with assistance; 3, sitting unaided; 4, weak hopping; and 5, normal hopping. Two individuals blinded to the treatment independently graded their neurological function.

Histological Study

To determine pathological changes in the spinal cord after ischemia, hematoxylin and eosin staining was performed on 5 sections per animal using light microscopy. The number of intact large motor neurons in the ventral gray matter region was visually counted. An observer unaware of the animal groups and neurological outcomes visually examined each slide (magnification $\times 100$). Neurons were considered “dead” if the cytoplasm was diffusely eosinophilic and “viable” if they demonstrated basophilic stippling (i.e., contained Nissl substance).

Western Blot Analysis

Western blot analysis was performed to investigate RIP1, RIP3, Bax, and caspase-8 expression changes. Tissue samples were homogenized in lysis buffer (Appendices A). Furthermore, the homogenates were centrifuged at 12,000 *g* for 15 min at 4°C . Assays to determine the protein concentrations of the samples were performed by comparing the results with a known concentration of bovine serum albumin using a Bicinchoninic Acid Protein Assay Kit (T9300A; Takara Bio Inc., Shiga, Japan). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed on a 12% polyacrylamide gel under nonreducing conditions. Briefly, protein samples were boiled at 100°C in 5% β -mercaptoethanol and 2.5% SDS, and lysates equivalent to 20 μg of protein from each sample were run on the gel for 30 min at 20 mA (Appendices B) with a size marker (dual color protein; Bio-Rad Laboratories, Hercules, CA, USA). Proteins on the gel were transferred to polyvinylidene fluoride membranes on a

semidry electrotransfer unit (Bio-Rad). Membranes were placed in 5% powdered milk in tris-buffered saline containing 0.05% Tween-20 (TBST) to block nonspecific binding after transfer. The membranes were incubated for 20 hr at 4°C with primary antibodies: goat polyclonal anti-RIP antibody (sc-1169; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:500 dilution; mouse monoclonal anti-RIP3 antibody (sc-374639; Santa Cruz Biotechnology) at 1:1,000 dilution; rabbit polyclonal anti-Bax antibody (BS-0127R; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 1:500 dilution; and goat polyclonal anticaspase-8 antibody (sc-6136; Santa Cruz Biotechnology) at 1:500 dilution. After washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-goat immunoglobulin G (IgG) (sc-2354; Santa Cruz Biotechnology), horseradish peroxidase-conjugated antimouse IgG (A90-105P; Bethyl Laboratories, Inc., Montgomery, TX, USA), or horseradish peroxidase-conjugated antirabbit IgG (bs-0295G-HRP; Bioss, Inc., Boston, MA, USA), at 1:5000 dilution in TBST for 30 min. Another membrane was similarly stained without primary antibodies to ascertain the specific antibodies binding to the proteins. Blotted protein bands were visualized with the Clarity Western ECL Substrate (Bio-Rad) and detected using the ImageQuant™ LAS 4000 Imaging System (Fujifilm Holding Corporation, Tokyo, Japan). Western blot images were quantified by plotting a two-dimensional densitogram using Image J software, version 1.53 (Research Services Branch, National Institute of Mental Health, National Institute of Health, Bethesda, MD, USA).

Immunohistochemical Study

We performed immunohistochemical analysis in 5 sections per animal to investigate the changes in RIP1, RIP3, Bax, and caspase-8 expression. Spinal cord sections were rinsed for 20 min in 0.1 M phosphate buffered saline (PBS). Sections were blocked in Blocking One Histo (Nacalai Tesque, Inc., Kyoto, Japan) for 30 min at room temperature and incubated with primary antibodies in 0.3% Triton-X 100 and 5% Blocking One Histo for 20 hr at 4°C . Primary antibodies were the same as those used for western blot analysis, as follows: anti-RIP at 1:100, anti-RIP3 at 1:200, anti-Bax at 1:100, and anticaspase-8 at 1:100. Then, the sections were incubated in quenching buffer (Appendices C) for 15 min to quench endogenous peroxidase activity, washed in PBS, and incubated for 1 hr with biotinylated anti-goat IgG (PK-6105; Vector Laboratories, Burlingame, CA, USA), biotinylated antimouse IgG (PK-6102;

Vector Laboratories), or biotinylated antirabbit IgG (sc-2491; Santa Cruz Biotechnology) at 1:200 dilution in PBS. Samples were then incubated with an avidin-biotin-horseradish peroxidase complex (30462-30; Nakarai Tesque, Inc.), colorized with diaminobenzidine/ H_2O_2 solution, and the cytoplasm was counterstained with hematoxylin. A set of sections was similarly stained without primary antibodies to determine specific antibody binding to proteins.

Double-Fluorescence Immunohistochemical Study

Double-fluorescence immunohistochemical analysis of both pairs was performed on 5 sections per animal to investigate the coexpression of RIP1 and RIP3 and that of Bax and caspase-8 in neuronal cells. Spinal cord sections were prepared as described above and blocked using Blocking One Histo before applying the primary antibodies. Sections were then incubated with RIP goat polyclonal antibody at 1:50 dilution and simultaneously with RIP3 mouse monoclonal antibody at 1:50 dilution or with Bax rabbit polyclonal antibody at 1:50 dilution and simultaneously with caspase-8 goat polyclonal antibody at 1:50 dilution overnight at 4°C. They were detected using donkey anti-goat IgG linked with red-orange fluorescent Alexa Fluor 555 at 1:500 dilution (A2142; Invitrogen) and goat antimouse IgG linked with green fluorescent Alexa Fluor 488 at 1:500 dilution (A11001; Invitrogen), or mouse antirabbit IgG linked with red-orange fluorescent Cruz Fluor 555 at 1:500 dilution (sc-516249; Santa Cruz), and mouse anti-goat IgG linked with green fluorescent Cruz Fluor 488 at 1:500 dilution (sc-516241; Santa Cruz). The slides were mounted in aqueous mounting media with 1,4-diazabicyclo[2.2.2]octane and observed using fluorescence microscopy (magnification $\times 400$).

Statistical Analysis

Parametric data are presented as mean $\pm$ standard deviation and nonparametric data as medians. For quantitative analyses, neurological scores were analyzed using the Kruskal-Wallis test with post hoc Steel-Dwass tests. The cell numbers and optical density values of western blots were analyzed using the one-way analysis of variance with post hoc Tukey's multiple comparison tests. Statistical significance was set at a P value of <0.05 . JMP version 16 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

RESULTS

Neurological Assessment

The results of neurological assessment are presented in [Figure 2](#) and [Supplementary Table](#). Neurological function significantly declined in the V and N groups compared with that in the S group 7 days after reperfusion (median: V = 0, $P = 0.013$; N = 3, $P = 0.021$). However, the neurological function in the N group was significantly preserved compared with that in the V group 7 days after reperfusion ($P = 0.025$).

Histological Study

The results of cell counting in the ventral gray matter region of the sections are illustrated in [Figure 3](#). In the S group, spinal cords were intact with many large motor neurons (24.2 ± 3.93 cells per slide; [Fig. 3A](#)). In the V group, although most motor neurons were intact 2 days after reperfusion ([Fig. 3B](#)), approximately 65% of the motor neurons were lost in the ventral gray matter 7 days after reperfusion (8.60 ± 3.38 cells per slide; $P < 0.001$; [Fig. 3C](#)). In the N group, most motor neurons in the ventral gray matter were preserved 2 days after reperfusion ([Fig. 3D](#)), and they significantly decreased 7 days after reperfusion compared with the S group (16.0 ± 2.83 cells per slide; $P < 0.001$; [Fig. 3E](#)). However, significantly more motor neurons survived 7 days after reperfusion in the N group compared with the V group ($P < 0.001$) ([Fig. 3F](#)). The dorsal horn neurons remained intact after transient ischemia (data not shown). Therefore, selective loss of motor neurons was confirmed, as previously reported.

Western Blot Analysis

The results of western blot analysis are presented in [Figure 4](#). In the S group, RIP1, RIP3, Bax, and cleaved caspase-8 were detected as weak bands of 74 kDa, 60 kDa, 21 kDa, and 43 kDa, respectively. These bands were strongly enhanced 8 hr after reperfusion in the V group ([Fig. 4A](#)). RIP1 and Bax enhancements were preserved until 1 day after reperfusion, and RIP3 enhancements were preserved until 2 days after reperfusion. In the N group, the RIP1 and RIP3 bands were similar to those in the S group until 2 days after reperfusion. However, the Bax and caspase-8 bands were strongly enhanced 8 hr after reperfusion ([Fig. 4B](#)). Control membranes without primary antibodies exhibited no bands (data not shown). Analysis of variance indicated that RIP1 (C), RIP3 (E), Bax (G), and caspase-8 (I)

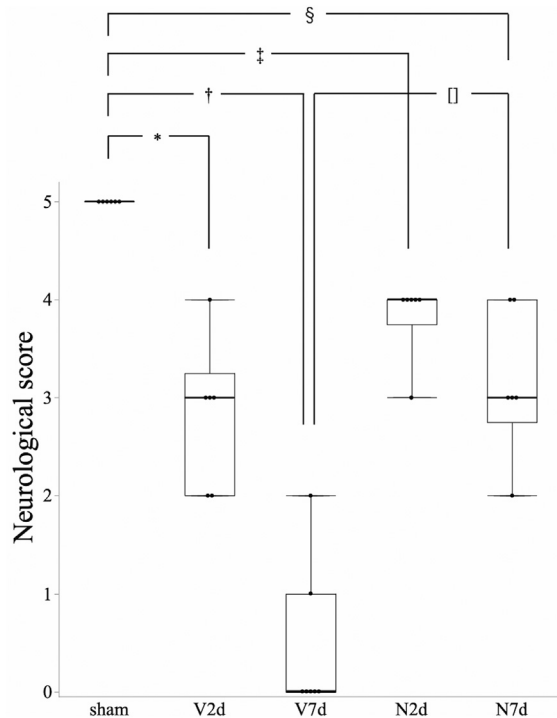


Fig. 2. Scatter plots and box plots of neurological scores of rabbits in the sham group and 2 and 7 days after transient spinal cord ischemia in the vehicle- (V2d and V7d) and necrostatin-1- (N2d and N7d) treated groups. Each dot represents an individual score. The boxes represent interquartile ranges, and the edges of the upper and lower whiskers represent maximum and minimum scores, respectively. The thick lines in the boxes represent median scores. Neurological function significantly declined at both 2 and 7 days after reperfusion in the V and N groups. However, neurological function in the N7d group was significantly preserved compared with that in the V7d group. $N = 6$ per group at each time point. * $P = 0.021$, † $P = 0.013$, ‡ $P = 0.016$, § $P = 0.021$, ¶ $P = 0.025$.

levels were significantly increased 8 hr after reperfusion in the V group (relative optical densities: RIP1, 2.28 ± 0.79 , $P = 0.001$; RIP3, 1.94 ± 1.03 , $P = 0.045$; Bax, 1.89 ± 0.65 , $P = 0.042$; caspase-8, 1.89 ± 0.51 , $P = 0.047$) compared with those in the S group. In addition, the RIP1 and Bax upregulations were preserved until 1 day after reperfusion, and the RIP3 upregulation was preserved until 2 days after reperfusion. In contrast, there were no significant increases in RIP1 (D) and RIP3 (F) levels in the N group at any time. However, Bax (H) and caspase-8 (J) levels in the N group significantly increased 8 hr after reperfusion (Bax, 1.95 ± 0.74 , $P = 0.029$; caspase-8, 2.03 ± 0.88 , $P = 0.021$) compared with that in the S group. In addition, in a comparison between the V and N groups, the

expression of RIP1 at 8 hr after reperfusion and RIP3 at all time points significantly decreased in the N group compared with that in the V group (RIP1 after 8 h, $P = 0.004$; RIP3 after 8 h, $P = 0.028$; RIP3 after 1 day, $P = 0.005$; RIP3 after 2 days, $P = 0.004$). In contrast, there were no significant differences in Bax and caspase-8 levels at any time point.

Immunohistochemical Study

The immunoreactivity of RIP1, RIP3, Bax, and caspase-8 in the spinal cord is illustrated in Figure 5 and Supplementary Figure. The spinal cords of the S group did not display any protein immunoreactivity in any cell (Fig. 5A, D, G, and J). In the V group, motor neurons selectively exhibited strong immunoreactivity for all proteins 8 hr after reperfusion (Fig. 5B, E, H, and K). Moreover, the immunoreactivity for RIP1 and Bax was preserved on the first day (Supplementary Figures A and I), and that for RIP3 was preserved for the first 2 days (Supplementary Figures E and F). In the N group, RIP1 and RIP3 immunoreactivity were negligible in the motor neurons (Fig. 5C, F). However, strong immunoreactivity was observed for Bax and caspase-8 8 hr after reperfusion (Fig. 5I, L).

Double-Fluorescence Immunohistochemical Study

The results of RIP1 and RIP3 or Bax and caspase-8 double staining immunohistochemistry in the V group are illustrated in Figure 6. RIP1 and Bax (Fig. 6A, D) were detected using red-orange fluorescence, and RIP3 and caspase-8 (Fig. 6B, E) were detected using green fluorescence. These molecules were expressed in the cytoplasm of motor neurons. Merged images revealed that the cells were double-positive. In the motor neurons, a combination of RIP1 and RIP3, Bax, and caspase-8 were expressed and respectively colocalized in the cytoplasm (Fig. 6C, F).

DISCUSSION

The major findings of this study are as follows: (i) Nec-1 reduced delayed motor neuron death and attenuated neurological dysfunction after transient spinal cord ischemia. (ii) Transient spinal cord ischemia increased RIP1, RIP3, Bax, and caspase-8 expressions 8 hr after reperfusion. (iii) Nec-1 suppressed the upregulation of RIP1 and RIP3, whereas that of Bax and caspase-8 was preserved even when Nec-1 was administered.

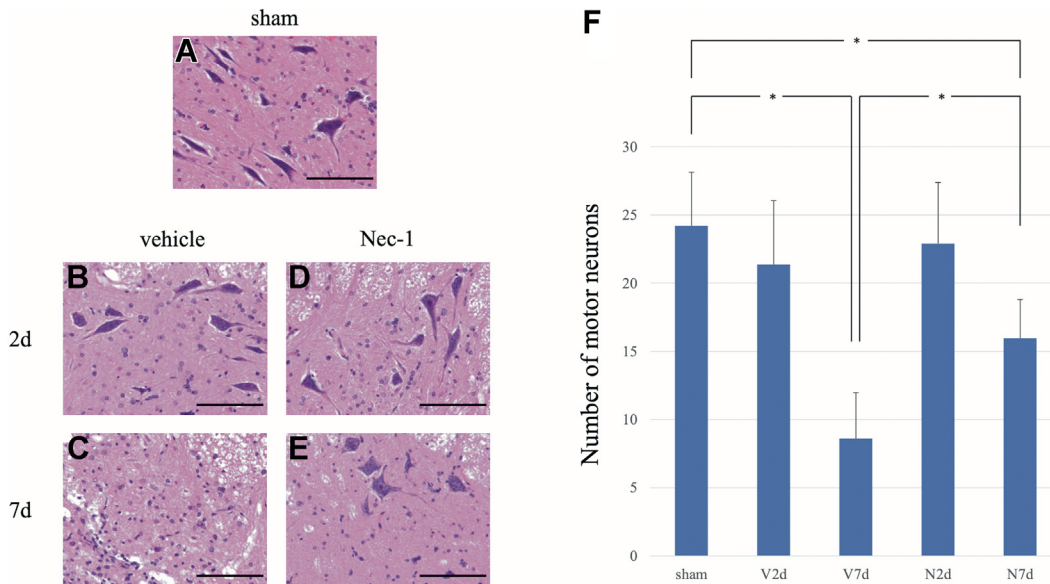


Fig. 3. **A–E**, Histological findings in spinal cords stained with hematoxylin and eosin after transient ischemia in rabbits. The spinal cord of the sham group (**A**), vehicle-treated group 2 days after reperfusion (V2d) (**B**), and the necrostatin-1-treated group 2 days after reperfusion (N2d) (**D**) remained unchanged histologically. Some motor neurons were lost in the necrostatin-1-treated group 7 days after reperfusion (N7d) (**E**), whereas most motor neurons were lost in the vehicle-treated group 7 days after reperfusion (V7d) (**C**). Motor neurons were

selectively lost without apparent gliosis or cellular infiltration. Scale bar = 100 μ m. **F**, number of cells observed per slide. In the V7d group, approximately 65% of the motor neurons in the spinal cord were damaged. In the N7d group, motor neurons significantly decreased compared to those in the sham group. However, motor neurons of the N7d group were significantly preserved compared with those of the V7d group. $N = 6$ per group at each time points. $*P < 0.001$.

Previous studies have demonstrated the involvement of necroptosis in several disease models related to ischemia-reperfusion injuries.^{18–20} In addition, our recent study revealed that transient spinal cord ischemia induced necroptosis in motor neurons in rabbit models.¹³ The serine/threonine kinase activity of RIP1 and its interaction with RIP3 are necessary for necroptosis.¹⁴ This study revealed the upregulation of these proteins in motor neurons during the early phase, as previously reported.¹³ Recent studies have revealed that Nec-1 effectively inhibits necroptosis and attenuates necroptosis-related diseases. Regarding spinal cord-related diseases, Nec-1 reduced neural cell damage after traumatic SCI in mice and rats,^{28,29} and protected against acute ischemic SCI in mice.²³ However, its efficacy in delayed motor neuron death following ischemia remains unclear. A novelty of this study is that Nec-1 also reduced delayed motor neuron death and attenuated delayed paraplegia following progressive neurological dysfunction after transient spinal cord ischemia in rabbits. In addition, suppressed RIP1 and RIP3 upregulation in the N group suggests successful inhibition of necroptosis by Nec-1.

Necroptosis and apoptosis are involved in several pathways. Studies have revealed that Nec-1 not only inhibited necroptosis, but also suppressed apoptosis in some models.^{21,22,29} However, some studies have observed opposite results—Nec-1 did not affect apoptosis.^{28,30} In this study, we investigated the effects of Nec-1 on apoptosis by assessing the expression of the apoptosis-related proteins—Bax and caspase-8, which play an important role in activating apoptosis pathways^{31,32} (Fig. 1). We revealed that these proteins were upregulated after reperfusion and the upregulation was preserved even after Nec-1 administration, although the upregulation of RIP1 and RIP3 was suppressed by Nec-1. Our results suggest that Nec-1 selectively inhibits necroptosis of motor neurons with minimal effect on apoptosis although necroptosis and apoptosis are both involved with delayed motor neuron death. Recent studies have demonstrated that the combined use of necroptosis and apoptosis inhibitors attenuates ischemia-reperfusion injuries more effectively in rat lung³³ and pig myocardial injury models.³⁴ Future studies using both inhibitors are expected to more strongly attenuate delayed paraplegia following ischemia.

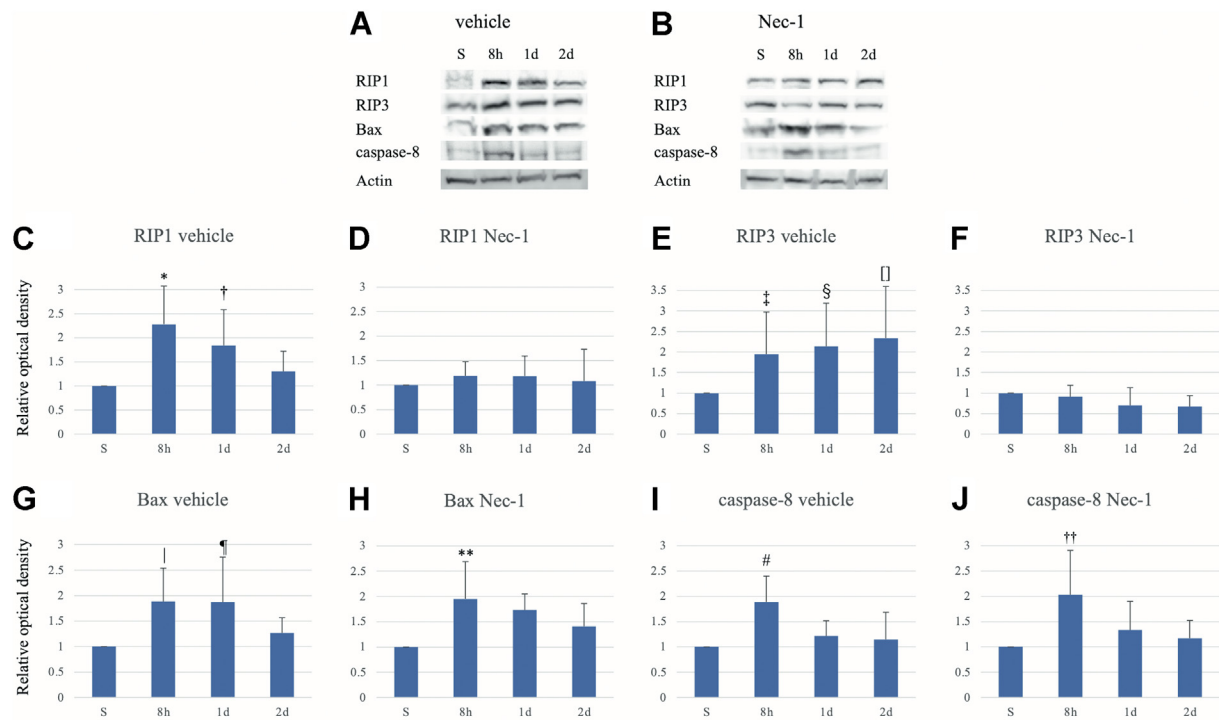


Fig. 4. Representative western blots for receptor-interacting protein kinase (RIP) 1, RIP3, Bax, and caspase-8 in rabbits. In the vehicle-treated group, RIP1, RIP3, Bax, and caspase-8 were strongly enhanced 8 hr after reperfusion. RIP1 and Bax enhancements were preserved until 1 day after reperfusion, and RIP3 enhancement was preserved until 2 days after reperfusion (**A**). In the necrostatin-1-treated group, RIP1 and RIP3 were not enhanced after reperfusion. In contrast, Bax and caspase-8 were strongly enhanced 8 hr after reperfusion (**B**). Quantitative analysis revealed that transient ischemia in the vehicle-treated group significantly

increased RIP1 (**C**), RIP3 (**E**), Bax (**G**), and caspase-8 (**I**) expression ($n = 6$; RIP1 after 8 h, $*P = 0.001$; RIP1 after 1 day, $†P = 0.023$; RIP3 after 8 h, $‡P = 0.045$; RIP3 after 1 day, $§P = 0.023$; RIP3 after 2 days, $¶P = 0.018$; Bax after 8 h, $‖P = 0.042$; Bax after 1 day, $¶P = 0.035$; caspase-8 after 8 h, $#P = 0.047$) compared with that in the sham group (S). In the necrostatin-1 treated group, RIP1 (**D**) and RIP3 (**F**) expression did not increase. In contrast, Bax (**H**) and caspase-8 (**J**) expression increased 8 hr after reperfusion (Bax, $**P = 0.029$; caspase-8, $††P = 0.021$) compared with that in the S group. The bars above each staple represent the standard deviation.

Several necroptosis inhibitors have been used in clinical trials for the treatment of psoriasis, ulcerative colitis, neurodegenerative diseases, and some types of cancers.^{35,36} A phase 2 multicenter randomized placebo-controlled study for psoriasis patients reported that a necroptosis inhibitor improved the severity of plaque lesions compared with placebo without any severe drug-related adverse effects.³⁵ Further studies are expected to apply this drug to patients with SCI while preserving the efficacy and ensuring safety.

Recently, studies found that SCI following TEVAR occurs more frequently in delayed phases as compared to the open surgeries.³⁷ Previous studies suggested the etiologies of SCI after TEVAR varies, including reduced flow to the collateral network due to postoperative hypotension³⁸ and intercostal artery thrombosis following

stent grafts deployment.³⁹ We believe that such hemodynamic instability can generate ischemia-reperfusion of the spinal cord even in the postoperative phase, potentially increasing necroptosis of motor neurons in a more delayed phase. Therefore, we expect that Nec-1 will contribute to reduce delayed paraplegia as TEVAR continues to increase in the future.

This study has some limitations. First, we assessed caspase-8 expression to determine its influence on apoptosis, but caspase-8 is a protein related to both necroptosis and apoptosis. We need to consider the possibility of its regulation by necroptotic changes for a precise analysis. Second, the effective dose of Nec-1 remains unclear. The efficacy of a single 1 mg/kg Nec-1 dose was limited in this study. However, additional studies are necessary to determine the best therapeutic

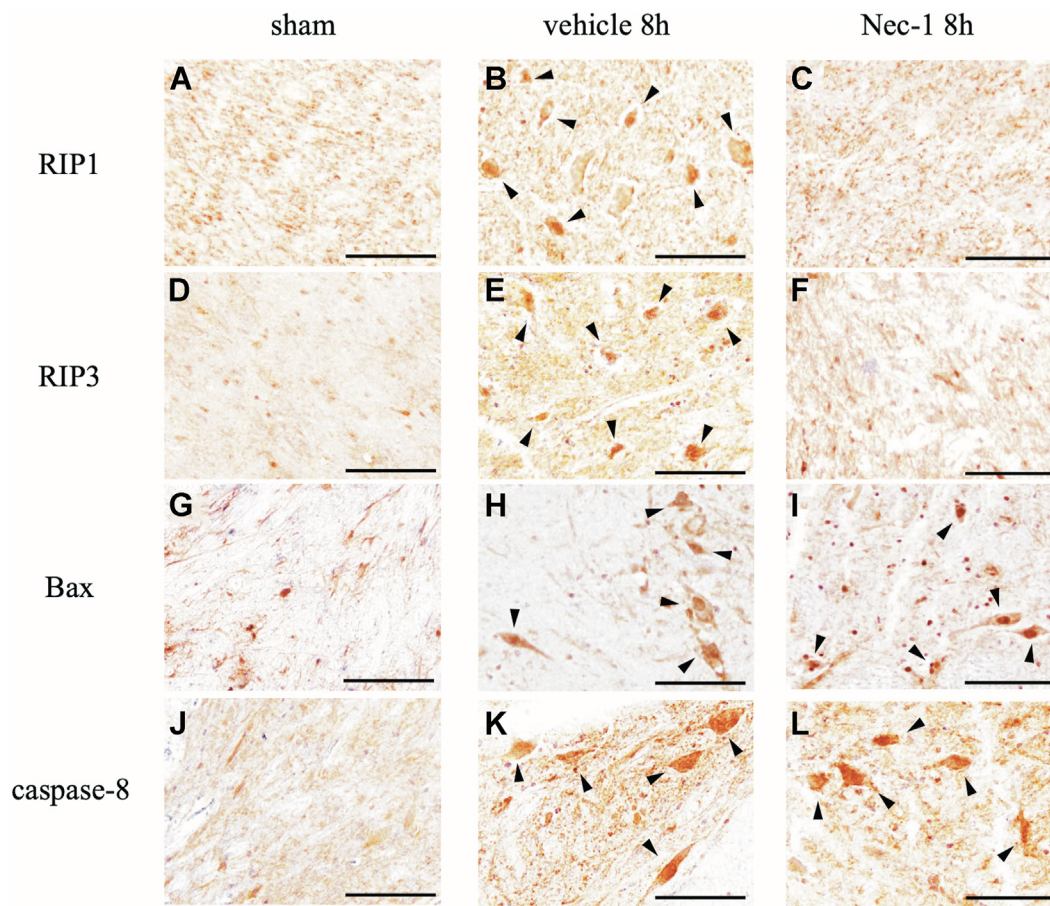


Fig. 5. Immunostaining of receptor-interacting protein kinase (RIP) 1, RIP3, Bax, and caspase-8 in the motor neurons of a sham spinal cord (RIP1, **A**; RIP3, **D**; Bax, **G**; caspase-8, **J**) and in the vehicle- (RIP1, **B**; RIP3, **E**; Bax, **H**; caspase-8, **K**) and necrostatin-1-treated groups

8 hr after reperfusion in rabbits (RIP1, **C**; RIP3, **F**; Bax, **I**; caspase-8, **L**). Motor neurons that express immunoreactive proteins are indicated using *arrows*. Scale bars = 100 μ m.

dose and side effects. Third, we cannot precisely compare the results of western blot analysis in the V and N groups because samples of each group are not run in the same gels; however, our main conclusions can be explained by comparisons of both groups with the sham group analyzed under the same conditions. Fourth, delayed paraplegia in this study occurred after progressive neurological dysfunction, whereas that in clinical cases usually occurs without such a worsening course. Although both pathologies can be explained by delayed motor neuron death, the difference should be verified in future studies. Lastly, SCI induced by our model is partially different from that in clinical cases. In TEVAR or graft replacement without intercostal artery reconstruction, reperfusion to the spinal cord cannot be obtained in the same way as in our model. In addition, TEVAR can

preserve distal perfusion, which guarantees intraoperative supplementary spinal cord perfusion from collateral networks. Further studies are expected to develop new models that more accurately reflect SCI following each clinical surgery.

CONCLUSIONS

This study demonstrated the efficacy of Nec-1 in reducing delayed motor neuron death and attenuating delayed paraplegia following transient spinal cord ischemia in rabbits. The upregulation of RIP1 and RIP3 immunoreactivity induced early after reperfusion in motor neurons was suppressed by Nec-1. At the same time, those of Bax and caspase-8 were preserved despite Nec-1 administration. Our results suggest that Nec-1 selectively inhibits necroptosis in

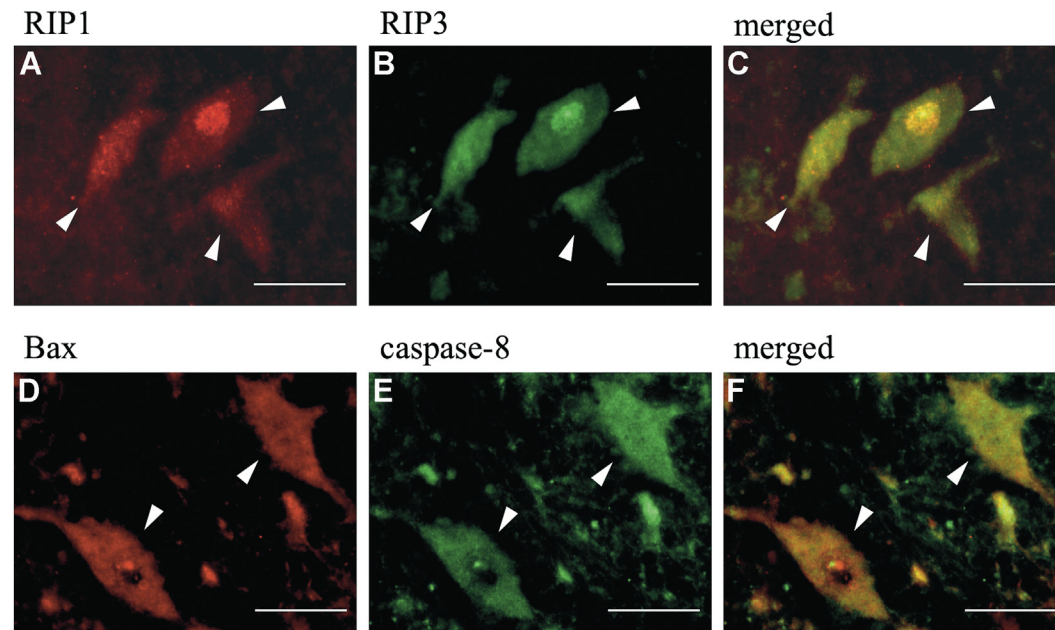


Fig. 6. Colocalization of receptor-interacting protein kinase (RIP) 1 with RIP3 (**A–C**), and that of Bax with caspase-8 in motor neurons 8 hr after transient ischemia in the vehicle-treated group (**D–F**). RIP1 was detected using red-orange fluorescent Alexa Fluor 555 (**A**), and RIP3 was detected using green fluorescent Alexa Fluor

488 (**B**). The merged image is presented in (**C**). Bax was detected using red-orange fluorescent Cruz Fluor 555 (**D**), and caspase-8 was detected using green fluorescent Cruz Fluor 488 (**E**). The merged image is presented in (**F**). The double-positive cells are yellow and indicated using arrows. Scale bar = 25 μ m.

motor neurons after transient spinal cord ischemia in rabbits with minimal effect on apoptosis.

AUTHOR CONTRIBUTIONS

T.N., S.F., and T.H. conceived the idea of the study. T.N., H.U., K.M., and H.M. performed the experiments and collected the data. S.F., T.U., M.K., G.S., Y.O., and H.S. contributed to the interpretation of the results. T.N. drafted the original manuscript. A.S. supervised the conduct of this study. All authors reviewed the manuscript draft and revised it critically for intellectual content. All authors approved the final version of the manuscript to be published.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.avsg.2023.05.011>.

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