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Endoplasmic Reticulum Stress is Involved in the Protective Effect of Sivelestat Sodium Hydrate (ONO-5046) in Spinal Cord Ischemia-Reperfusion Injury

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Background: Postoperative complications of thoracoabdominal aortic aneurysm include paraplegia due to impaired blood flow in the spinal cord. Sivelestat sodium hydrate (ONO-5046), a specific neutrophil elastase inhibitor, can prevent neuropathy after ischemia-reperfusion of the spinal cord; however, the underlying mechanism remains unclear. Here, we examined whether ONO-5046 elicits its protective effects in spinal cord ischemia by affecting endoplasmic reticulum (ER) stress.

Methods: Forty-five male Japanese white rabbits (weight 2.5-3.0 kg) were assigned to three groups: a sham control group (n = 5), and two other groups (n = 20, respectively; n = 5 each time point) that were subjected to spinal cord ischemia-reperfusion for 15 min and administered saline or ONO-5046 intravenously. From 8 h to 7 d after resumption of blood flow, a neurological evaluation, histological evaluation of the spinal cord, and immunohistochemical evaluation based on the expression of GRP78 and caspase12 were performed.

Results: Rabbits treated with ONO-5046 had fewer functional deficits and more surviving motor neurons after ischemia than did rabbits in the saline and control groups. In rabbits treated with ONO-5046, histological findings of the spinal cord showed a high number of viable motor nerves, whereas induction of GRP78, an ER stress response-related protein, was prolonged. Furthermore, caspase12 expression was activated by excessive ER stress and was downregulated in rabbits treated with ONO-5046, as compared with that in rabbits administered saline.

Conclusions: ONO-5046 exerts a protective effect on the spinal cord by relieving ER stress during spinal cord ischemia. (J Nippon Med Sch 2023; 90: 50–57)

Key words: sivelestat, endoplasmic reticulum stress, rabbits

Introduction

The incidence of ischemic spinal cord injuries such as paraplegia after surgical repair of thoracic and thoracoabdominal aortic aneurysms is 3%-23.5%[1,2]; the incidence of permanent paraplegia is 1.2%-4%[3,4]. Clinically, drainage of cerebrospinal fluid, maintenance of spinal artery pressure, mild hypothermia, and adequate supply of oxygen have been shown to be effective in alleviating paralysis[5,6], but the spinal cord-protective effect of drugs has not been demonstrated[7]. In contrast, animal studies have shown that sivelestat sodium hydrate (ONO-5046), a potent specific neutrophil elastase inhibitor used to treat acute lung injury, has a spinal cord-protective effect[8–10]. However, the underlying mechanism has not been elucidated.

In a study using a transient spinal cord ischemia model, 20 min of ischemia caused necrosis of the spinal cord, whereas 15 min of ischemia resulted in delayed motor neuron death in the anterior horn of the spinal cord[11]. Furthermore, so-called "selective motor neuron death" occurred, indicating that delayed motor neuron death is most likely due to apoptosis[12]. A 15-min tran-

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sient ischemia model showed immune reactivity of glucose regulated protein 78 (GRP78) and caspase12 at 8 h after ischemia, which returned to baseline levels 1 d later[13]. GRP78 and caspase12 are expressed under endoplasmic reticulum (ER) stress. GRP78 activates the unfolded protein response (UPR) pathway and is involved in normalizing ER function[14–16], and caspase12 induces apoptosis[17], indicating that ischemia-reperfusion causes ER stress. These results suggest that transient spinal cord ischemia causes ER stress in motor neurons and induces apoptosis.

Neutrophils play an important role in ischemia-reperfusion injury. Studies using a cerebral ischemia model have shown that neutrophils accumulate in the ischemic region and are important in central nervous system ischemia-reperfusion injury[18–21]. Neutrophil elastase released from neutrophils is a potent proteolytic enzyme, and excessive release of neutrophil elastase causes tissue damage[22,23].

ONO-5046 is a potent specific neutrophil elastase inhibitor and is used to treat acute lung injury associated with systemic inflammatory response syndrome. Because it is a pharmacological neutrophil elastase inhibitor, ONO-5046 is also effective as a therapeutic agent for inflammation caused by neutrophil elastase in organs other than the lungs[24–26]. The spinal cord-protective effect of ONO-5046 in ischemic spinal cord injury has also been reported, but there are only a few studies of its action mechanism[8–10].

ER has an important role in ischemia-reperfusion injury-induced apoptosis, and excessive ER stress causes apoptosis[27], but the effect of ONO on ER stress has not been investigated. Therefore, in this study, we used a rabbit model of transient spinal cord ischemia to examine whether ER stress is involved in the protective effect of ONO-5046 on motor nerve neurons against spinal cord ischemia.

Materials and Methods

The experimental and animal care protocols were approved by the Animal Care Committee of the National Hospital Organization Sendai Medical Center (permission number: 200802). N-{2-[4-(2,2-Dimethylpropionyloxy) phenylsulfonylamino]benzoyl}aminoacetate tetrahydrate (Sivelestat sodium hydrate: ONO-5046) was provided by Ono Pharmaceutical Company (Osaka, Japan).

Animal Preparation

Forty-five, 3-month-old male domesticated white rabbits weighing 2.5-3.0 kg (Kumagai-shigeyasu Company, Sendai, Japan) were used. All rabbits were allowed free access to food and water before and after the procedure and were treated in accordance with the National Research Council (US) Committee's Guide for the Care and Use of Laboratory Animals[28]. A transient spinal cord ischemia model was created using the method reported by Sakurai et al.[18] The rabbits were allocated to three groups: a sham-operated control group (Group S, n = 5), ischemic group (Group I, n = 20, n = 5 at each time point), and ONO-5046 treatment group (Group O, n = 20, n = 5 at each time point). On the basis of the results of previous studies, we predicted that the significance level was 5%, the power was 80%, the intergroup variance was 0.8, and the intragroup variance was 0.5; power analysis was performed to determine the number of rabbits required per group. To reduce the number of experimental animals, only five rabbits were used in the sham control group (Group S) without ischemic invasion (i.e., no change in findings was expected). Anesthesia in all rabbits was induced by intramuscular injection of ketamine (50 mg/kg) and was maintained by inhalation of 2% halothane and oxygen (AM200; Aika, Tokyo, Japan). Respiratory management was performed via spontaneous breathing. A 24 G catheter was placed in the left femoral artery to monitor blood pressure and heart rate, and rectal temperature was continuously measured. A 24 G catheter was placed in the posterior auricular vein for drug administration. A 5 French catheter scale arterial embolectomy catheter (E-080-5F; Edwards Lifesciences LLC, Irvine, CA, USA) was inserted through the right femoral artery, advanced 15 cm, and the tip of the catheter was placed in the abdominal aorta. With this procedure, the position of the balloon at the tip of the catheter was located 0.5-1.5 cm distal to the left renal artery. In Group S, the catheter was quickly removed without balloon dilation. In Groups I and O, the balloon was inflated until the arterial pressure waveform in the femoral artery disappeared, blocking blood flow to the spinal cord. After 15 min, the balloon was deflated and the artery reperfused. Our previous experiments confirmed that 15 min of transient spinal cord ischemia was sufficient for selective and delayed motor neuron death[12]. Saline (2 mL/kg/h, Group I) or ONO-5046 (5 mg/mL in saline; 10 mg/kg/h, Group O) was continuously intravenously infused from the induction of ischemia to 60 min after the end of ischemia. After 60 min of reperfusion, all catheters were removed, and the rabbits were awakened from anesthesia.

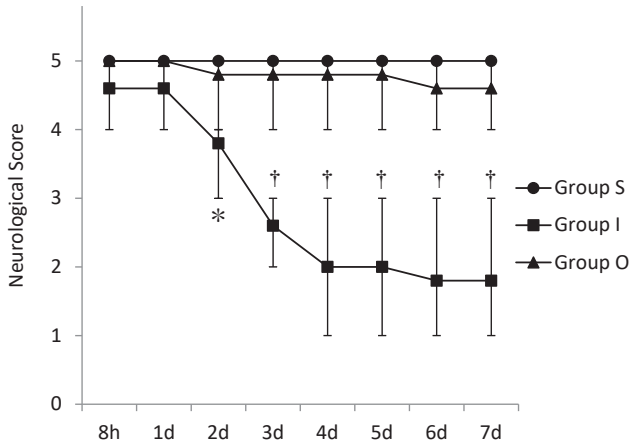

Fig. 1 Changes in neurological score in a 15-min spinal cord transient ischemia model of rabbits
In Group S, there was no decrease in the scores 7 days after ischemia. In Group I, the score dropped significantly from day 2 to day 4. In Group O, the score dropped slightly from day 2.
*$p < 0.05$ vs. Group O, †$p < 0.01$ vs. Group O. Data are expressed as median (maximum–minimum). Group S: sham-operated control group; Group I: ischemic group; Group O: sivelestat sodium hydrate (ONO-5046) treatment group

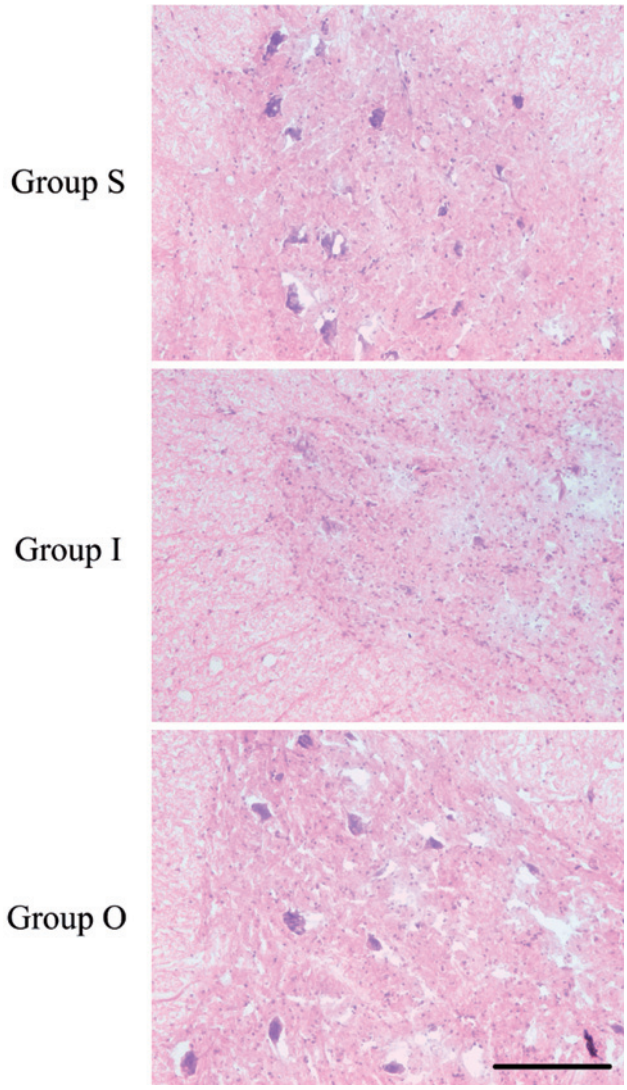

Fig. 2 Histological findings in ventral gray matter of the spinal cord 7 days after 15-min spinal cord transient ischemia in rabbits (hematoxylin & eosin staining)
Group S: Normal tissue structure; Group I: Most motor neurons were damaged; Group S: There were a few damaged motor neurons and no glial changes; scale bar = 200 μm; Group S: sham-operated control group; Group I: ischemic group; Group O: sivelestat sodium hydrate (ONO-5046) treatment group

Neurological Assessment

Neurological function was assessed at 8 h, or 1, 2, or 7 days after ischemia (n = 5 at each time point). Neurological assessment involved evaluation of lower limb function on a 6-point scale using a modified Tarlov score used by Johnson et al.[29]: 0 = hind limb paralysis; 1 = severe paraparesis; 2 = functional movement, no hop; 3 = ataxia, uncoordinated hop; 4 = minimal ataxia; 5 = normal function.

Histological Assessment

The animals were sacrificed by deep anesthesia with sodium pentobarbital (100 mg/kg intravenously) after neurological evaluation. Immediately after death, the spinal cord was removed quickly and carefully. All removed spinal cords were immediately frozen in powdered dry ice and stored in a freezer at −80°C.

To histologically evaluate the spinal cord, the spinal cord was cut laterally at the level of L2 or L3 to 20-μm-thick sections, which were mounted on glass slides. Hematoxylin and eosin staining was performed and observed using an optical microscope (Nikon Corp. Tokyo, Japan) to evaluate pathological changes after spinal cord ischemia. Uninjured normal motor neurons in the ventral gray matter were counted in five sections per animal. Ischemic motor neuron death was defined as the presence of cytoplasmic acidophilic staining and pyknosis with a lack of Nissl bodies.

Immunohistochemical Assessment

Immunohistochemical techniques were used to evaluate changes in the expression of GRP78 and caspase12 after reperfusion. First, spinal cord sections were rinsed with 0.1 M phosphate-buffered saline for 20 min and blocked with 2% normal horse serum for 2 h at 20 to 25°C. The sections were then incubated in 10% normal

Table 1 Summary of immunohistochemical analysis to evaluate the changes in the expression of GRP78 and caspase12 after reperfusion

Period after ischemia	8 h N = 5 for each	1 day N = 5 for each	2 days N = 5 for each
GRP78			
Group S	−−−−−		
Group I	+++++	±±±±±	−−−−−
Group O	±±−−−	±±±±±	±±±±−
Caspase12			
Group S	−−−−−		
Group I	+++++	±−−−−	−−−−−
Group O	±−−−−	±−−−−	−−−−−

+: strong staining; ±: weak staining; −: no staining; Group S: sham-operated control group; Group I: ischemic group; Group O: sivelestat sodium hydrate (ONO-5046) treatment group

horse serum or 10% normal rabbit serum for 20 h at 4°C, as needed. The primary antibodies used were goat polyclonal anti-GRP78 antibody (SC-1050; Santa Cruz Biotechnology, Inc., Paso Robles, CA, USA) and mouse monoclonal anti-caspase12 antibody (SC-21747; Santa Cruz Biotechnology, Inc.). The antibodies were used at a dilution of 1:200.

Subsequently, the sections were exposed to 0.3% H_2O_2 and 10% methanol for 20 min to quench the endogenous peroxidase activity and then washed again with phosphate-buffered saline. The sections were then incubated with biotinylated anti-goat IgG (PK-6105; Vector Laboratories, Burlingame, CA, USA) or biotinylated anti-mouse IgG (PK-6102; Vector Laboratories) for 3 h. The antibodies were diluted 1:200 with phosphate-buffered saline containing 0.018% normal horse or rabbit serum. The sections were then incubated with an avidin-biotin-horseradish peroxidase complex (PK-6102; Vector Laboratories). Immunostaining was performed using DAB/H_2O_2 solution, and the cytoplasm was counterstained with hematoxylin. To confirm specific antibody binding, a set of sections was stained in a similar manner without the primary antibody. Expression levels of GRP78 and caspase 12 were evaluated on a three-point scale of strong staining (+), weak staining (±), and no staining (−), based on a pathologist's comprehensive assessment of the staining intensity and number of positive cells. Histological and immunohistochemical assessments were performed by observers who were blinded to the laboratory animal population information and neurological outcomes.

Statistical Analysis

All statistical analyses were performed using R software (version 4.0.5; R Development Core Team). The Kruskal-Wallis test was used to compare neurological scores and cell numbers, and statistical significance was set at $p < 0.05$. Nonparametric data are presented as median (maximum-minimum).

Results

Neurological Assessment

All rabbits survived until the end of the observation period. In Group S, all rabbits showed normal neural function (score 5) throughout the observation period. In Group I, lower limb dysfunction worsened from 2 to 4 d after ischemia. The score on day 4 and thereafter was stable at -2, and the score on day 7 was 1.8 (3-1) (median [maximum-minimum]). In Group O, individuals with a score of 4 and mild dysfunction were observed from day 2 after ischemia, but no individual had a score of less than 4, and the score on day 7 after ischemia was 4.6 (5-4) (median [maximum-minimum]), which was significantly higher than the value in Group I ($p < 0.01$) (**Fig. 1**).

Histological Assessment

Figure 2 shows typical histological findings 7 d after ischemia. Normal tissue was observed in Group S, and motor neurons with darkly stained nuclei and Nissl bodies were observed. In Group I, gray matter vacuolization was observed, nuclei were shed, and a mixture of ischemic and normal motor neurons with deeply stained cytoplasm was observed. In Group O, a few ischemic motor neurons were observed; however, in Group S, many normal motor neurons were observed. The number of normal motor neurons was lower in Group I than in the other groups (Group S: 35 [64-10]; Group I: 6 [13-1], $p < 0.01$; Group S and O, Group O: 27 [47-5]; all data are

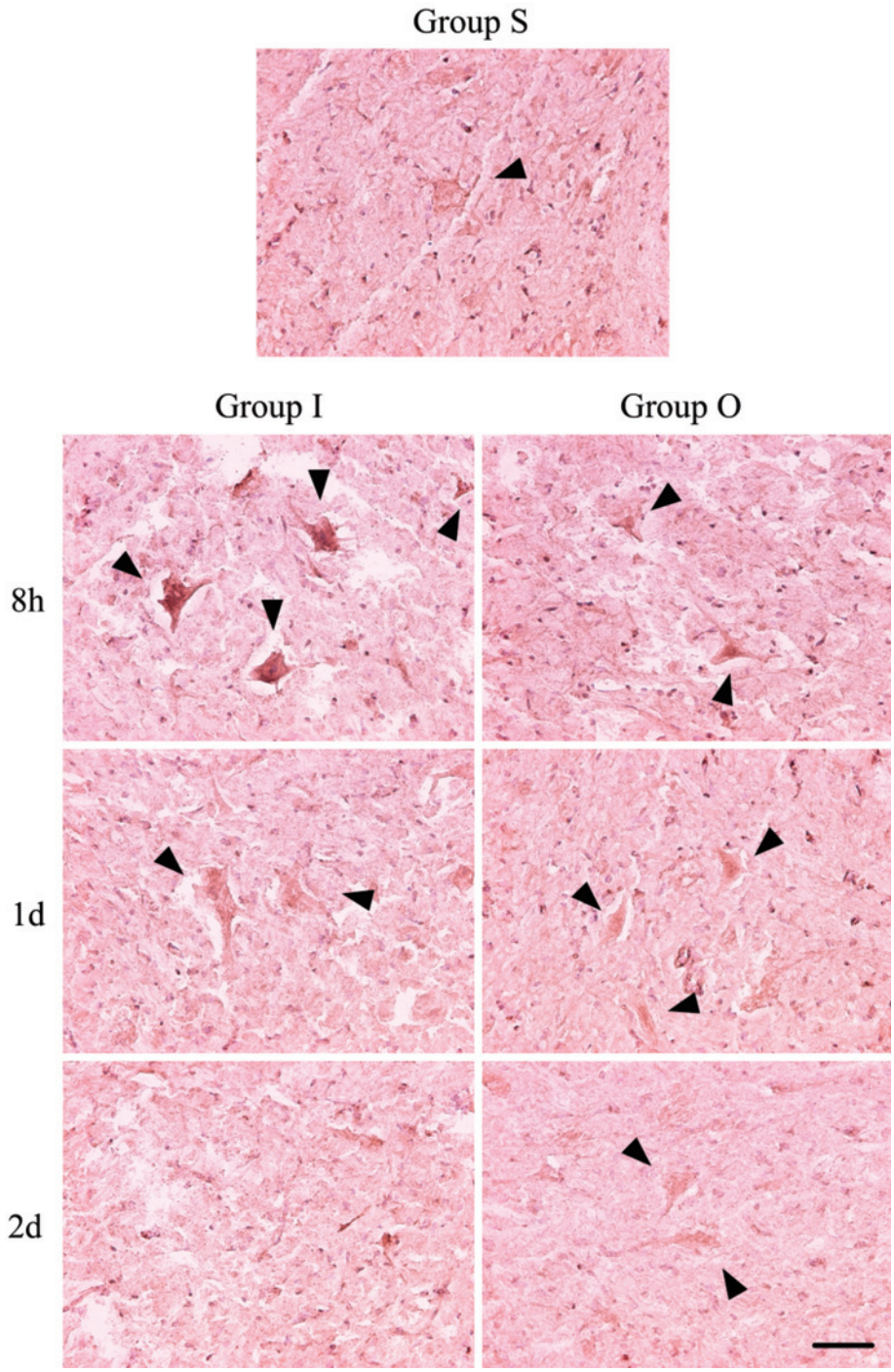

Fig. 3 Expression of GRP78 in spinal motor neurons up to 2 days after 15-min spinal cord transient ischemia in rabbits
Group S: GRP78 was weakly expressed in undamaged tissue; Group I: GRP78 was strongly expressed 8 h after ischemia, but expression decreased rapidly, and GRP78 was not expressed 2 days after ischemia; Group O: GRP78 was weakly expressed at 8 h after ischemia, and expression continued until 2 days later. Black arrowheads indicate expression of GRP78; scale bar = 50 μm; Group S: sham-operated control group; Group I: ischemic group; Group O: sivelestat sodium hydrate (ONO-5046) treatment group

expressed as median [maximum-minimum]).

Immunohistochemical Assessment

Table 1 shows the results of the immunohistochemical analysis of each group. A typical tissue image of GRP78 and caspase12 expression is shown in **Figures 3, 4**. In Group S, no induction of GRP78 or caspase12 expression

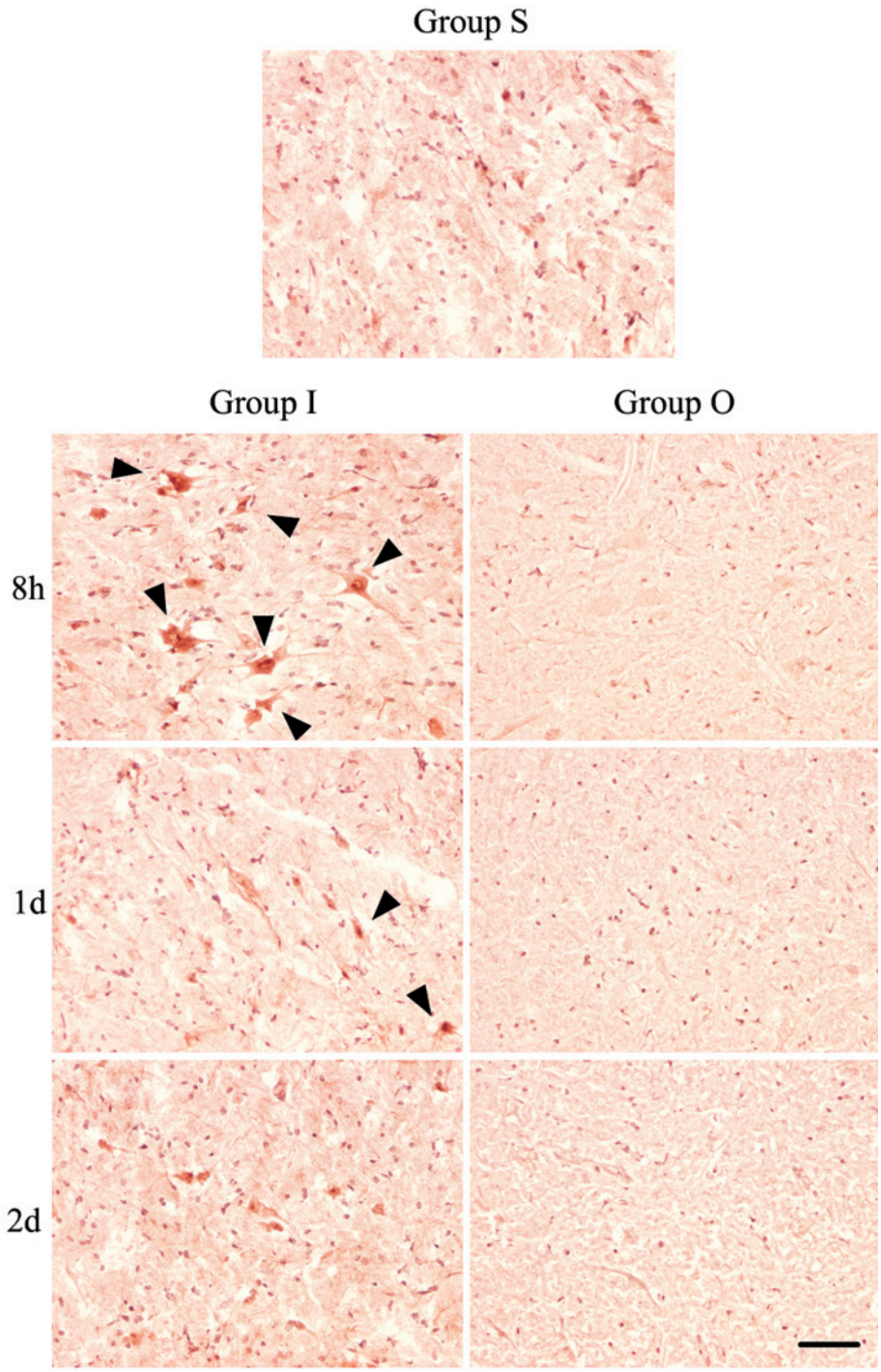

Fig. 4 Expression of caspase12 in spinal motor neurons up to 2 days after 15-min spinal cord transient ischemia in rabbits
Group S, Group O: No expression of caspase12 was observed; Group I: Caspase12 was expressed 8 h after ischemia but was not expressed 2 days later. Black arrowheads indicate expression of caspase12; scale bar = 50 μm; Group S: sham-operated control group; Group I: ischemic group; Group O: sivelestat sodium hydrate (ONO-5046) treatment group

was observed. In Group I, strong expression of GRP78 and caspase12 was observed in motor neurons 8 h after reperfusion, which had almost disappeared after 1 d; induction of GRP78 and caspase12 was not observed after day 2. In Group O, GRP78 expression started slowly 8 h after reperfusion and was maintained until day 2. Intensity of GRP78 expression was weaker than in Group I. Caspase12 expression was weak in only one rabbit.

Discussion

This study examined whether the spinal cord-protective effect of sivelestat was associated with ER stress. In a 15-min spinal cord transient ischemia model of rabbits, continuous administration of ONO-5046 from the time of spinal cord ischemia to 60 min after reperfusion resulted in improvement in neurological scores and histological findings after reperfusion, as compared with findings for the ischemic group. The improvement was maintained even after 7 d of reperfusion. In addition, immunohistochemical analysis showed prolonged induction of GRP78 and suppression of caspase12 expression.

Sakurai et al.[12,13] reported that a 15-min transient spinal cord ischemia model expressed GRP78, an ER stress-induced molecule chaperone, and caspase12, an ER stress-induced apoptosis-related caspase. As a result, delayed motor neuronal cell death occurred. Similarly, in the present ischemic group, expression of GRP78 and caspase12, as well as motor neuron death, were observed. Administration of ONO-5046 during spinal cord ischemia-reperfusion prolonged GRP78 expression and suppressed caspase12 expression. These results suggest that ONO-50456 suppressed ER stress in spinal cord ischemia. In the ischemic group, the ER stress response was caused by ischemic stress, but the excessive UPR triggered activation of the stress sensor and caspase12-induced apoptosis. In the treated group, it is plausible that ONO-5046 reduced ER stress, resulting in a normal ER stress response. GRP78 expression was mild, and its expression duration was prolonged; caspase12 expression was suppressed, and consequently, damaged motor neurons could recover. To our knowledge, no study has demonstrated that sivelestat directly suppresses ER stress.

On the contrary, studies have shown that neutrophils accumulate in the ischemic area during spinal ischemia[30] and that sivelestat prevents diseases caused by neutrophil elastase[31–33]. In the current study, we hypothesized that ONO-5046 inhibited elastase released from neutrophils accumulating in the ischemic region of the spinal cord, resulting in less damage to motor neurons. Administration of ONO-5046 for ischemic spinal cord injury inhibited neutrophil elastase activity released to the ischemic region and reduced cell damage, that is, ER stress. Thus, it is considered that GRP78 expression is prolonged and the ER stress response is activated for survival, thereby inhibiting the expression of lethal caspase 12.

Ischemic motor neurons were observed histologically, even though the results of a neurological evaluation were normal in Group O. ONO-5046 has a neuroprotective effect in transient spinal cord ischemia; however, it was unable to completely suppress delayed cell death. It is unclear whether this is a problem with the method of administering ONO-5046 or a limitation in drug efficacy. Further research is needed to determine the dose and method of ONO-5046 administration and identify the part of the ER stress response that is affected.

The current study has some limitations. First, ONO-5046 was continuously administered at 10 mg/kg/h from the time of ischemia to 60 min after reperfusion, but it is unclear, because of species differences, whether this method of administration yields results than can be extrapolated to humans. Second, the observation period for each group may not have been long enough to assess the delayed death of motor neurons. Third, a precise electrophysiological evaluation of motor function of the hind limbs was not performed; thus, a minute or potential motor dysfunction may have been overlooked. Finally, evaluation of GRP78 and caspase12 expression levels has not been quantified. Quantification could provide a more accurate assessment.

In conclusion, ONO-5046 prolonged GRP78 expression, suppressed apoptosis-promoting caspase12 expression, and protected motor neurons from ischemic spinal cord injury. These findings suggest that administration of ONO-5046 relieves ER stress during spinal cord ischemia and exerts a spinal cord-protective effect. Future studies should examine methods of administration, such as drug dose and time, and the long-term neuroprotective effects of ONO-5046.

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Conflict of Interest: This study was facilitated by ONO Pharmaceutical Company (Osaka, Japan), which was responsible for bulk provision of ONO-5046.

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