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Research report

Free radical scavenger edaravone produces robust neuroprotection in a rat model of spinal cord injury

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ABSTRACT

We used a multimodal approach to evaluate the effects of edaravone in a rat model of spinal cord injury (SCI). SCI was induced by extradural compression of thoracic spinal cord. In experiment 1, 30 min prior to compression, rats received a 3 mg/kg intravenous bolus of edaravone followed by a maintenance infusion of 1 (low-dose), 3 (moderate-dose), or 10 (high-dose) mg/kg/h edaravone. Although both moderate- and high-dose edaravone regimens promoted recovery of spinal motor-evoked potentials (MEPs) at 2 h post-SCI, the effect of the moderate dose was more pronounced. In experiment 2, moderate-dose edaravone was administered 30 min prior to compression, at the start of compression, or 10 min after decompression. Although both preemptive and coincident administration resulted in significantly improved spinal MEPs at 2 h post-SCI, the effect of preemptive administration was more pronounced. A moderate dose of edaravone resulted in significant attenuation of lipid peroxidation, as evidenced by lower concentrations of the free radical malonyldialdehyde in the spinal cord 3 h post-SCI. Malonyldialdehyde levels in the high-dose edaravone group were not reduced. Both moderate- and high-dose edaravone resulted in significant functional improvements, evidenced by better Basso-Beattie-Bresnahan (BBB) scores and better performance on an inclined plane during an 8 week period post-SCI. Both moderate- and high-dose edaravone significantly attenuated neuronal loss in the spinal cord at 8 weeks post-SCI, as evidenced by quantitative immunohistochemical analysis of NeuN-positive cells. In conclusion, early administration of a moderate dose of edaravone minimized the negative consequences of SCI and facilitated functional recovery.

1. Introduction

Spinal cord injury (SCI) can lead to severe neurological sequelae. SCI pathophysiology is described as biphasic, consisting of primary and secondary damage. Primary damage involves initial mechanical injury that directly disrupts the spinal cord, which is followed by secondary damage that involves vascular dysfunction, edema, ischemia, free radical production, inflammation, and delayed apoptotic cell death (Rowland et al., 2008). Primary and secondary damage mechanisms also activate inflammatory cascades and result in glial reactivity associated with a robust immune response to damage (Dietrich, 2015; Mortazavi et al., 2015).

Free radicals produced immediately after SCI cause cell membrane injury via peroxidation of unsaturated fatty acids in phospholipids (lipid peroxidation), resulting in further damage. The post-SCI stages can be categorized into multiple contiguous phases: immediate (0–2 h), early acute (2–48 h), subacute (2–14 d), intermediate (14 d–6 mo), and chronic (>6 mo) (Rowland et al., 2008; Witiw and Fehlings, 2015). The immediate and early acute

Abbreviations: ANOVA, analysis of variance; BBB, Basso-Beattie-Bresnahan; ChAT, choline acetyltransferase; DAPI, 4′,6-diamidino-2-phenylindole; IL, interleukin; MDA, malonyldialdehyde; MEP, motor-evoked potential; MMP, matrix metalloproteinase; MPS, methylprednisolone; NeuN, neuronal nuclear antigen; SCI, spinal cord injury; TNF, tumor necrosis factor.

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phases are characterized by increased free radical production (Siddiqui et al., 2015).

Previous studies have focused on the neuroprotective effects of various agents that scavenge free radicals that induce secondary injury. Large-dose methylprednisolone (MPS) is the only current clinical treatment that has been used in humans following SCI and the results are contradictory (Bains and Hall, 2012; Evaniew et al., 2016; Hall, 2011). Besides the potent anti-inflammatory effect (Constantini and Young, 1994), the mechanisms of neuroprotection by MPS may include reduced lipid peroxidation and inhibition of free radical action (Anderson et al., 1985; Koc et al., 1999). However, a single large MPS dose was reported to have no beneficial effects during the acute SCI phase and also led to an increased risk of infection and gastrointestinal bleeding (Evaniew et al., 2016; Ito et al., 2009; Suberviola et al., 2008).

Edaravone is a potent free radical scavenger that has been clinically used to reduce neuronal damage induced by acute cerebral infarction (Edaravone Acute Infarction Study, 2003). Edaravone provides neuroprotection in a cerebral ischemia model by inhibiting lipid peroxidation (Watanabe et al., 1994), which has been reported to begin within 5 min after injury (Vagnozzi et al., 1999). Edaravone also exerts cerebral neuroprotection and improves neurological deficits in SCI models (Aoyama et al., 2008; Ohta et al., 2005).

However, it remains unclear whether therapeutic strategies using edaravone could effectively prevent and/or treat SCI sequelae. Therefore, we hypothesized that edaravone administration, before or as early as possible after SCI, might effectively inhibit the lipid peroxidation that occurs immediately after SCI. We also hypothesized that different edaravone administration doses might exert different effects on recovery following SCI. Accordingly, we performed electrophysiological analyses to determine whether edaravone acutely reverses spinal cord conduction failure. We also performed biochemical and behavioral analyses to determine whether different doses and commencement times of edaravone administration affect the degree of improvement during the acute and intermediate post-SCI phases.

2. Results

Three rats were excluded from analysis due to severe hypovolemic shock resulting from major hemorrhage after cannulation failure, four rats were excluded due to unmeasurable spinal motor-evoked potentials (MEPs) before compression, and one rat was excluded due to a failure to excise the spinal cord specimen. Data from 49 rats used in the electrophysiological and biochemical experiments, 28 rats used in behavioral experiments, and 19 rats used in immunohistochemical experiments were analyzed.

2.1. Electrophysiological analysis: the effect of edaravone on MEP recovery during the acute phase of SCI

Cervical MEPs at 2 h after compression showed no amplitude changes compared with baseline (Fig. 1). However, lumbar MEP amplitudes were completely abolished during compression. As shown in Fig. 2 and Fig. 3, different administration doses and time regimens resulted in variable degrees of improvement in signal conduction. The results of experiment 1, which compared the effects of three edaravone dosing regimens, showed that amplitude recovery at 2 h post-SCI was variable (Fig. 2A). The relative amplitudes in the sham group were significantly greater than in the saline group (98.1 ± 2.2 vs. 0.02 ± 0.04, respectively; $P < .0001$) (Fig. 2B). The low-dose edaravone group showed no appreciable MEP recovery (relative amplitude 0.22 ± 0.18, $P = .0512$, vs. saline group). The moderate-dose edaravone group (relative amplitude 0.59 ± 0.25) showed significantly improved conduction compared with the saline group ($P < .0001$), and this improvement was more pronounced than in the high-dose edaravone group (relative amplitude 0.31 ± 0.12, $P < .01$ vs. saline group).

Results from experiment 2, which compared the effects of three different commencement times for moderate-dose edaravone administration, showed that changing the commencement time also affected amplitude recovery at 2 h post-SCI (Fig. 3A). As shown in Fig. 3B, edaravone administration at 30 min prior to compression resulted in significantly improved MEP amplitude compared with the saline group (relative amplitudes 0.59 ± 0.43 vs. 0.02 ± 0.04, respectively; $P < .0001$). Starting edaravone administration simultaneously with compression also resulted in a significantly greater relative MEP amplitude compared with the saline group (0.23 ± 0.18, $P < .05$), but this beneficial effect was less pronounced than with preemptive administration. However, commencement of edaravone administration at 10 min after decompression did not result in an improved MEP amplitude (relative amplitude 0.05 ± 0.06, $P = .9819$ vs. saline group).

2.2. Biochemical analysis: the effect of edaravone on lipid peroxidation during the acute phase of SCI

As shown in Fig. 4A, there was no significant difference in plasma malonyldialdehyde (MDA) levels between the sham and saline groups (0.65 ± 0.44 μmol/l and 0.52 ± 0.38 μmol/l, respectively; $P = .9423$), suggesting that SCI did not appreciably affect blood MDA levels. The moderate edaravone dose resulted in lower plasma MDA levels (0.23 ± 0.07 μmol/l) than the saline group, although the difference was not statistically significant ($P = .5441$). The low-dose edaravone resulted in slightly greater plasma MDA levels (0.60 ± 0.21 μmol/l) than the saline group, although the difference was not statistically significant ($P = .9897$). Plasma MDA levels in the high-dose edaravone group were significantly greater than in the saline group (1.15 ± 0.68 μmol/l; $P < .05$).

As shown in Fig. 4B, spinal cord MDA levels in the saline group were significantly greater than in the sham group (1.04 ± 0.18 nmol/mg protein vs. 0.66 ± 0.15 nmol/mg protein, respectively; $P < .01$). This confirmed that SCI causes increased free radical production in spinal cord tissue. However, moderate-dose edaravone resulted in significantly lower spinal cord MDA levels (0.43 ± 0.16 nmol/mg protein; $P < .0001$) compared with the saline group. The ability of moderate-dose edaravone to decrease free radical production in spinal tissue suggested it could be used effectively to treat SCI. Additionally, spinal cord MDA levels in the low-dose (0.95 ± 0.13 nmol/mg protein) and high-dose (1.14 ± 0.26 nmol/mg protein) edaravone groups were not significantly different from the saline group ($P = .8661$ and $P = .7714$, respectively). Of note, high-dose edaravone resulted in significantly greater spinal cord MDA levels compared with the sham group ($P < .001$). There was also a slight, but significant, increase in spinal cord MDA levels in the low-dose edaravone group (0.95 ± 0.13 nmol/mg protein) compared with the sham group ($P < .05$, vs. sham group).

2.3. Behavioral analysis: the effect of edaravone on motor function recovery following SCI

Fig. 5A shows results from open-field locomotion assessment using Basso-Beattie-Bresnahan (BBB) scores from 1 d post-SCI to 8 wk post-SCI. The sham group showed preservation of motor function (BBB score of 21) throughout the entire period. Mean BBB scores in the moderate-dose edaravone group were significantly greater than in the saline group (5.9 ± 2.3 versus 0.3 ± 0.5, respectively; $P < .0001$) at 1 d post-SCI, although rats in the high-dose edaravone group did not exhibit similar improvement at 1 d post-SCI (4.1 ± 0.9; $P = .1428$ vs. saline group). Improvement in this

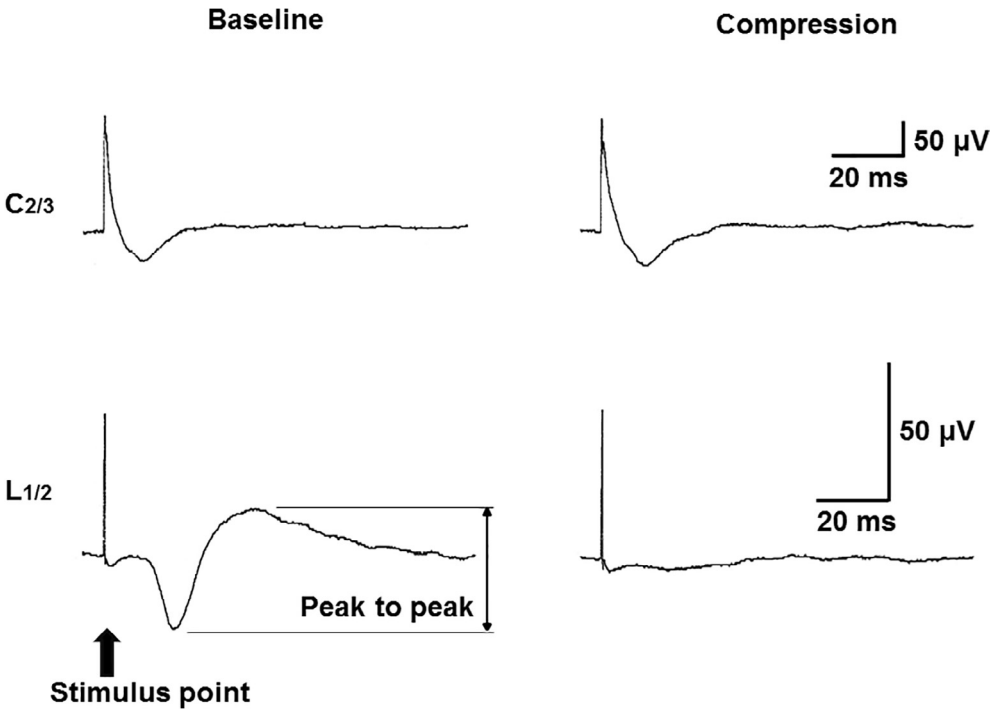

Fig. 1. Representative motor-evoked potentials (MEPs) recorded at cervical and lumbar levels at different time points relative to SCI. Peak-to-peak MEP amplitudes were measured at each recording. The stimulus point is indicated by an arrow.

group was first observed one week later. BBB scores of 2–6 describe rats that cannot support weight and with slight or extensive movement of some hindlimb joints (Basso et al., 1995). Mean BBB scores for moderate-dose (16.0 ± 2.2) and high-dose (15.3 ± 1.1) edaravone groups were significantly greater than for the saline group (6.3 ± 2.9) throughout the remaining observation period, as well as at the end of the 8 wk period ($P < .01$ for both comparisons). BBB scores of 15–16 represent walking with progressive improve ments in foot placement and toe clearance (Basso et al., 1995).

Fig. 5B shows results of rat performance assessment in the inclined plane test from 1 d post-SCI to 8 wk post-SCI. Improvements observed in the inclined plane test were similar to the BBB score results. The moderate-dose edaravone resulted in significant improvement compared with the saline group at 1 d post-SCI (61.2° ± 2.0° vs. 52.2° ± 2.9°, respectively; $P < .001$), although high-dose edaravone resulted in no significant change at 1 d post-SCI (58.1° ± 1.7°, $P = .5676$). The moderate-dose (71.5° ± 2.8°) and high-dose (71.3° ± 2.4°) edaravone resulted in significantly improved inclination angles compared with the saline group (61. 4° ± 4.5°) throughout the remaining observation period, as well as at the end of the 8 wk observation period ($P < .01$, $P < .001$ and $P < .0001$, respectively). Thus, results from the two behavioral tests indicated that edaravone administration might result in motor function recovery after SCI.

2.4. Immunohistochemical analysis: the effect of edaravone on neuronal cell loss following SCI

Neurons in the ventral horn were identified by the presence of neuronal nuclear antigen (NeuN), a neuron-specific marker. A severe loss of neurons occurred at the epicenter (Fig. 6A) and extended rostrally and caudally. Large cavities were observed in the saline group. Greatest neuronal cell loss was observed at the epicenter of the ventral horn in the saline group (15.20 ± 3.11 neurons compared to 105.0 ± 11.3 in the sham group, $P < .0001$). Neuronal cell loss was significantly attenuated in the moderate-dose (49.40 ± 9. 45 neurons; $P < .0001$ vs. saline group) and high-dose (44.60 ± 8.2 6 neurons; $P < .001$ vs. saline group) edaravone groups (Fig. 6C). The cell counts in the rostral and caudal regions showed a pattern similar to that of the epicenter. In the rostral region, the neuronal counts in the moderate-dose (54.20 ± 10.71 neurons; $P < .001$) and high-dose (56.00 ± 11.02 neurons; $P < .01$) edaravone groups were significantly greater than in the saline group (22.60 ± 5.90 neurons) (Fig. 6B). Furthermore, in the caudal region, the neuronal counts in the moderate-dose (60.60 ± 11.80 neurons; $P < .001$) and high-dose (49.20 ± 9.12 neurons; $P < .01$) edaravone groups were significantly greater than in the saline group (25.80 ± 6.10 neurons) (Fig. 6D). Thus, the analysis of NeuN immunoreactivity revealed a significant neuroprotective effect of edaravone in rat SCI.

3. Discussion

3.1. Effect of edaravone on electrophysiological recovery immediately post-SCI

Conventionally, the BBB locomotor rating scale is used to evaluate motor function recovery several weeks after SCI (Basso et al., 1995; Ohta et al., 2011). However, there is currently no method to directly evaluate the therapeutic effect of an experimental drug on motor recovery immediately after SCI as animals are administered anesthesia during this phase. The BBB test is not suitable for anesthetized rats because it is used to evaluate locomotor performance in awake and spontaneously walking animals. However, electrophysiological analysis can be utilized to investigate the neural substrates underlying functional deficits and evaluate functional recovery in anesthetized rats immediately following spinal insult (Onifer et al., 2007). In fact, electrophysiological analysis of spinal MEPs may be a more sensitive test for revealing subtle changes than behavioral analysis, especially during the immediate phase or under general anesthesia.

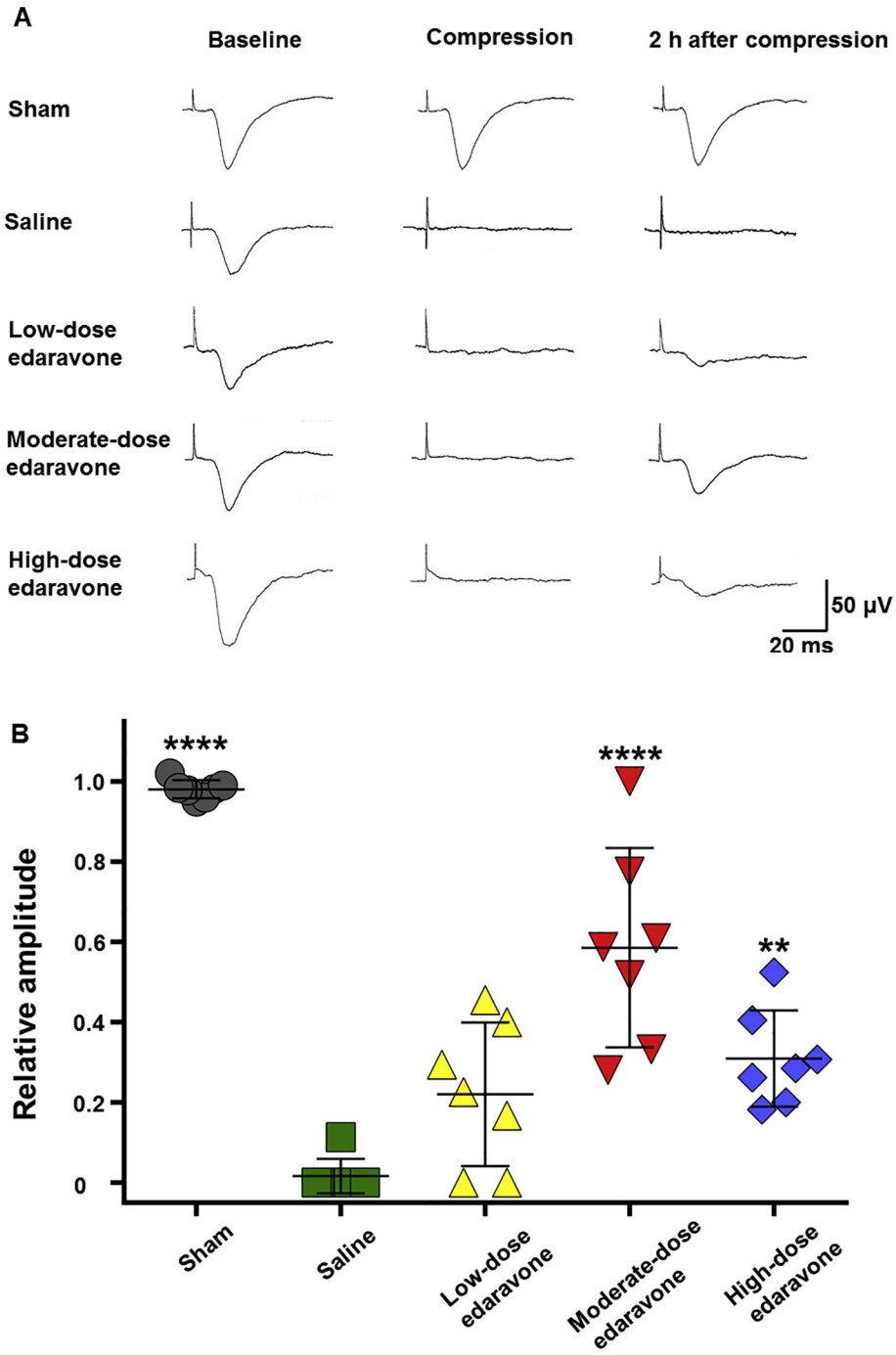

Fig. 2. Determination of the optimal edaravone dose during electrophysiological recording of spinal motor-evoked potentials (MEPs). (A) Representative spinal motor-evoked potentials (MEPs). Infusion rates for low-, moderate-, and high-dose edaravone were 1, 3, and 10 mg/kg/h, respectively. All infusions were started after a 3 mg/kg intravenous bolus of edaravone administered 30 min before spinal cord injury (SCI). (B) Graphical representation of relative amplitudes measured at 2 h in each experimental group. Data are presented as mean ± SD; $n = 7$ per group; $^{**}P < .01$, $^{****}P < .0001$ compared with the saline group; Dunnett's test following one-way ANOVA.

Here, we tested three different edaravone doses and hypothesized that the highest dose would exert the greatest therapeutic effect. However, improved electrophysiological function was not observed after administration of the edaravone dose that exceeded the moderate dose range. In fact, as our results show, moderate-dose edaravone (3 mg/kg/h) was the optimal dose for SCI treatment. We also determined the optimal therapeutic time window for the moderate edaravone dose, and results showed that preemptive administration (30 min prior to SCI) or simultaneous administration (at the start of SCI) were both superior to the saline control. However, edaravone administration shortly after SCI (10 min after decompression) did not result in significant improvement. These results suggest that moderate edaravone doses should be administered as soon as possible after spinal insult to restore axonal conduction. The timing of neuroprotective treatment would influence the dose-response for edaravone. Lipid peroxidation increases significantly within the first 5 min after SCI (Carrico et al., 2009; Vagnozzi et al., 1999; Xiong and Hall, 2009). Withholding edaravone until after injury would shift the neuroprotective dose-response curve to the right. Accordingly, edaravone adminis-

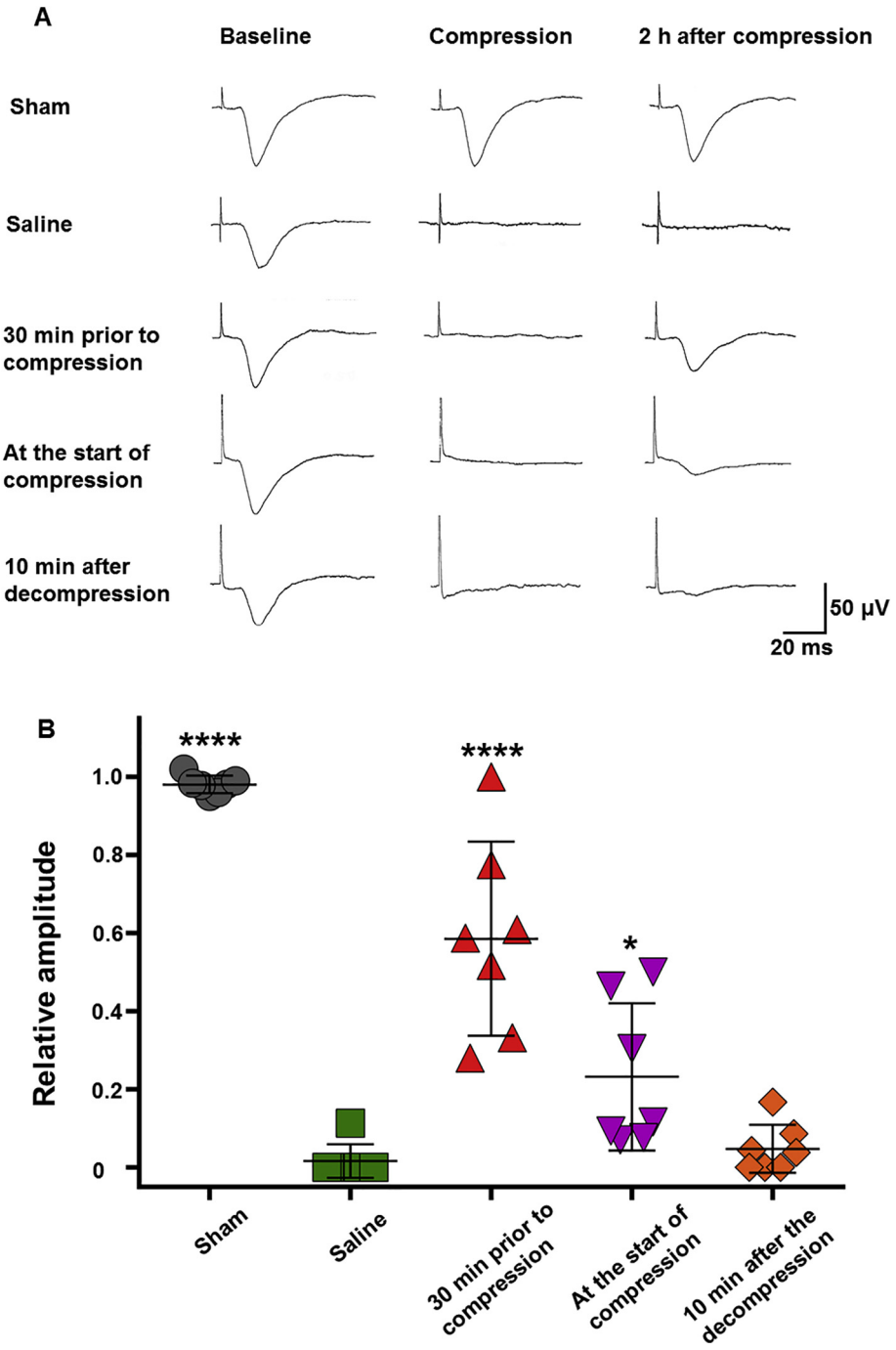

Fig. 3. Determination of the optimal commencement time of moderate-dose edaravone during electrophysiological recording of spinal motor-evoked potentials (MEPs). (A) Representative spinal motor-evoked potentials (MEPs) of moderate-dose edaravone administered at three different time points relative to spinal cord injury (SCI): 30 min before compression, at the start of compression, and 10 min after decompression. (B) Graphical representation of relative amplitudes measured 2 h following moderate-dose edaravone administered at three different time points relative to compression. Data are presented as mean ± SD; $n = 7$ per group; $^{*}P < .05$, $^{****}P < .0001$ compared with the saline group; Dunnett's test following one-way ANOVA.

tration after SCI would require a higher dose of edaravone to inhibit lipid peroxidation compared with when it is given before SCI. Further studies are required to elucidate the relationship between the timing of edaravone administration after injury and the optimum dose. In addition, lipid peroxidation in rat SCI models does not peak until at least 24 h after SCI and remains elevated for as much as 1–2 wks (Carrico et al., 2009; Vagnozzi et al., 1999; Xiong and Hall, 2009). Hence, although we administered maintenance infusion for only 2 h, longer administration duration, as well as a higher dose, might have a better effect on electrophysiological recovery. However, it is technically difficult to keep the electrodes in the same position for 1–2 wks and we could not experiment with long-term administration.

In a clinical setting, prophylactic and preemptive edaravone could be used in high-risk surgery cases, which can lead to iatrogenic SCI, such as surgery to correct scoliosis or a thoracoabdominal aortic aneurysms graft replacement (Denda et al., 2006; Ishii et al., 2015). To this end, intraoperative monitoring of MEPs is useful to detect early spinal cord dysfunction (Tanaka et al., 2016), allowing the clinician to provide earlier treatment. Although it is

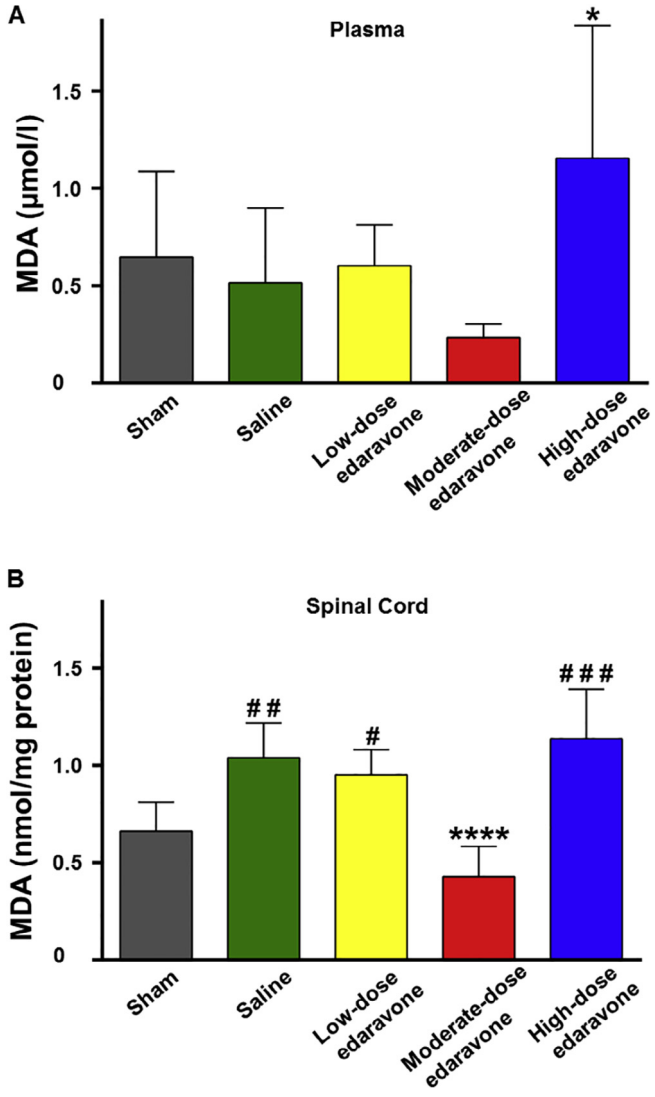

Fig. 4. Effect of edaravone administration on malonyldialdehyde (MDA) concentrations measured in the plasma (A) and spinal cord tissue (B) 3 h after spinal cord injury (SCI). Data are presented as mean ± SD; $n = 5–7$ per group; $^{*}P < .05$, $^{****}P < .0001$ compared with the saline group, $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ compared with the sham group; Dunnett's test following one-way ANOVA.

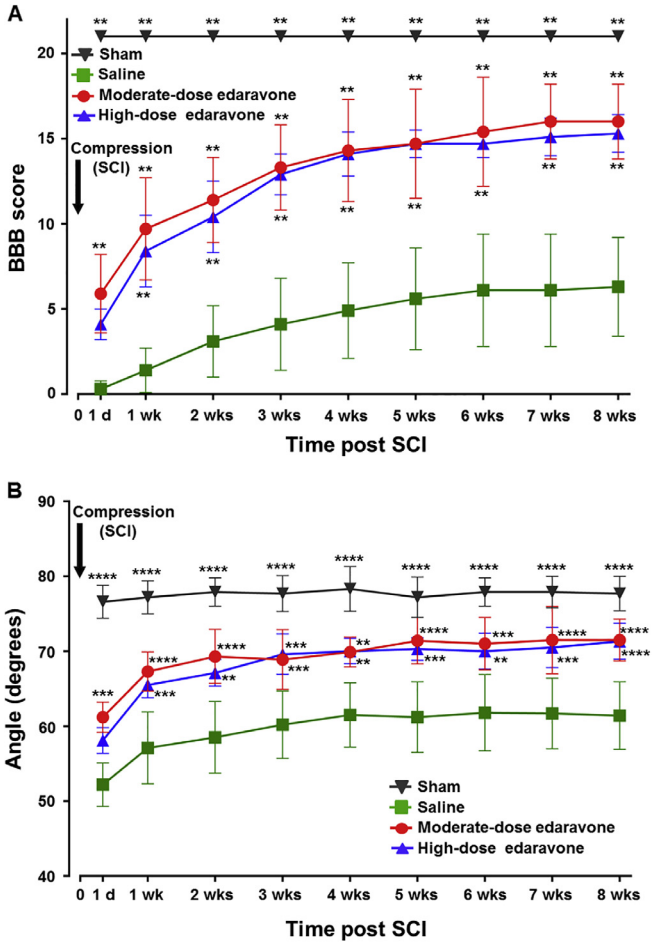

Fig. 5. The effect of edaravone administration on the recovery of motor function evaluated during 1 d–8 wk period following spinal cord injury (SCI) by (A) the open-field locomotion test with Basso-Beattie-Bresnahan (BBB) score rating and (B) the inclined plane test. Data are presented as mean ± SD; $n = 7$ per group; $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$ compared with saline; Bonferroni's test following two-way ANOVA.

impossible to preventively treat traumatic SCI, higher dose edaravone to inhibit lipid peroxidation after injury might produce a better recovery effect.

3.2. Effect of edaravone on lipid peroxidation immediately post-SCI

Free radicals play an important role in secondary damage during the immediate and early acute phase of SCI. Free radicals induce lipid peroxidation, which leads to apoptosis. As described above, lipid peroxidation begins within 5 min after injury and progressively increases and persists for 24–48 h after injury (Carrico et al., 2009; Vagnozzi et al., 1999; Xiong and Hall, 2009). MDA is an indicator of lipid peroxidation, and an increase in MDA levels in the spinal cord can be detected immediately after SCI. Peak MDA values persist for several hours and gradually decrease to control levels around 1 wk post-SCI (Ohta et al., 2011). Therefore, we investigated MDA levels in the blood and spinal cord tissue at 3 h post-SCI. In our study, plasma MDA levels in the moderate-dose edaravone group were lower than in the saline group, but the difference between the groups was not significant. Conversely, high-dose edaravone resulted in significantly increased plasma MDA levels compared with the saline group. The moderate-dose edaravone group also showed a significant decrease in spinal MDA levels compared with the saline group. These results were supported by previously reported findings of significantly decreased MDA levels in spinal cord samples in edaravone-treated vs. untreated animals at 48 h post-SCI (Ozgiray et al., 2011). According to the same report, MDA levels in serum samples were not significantly reduced by edaravone treatment at 48 h post-SCI compared with no treatment (Ozgiray et al., 2011).

Results from our study showed that high-dose edaravone resulted in significantly increased spinal MDA levels compared with sham animals. This may be explained by the fact that when edaravone reacts with free radicals, it changes into edaravone radical intermediates (Abe et al., 2004; Yamamoto et al., 1996) and the accumulation could affect MDA measurements (leading to an increase in the measured MDA concentration) in blood plasma and spinal cord samples. Edaravone radical intermediates (edaravone radical and edaravone peroxyl radical) exhibit less reactivity as oxidants than typical reactive oxygen species (Ono et al., 1997). However, it remains to be determined whether these intermediates increase measured MDA levels and whether the increased MDA levels in the high-dose edaravone group were responsible for (i) the decreased spinal MEP amplitude recovery in the early

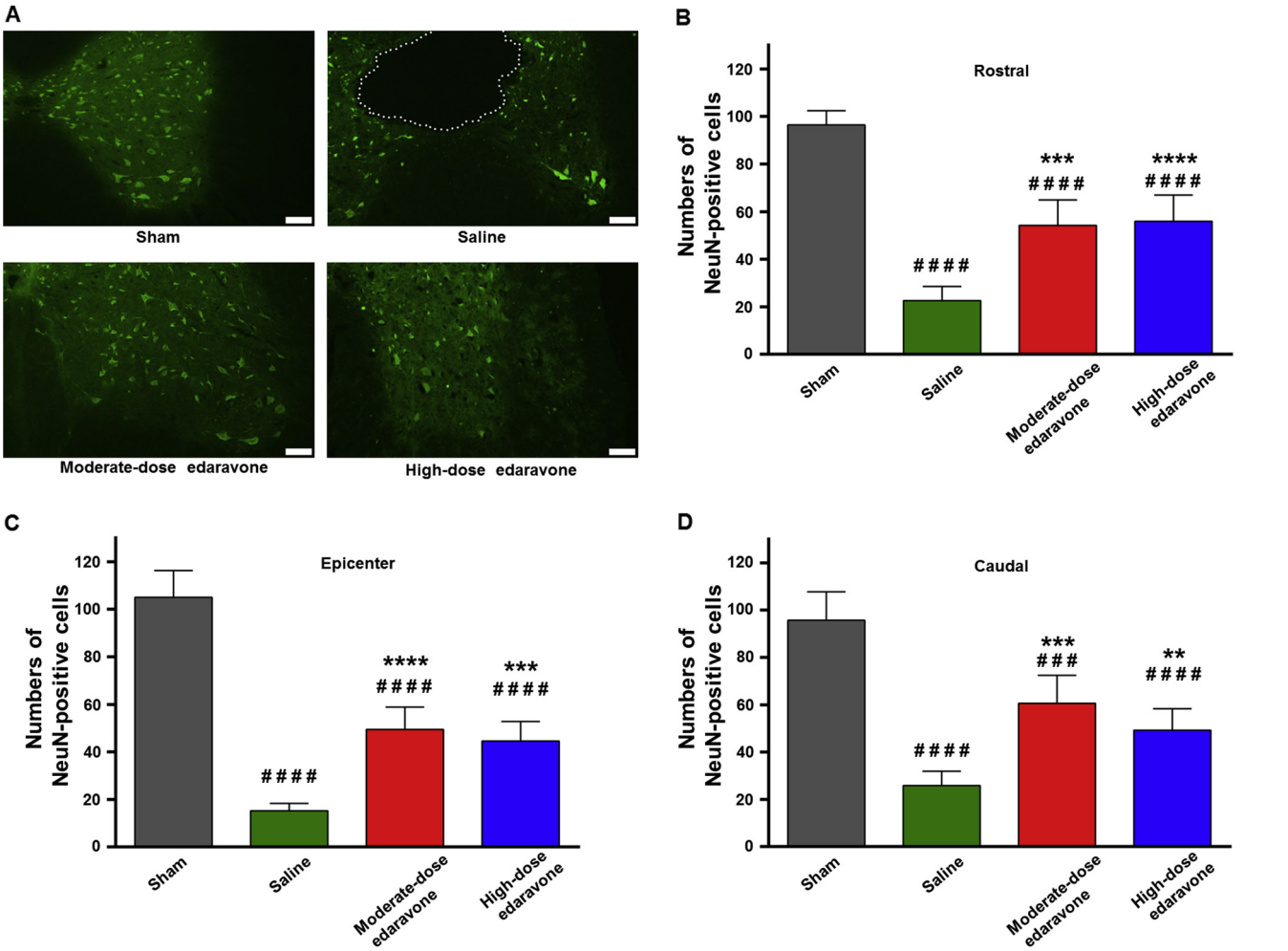

Fig. 6. Immunohistochemical evaluation of neuronal nuclear antigen (NeuN)-positive cells in the rat spinal cord. (A) Representative Anti-NeuN-stained transverse sections of the epicenter regions at 8 wk following spinal cord injury (SCI). Micrographs show the right ventral horn in each experimental group. A large cavity (dotted line) is visible in a representative slide from the saline group. Scale bar: 100 μm. (B–D) Quantification of NeuN-positive neuronal cells in the rostral, epicenter and caudal regions of bilateral ventral horns. Rostral and caudal regions are located 2 mm away from the epicenter. Data are presented as mean ± SD; $n = 4–5$ per group; $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$ compared with the saline group, $^{\#\#\#}P < .001$, $^{\#\#\#\#}P < .0001$ compared with the sham group; Dunnett's test following one-way ANOVA.

acute phase of SCI and (ii) the delayed motor function recovery during the first week post-SCI. As discussed in the following section, motor function recovery in the high-dose edaravone group after 1 wk and throughout the 8 wk observation period was similar to results from the moderate-dose edaravone group. Therefore, the use of high-dose edaravone was unnecessary from a practical point of view.

As an underlying mechanism for SCI, lipid peroxidation may also be involved in clinical cases of lumbar spinal canal stenosis, where reduction in intraneural blood flow and a state of relative ischemia in nerve tissues are present (Acker et al., 2016; Yoshihara, 2016). This suggests that edaravone could also serve as a therapeutic option in conservative treatment of this debilitating condition.

3.3. Effect of edaravone on behavioral recovery of motor function post-SCI

Ohta et al. (Ohta et al., 2011) reported that a moderate dose bolus of edaravone (3 mg/kg) twice daily for 3 d resulted in significantly higher BBB scores at 6 wk compared with saline controls, although there was no improvement with high-dose (10 mg/kg) edaravone. In the present study, we observed an initial delay in behavioral recovery in the high-dose edaravone group. However, motor task results throughout the rest of the observation period were similar and significantly better in the moderate- and high-dose edaravone groups compared with the saline group. Results from the inclined plane test were similar to BBB scores from the open-field locomotion test. The observed pattern of behavioral recovery was consistent with the electrophysiological study: both moderate- and high-dose edaravone resulted in significantly improved MEP amplitudes compared with saline in the acute SCI phase, although recovery after the moderate dose was slightly better.

Ohta et al. (Ohta et al., 2011) also reported that continuous edaravone infusion yields better results than twice daily bolus administration in terms of inhibiting persistent lipid peroxidation. Accordingly, we assumed that 10 mg/kg/h continuous edaravone infusion would result in superior behavioral outcomes compared with a 10 mg/kg bolus treatment. However, we found that high-dose edaravone did not have superior effects on motor function recovery (and was slightly worse) compared with the moderate edaravone dose. Therefore, because of the possibility of high-dose edaravone side effects, such as hepatic and renal dysfunction (Higashi et al., 2006), the use of high-dose edaravone cannot be recommended. As shown by the biochemical analysis, although

moderate-dose edaravone significantly lowered spinal cord MDA levels following SCI, high-dose edaravone significantly raised MDA levels in the spinal cord and plasma. Despite this, BBB scores and performance on the inclined plane were, for the most part, improved in these two edaravone groups compared with the saline group. Therefore, it is difficult to explain the observed improvement by a single mechanism of action, such as a beneficial effect of edaravone on lipid peroxidation. Anti-inflammatory effects of edaravone have been reported in recent studies. For example, edaravone suppresses expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) mRNA synthesis in neuroinflammation induced by middle cerebral artery occlusion (Wu et al., 2013). Also, stereotactic injection of edaravone for targeting intracerebral hemorrhagic lesions inhibits proinflammatory (TNF-α and IL-1β) and promotes anti-inflammatory (IL-4 and IL-10) responses (Zhang et al., 2016). Edaravone also decreases serum levels of the inflammatory biomarker matrix metalloproteinase (MMP)-9 in patients with acute cerebral infarction (Isahaya et al., 2012). Finally, anti-inflammatory treatments for SCI can improve locomotor function (Coll-Miro et al., 2016; Machova Urdzikova et al., 2015; Zhang et al., 2015). Therefore, it is possible that the anti-inflammatory effects of moderate- and high-dose edaravone were involved in the improved behavioral recovery observed in this study. Further studies are needed to better understand the beneficial effect of edaravone in SCI.

3.4. Effect of edaravone on neuronal cell loss post-SCI

The results of our immunohistochemical study showed that significantly greater numbers of neurons survived in the spinal cord after SCI as a result of edaravone treatment.

A neuroprotective effect of edaravone was previously shown in mice during permanent focal cerebral ischemia using the superoxide-sensitive dye dihydroethidium and neuronal marker NeuN staining in the boundary zone of the infarct (Shichinohe et al., 2004). Pearse et al. studied neuronal preservation by comparing the number of NeuN-positive neurons in cervical SCI rats (Pearse et al., 2005). The authors found greater neuronal cell loss distant from the injury epicenter in the severely injured group compared to lesser injury groups at 9 wk post-injury. A profound loss of neurons extended rostrally and caudally for distances proportional to the degree of injury. In agreement, in the present study we also found significant loss of neurons occurring 2 mm rostrally and caudally to the epicenter at 8 wk post-injury.

In another study, to assess motoneuronal loss, Wen et al. counted NeuN-stained cells in the ventral horn that met the criteria for motoneurons: a clearly identifiable nucleus and a cell soma larger than 100 μm^2 (Wen et al., 2015). Further, other authors quantified motor neurons by counting the number of choline acetyltransferase (ChAT)-positive cells (cholinergic neurons), whereas the total number of neurons was quantified by counting NeuN-positive cells with characteristic morphology (Wilcox et al., 2017). In this study, although we did not examine the number of motoneurons specifically, our neuronal cell counts based on NeuN-staining certainly included motoneurons of the ventral horn.

3.5. Conclusion

Results from our electrophysiological, behavioral and immunohistochemical analyses in a rat model of SCI suggest that moderate-dose edaravone administration may be beneficial for minimizing SCI progress and promoting functional recovery after injury. Our results also suggest that edaravone may be more effective if administered as early as possible after injury to minimize secondary damage. Edaravone may, therefore, provide a viable therapeutic strategy for spinal trauma and other related conditions in a clinical setting.

4. Experimental procedure

All protocols used in this study were approved by the Animal Use and Care Committee of Niigata University (Niigata, Japan; approval number: 233–2). Animal care and all procedures were in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. All efforts were made to minimize the number of animals used and to decrease animal suffering.

4.1. Animals

A total of 104 male Wistar rats (aged 8–12 weeks, weighing 370–420 g) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Older rats were used to facilitate the surgical procedure, according to a previous report (Shimoji et al., 1990). The rats were housed two per cage under a standard 12 h light-dark cycle; water and food were available *ad libitum*. The temperature of the experimental room was maintained at 24 ± 2 °C.

4.2. Anesthesia and monitoring

Anesthetic procedures for electrophysiological analysis were performed according to previously described methods with some modifications (Shimoji et al., 1990). Urethane is routinely used in acute electrophysiological experiments as an anesthetic agent, because it does not negatively affect neural electrical activity (Bolay and Dalkara, 1998). Following an intraperitoneal injection of 1.5 g/kg urethane (Sigma-Aldrich Co., St. Louis, MO, USA) to induce and maintain anesthesia, and after immobilizing the animal with intravenous infusion of 10 mg/kg/h rocuronium bromide (Eslax®; MSD, Tokyo, Japan), a tracheal tube was introduced through a tracheotomy and connected to a Harvard animal ventilator (Model 683; Harvard Apparatus Co., South Natick, MA, USA). A tidal volume of 2.5–3.5 ml and a frequency of 60–65 breaths/min were adjusted to maintain $PaCO_2$ at 35–45 mmHg. The rectal temperature was maintained at 37.0–38.0 °C using a warmed water circulator pad placed under the rat. The left femoral artery was cannulated to monitor arterial blood pressure or to collect blood. The left femoral vein was used to infuse acetated Ringer's solution (7–8 ml/kg/h) through an infusion pump (FP-1000, Melquest, Toyama, Japan).

Anesthesia for rats aroused after SCI to undergo behavioral testing was induced and maintained by inhalation of 3.0–4.0% sevoflurane (Sevofrane®; Maruishi Pharmaceutical, Osaka, Japan) administered in a continuous oxygen flow of 1 L/min under spontaneous respiration. We switched to sevoflurane in behavioral experiments, because urethane is carcinogenic to rats (Tannenbaum et al., 1962). The left femoral vein was used to infuse acetated Ringer's solution (7–8 ml/kg/h). These rats were also administered subcutaneous buprenorphine (Lepetan®; Otsuka Pharmaceutical, Tokyo, Japan) at a dose of 0.03 mg/kg before awakening to provide analgesia.

4.3. Surgical procedures

After the rats were mounted onto a stereotactic frame, laminectomies were performed by drilling at the T_{10} vertebrae to expose the spinal cord. The dura was not opened. The drilled opening was made wide enough to gently place a cylindrical weight 1.8 mm in diameter exactly onto the spinal cord. The extradural placement of a 10 g weight for 10 min on the spinal cord (spinal cord compression) was done to induce SCI after a stabilization period of 30 min

following laminectomy, which was needed to confirm that hemodynamic and spinal electrophysiological parameters were stable, according to a previous report (with slight modification) (Fujimoto et al., 2000). In the sham groups, laminectomy and cannulation were performed similarly to the methods described above but weight placement on the spinal cord (compression) was not done.

4.4. Recording of MEPs

Signal conduction in the motor pathways was assessed using transcranial spinal MEPs. A pair of ball electrodes (Ag-AgCl) was implanted for electrical stimulation into two skull openings that were drilled 1.5 mm posterior to bregma and 1 mm lateral to the midline, with a 1.5 mm interelectrode distance. Spinal MEPs were recorded from needle electrodes (300 μm in diameter, 10 mm in length) placed on cervical ($C_{2/3}$) and lumbar ($L_{1/2}$) cord surfaces at the midline (Fig. 7) (Shimoji et al., 1990). The position of the needle tip was determined by searching for the site where the MEP amplitudes became maximal. A reference electrode was placed on the nearby lumbar (L_1) bone. The motor cortex was stimulated with monophasic pulses at 0.9–1 Hz, 0.2 ms duration, 100–150 V, with a 0.5 ms delay, using a stimulator (SEN-3301, Nihon Kohden, Tokyo, Japan). All evoked potentials were amplified with a bioelectric amplifier (AB-621G, Nihon Kohden, Tokyo, Japan) using a high-cut of 300 Hz and a 0.1 s time constant. All responses to 20 stimulations were averaged (DAT-1100, Nihon Kohden, Tokyo, Japan). When the amplitude of the lumbar spinal MEPs disappeared after compression, it was always confirmed that the amplitude of the cervical spinal MEPs after compression did not change compared with the baseline amplitude. This showed that no damage occurred in the neural tracts from the motor cortex to the cervical cord, suggesting that electrophysiological measurements were normal (Fig. 1). Peak-to-peak MEP amplitudes were measured during each recording. The relative amplitude was calculated as the amplitude measured 2 h after SCI divided by the baseline amplitude.

4.5. Electrophysiological analysis: the effect of edaravone on MEP recovery during the acute phase of SCI

In experiment 1 (Fig. 8A), the appropriate edaravone (Radicut®; Mitsubishi Tanabe Pharma Corporation, Tokyo, Japan) dose was determined by randomly assigning rats to 5 groups: sham, saline, low-dose (1 mg/kg/h), moderate-dose (3 mg/kg/h), and high-dose (10 mg/kg/h). The edaravone doses were selected based on previous studies (Ohta et al., 2011; Suzuki et al., 2005). Rats in each edaravone group received an intravenous bolus of 3 mg/kg edaravone 30 min prior to compression, followed by continuous infusion for 2 h at the respective rates indicated above (Suzuki et al., 2005). In experiment 2 (Fig. 8B), the optimal commencement time of the moderate-dose of edaravone was determined by randomly assigning rats to 5 groups: sham, saline, edaravone 30 min prior to compression, edaravone at the start of compression, and edaravone 10 min after decompression. Rats in each edaravone group received an intravenous bolus of 3 mg/kg edaravone, followed by continuous infusion for 2 h at a rate of 3 mg/kg/h. Rats in the saline group received an intravenous bolus of 1 ml/kg normal saline 30 min prior to compression, followed by continuous infusion for 2 h at a rate of 1 ml/kg/h (Ohta et al., 2011; Suzuki et al., 2005). Sham rats were administered neither saline nor edaravone.

The moderate-dose edaravone group rats (experiment 1) and 30 min before compression group rats (experiment 2) received the same treatment. Therefore, they were pooled into the same group to minimize the number of animals used. The sham and saline rats from each experiment were also pooled for the two respective experimental groups.

4.6. Biochemical analysis: the effect of edaravone on lipid peroxidation during the acute phase of SCI

Biochemical analysis of blood and spinal cord tissue samples was performed in randomly selected animals from sham, saline and low-, moderate- and high-dose edaravone groups from experiment 1 (Fig. 8A). Blood was collected through the femoral artery at 3 h post-SCI following MEP recordings. An approximately 12 mm specimen of the spinal cord at the T_{10} vertebrae was excised following cardiac puncture and blood removal by means of perfusion with normal saline; the tissue was promptly placed in liquid nitrogen. Blood samples were centrifuged for 3 min at 10,000×*g* and rapidly frozen in liquid nitrogen. Plasma and spinal cord tissue samples were stored in a freezer at −70 °C until further biochemical analysis. For quantitative assessment of SCI-induced oxidative stress, plasma and spinal cord tissue samples were homogenized

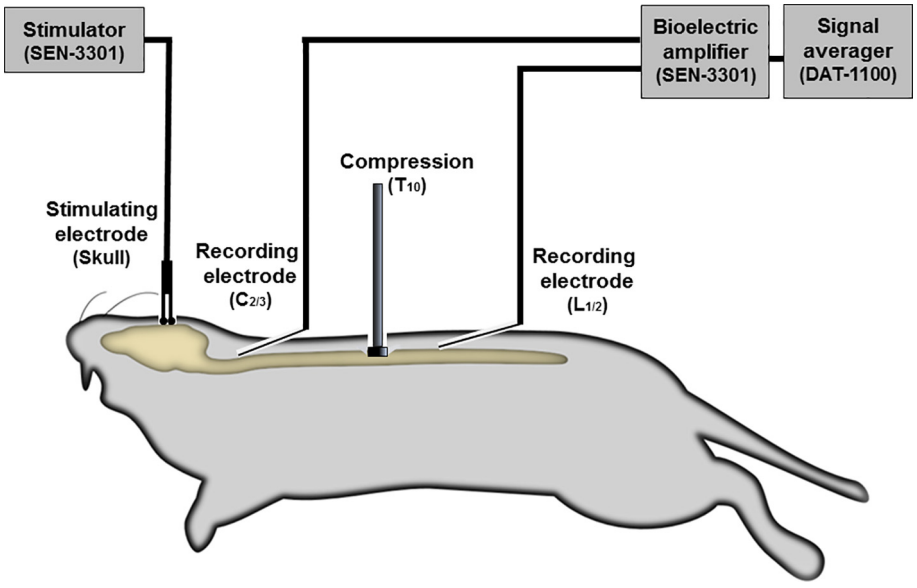

Fig. 7. Electrophysiological recording of spinal motor-evoked potentials (MEPs). Positioning of stimulating (skull) and recording (cervical ($C_{2/3}$) and lumbar ($L_{1/2}$)) electrodes. Spinal cord injury (SCI) was induced by gently placing a 10 g weight extradurally on the spinal cord at the T_{10} vertebrae for 10 min.

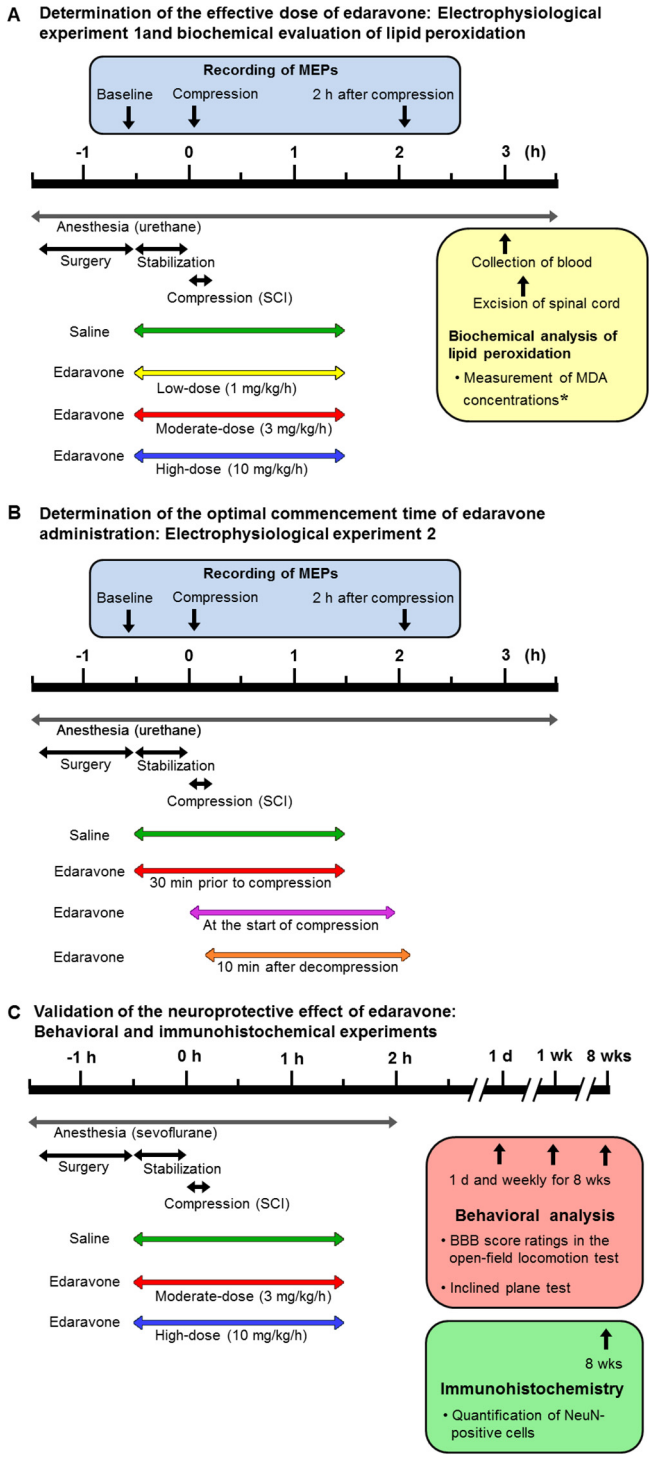

Fig. 8. Diagrammatic representation of the entire study, showing procedure timelines for electrophysiological experiments 1 (A) and 2 (B), and behavioral experiments (C). Asterisk indicates MDA measurement in the sham, saline, low-dose, moderate-dose, and high-dose edaravone groups. SCI, spinal cord injury; MEPs, motor-evoked potentials; MDA, malonyldialdehyde; BBB score, Basso-Beattie-Bresnahan score; NeuN, neuronal nuclear antigen.

and incubated at 60 °C for 1 h. The tissues were then used in a thiobarbituric acid test for MDA, an indicator of lipid peroxidation (Hall and Braughler, 1981). MDA concentrations in the plasma and spinal cord were determined using an assay kit (NWK-MDA01, Northwest Life Science Specialties, LLC, Vancouver, WA, USA) according to the hemoglobin back-extraction protocol provided by the manufacturer. The results were analyzed using a two-point calculation and normalized to homogenate protein concentration determined using a BCA Protein Assay Kit (Novagen, Merck KGaA, Darmstadt, Germany).

4.7. Behavioral analysis: the effect of edaravone on motor function recovery following SCI

The rats that underwent behavioral testing were divided into 4 groups: sham, saline, moderate-, and high-dose edaravone (Fig. 8C). Edaravone was administered according to the protocol of Experiment 1. Because administration of low-dose edaravone did not result in significant improvement of MEP amplitude in the electrophysiological study, this dose was not used in the behavioral analysis. As previously mentioned, SCI was induced under sevoflurane anesthesia. Each edaravone group received an intravenous bolus of 3 mg/kg edaravone 30 min prior to SCI, followed by intravenous infusion of moderate- or high-dose edaravone for 2 h, at which time the rats were anesthetized by inhalation of sevoflurane delivered in 100% oxygen. At the end of infusion, the rats were transferred to a cage and allowed to recover from anesthesia. Bladders were manually expressed twice daily until micturition control was recovered. Motor function was assessed using the BBB locomotor rating scale (Basso et al., 1995) and an inclined plane test (Taoka et al., 1997) performed at 1 d, and then weekly for 8 weeks post-SCI. Behavior tests were performed at the same time of day for each rat. In each session, motor recovery was first evaluated by degree of hindlimb performance using BBB scores, followed by evaluation of rat performance in the inclined plane test.

For assessment of BBB scores, which can range from 0 (complete paralysis) to 21 (normal movement), the rats were individually placed into a circular open field (90 cm diameter, 25 cm wall height), where they were able to move freely while being observed for 5 min by two experimenters. For the inclined plane test, a smooth-surfaced rubber mat was secured to a flat board, the angle of which could be adjusted (Taoka et al., 1997). The maximum inclination angle of the plane at which the rat maintained its position for 5 s without slipping was recorded. After the behavioral experiments were completed, animals were euthanized under deep anesthesia using sevoflurane.

4.8. Immunohistochemical analysis: the effect of edaravone on neuronal cell loss following SCI

The rats that underwent immunohistochemical evaluation were divided into 4 groups: sham, saline, moderate-, and high-dose edaravone (Fig. 8C). Edaravone was administered according to the protocol of Experiment 1; because administration of low-dose edaravone did not result in significant improvement of MEP amplitude in the electrophysiological study, this dose was not used in the immunohistochemical analysis.

At 8 wk post-SCI, animals (4–5 of each experimental group) were perfused with normal saline followed by 10% neutralized formalin (Wako, Osaka, Japan) under terminal deep anesthesia with isoflurane. The 12 mm specimens of spinal cord centered on the injury area were excised and post-fixed for 1 h in the same fixative solution, then cryoprotected in 20% sucrose in 0.1 M phosphate buffer for 24 h at 4 °C (Sasaki et al., 2014). Tissues were then stored frozen at − 80 °C and cut into 16 μm-thick transverse sections using a cryostat (Leica Biosystems, Nussloch, Germany). Sections mounted on Silane-coated glass slides were washed twice with TNTB (0.1 M Tris-HCl buffered saline, pH 7.5, containing 0.3% Tween 20), and then incubated for 48 h at 4 °C with primary antibody: mouse anti-NeuN (1:800, Millipore, Billerica, MA, USA) in 0.1% Tween 20 in TNB buffer (0.1 M Tris-HCl buffered saline, pH

7.5, containing 1% blocking reagent). Then sections were again washed twice with TNTB and incubated for 24 h at 4 °C with FITC-conjugated anti-mouse antibody (1:400, Millipore, Billerica, MA, USA) used as secondary antibody.

The sections were visualized using a fluorescence microscope equipped with a digital camera system (Olympus, Tokyo, Japan). For neuronal counting, one section of the injury epicenter and two other sections, one rostral and one caudal, located 2.0 mm from the epicenter were selected. Anti-NeuN antibody was used as a marker to identify neurons in the ventral horn. The numbers of NeuN-positive neurons were counted bilaterally in left and right ventral horns and averaged in each animal as previously described (Pearse et al., 2005).

4.9. Statistics

Statistical analysis was performed using GraphPad Prism 6 (GraphPad, San Diego, CA, USA). For statistical analysis of data from electrophysiological, biochemical and immunohistochemical experiments, we used one-way analysis of variance (ANOVA), followed by Dunnett's test. For statistical analysis of data from behavioral experiments, we used repeated measures two-way ANOVA, followed by Bonferroni's test. The data are presented as mean ± S. D. Statistical significance was set at $P < .05$.

Author disclosure statement

Declarations of interest: none.

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