Laboratory Investigation

The Neuroprotective Effect of Treatment of Valproic Acid in Acute Spinal Cord Injury

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Objective : Valproic acid (VPA), as known as histone deacetylase inhibitor, has neuroprotective effects. This study investigated the histological changes and functional recovery from spinal cord injury (SCI) associated with VPA treatment in a rat model.
Methods : Locomotor function was assessed according to the Basso-Beattie-Bresnahan scale for 2 weeks in rats after receiving twice daily intraperitoneal injections of 200 mg/kg VPA or the equivalent volume of normal saline for 7 days following SCI. The injured spinal cord was then examined histologically, including quantification of cavitation.
Results : Basso-Beattie-Bresnahan scale scores in rats receiving VPA were significantly higher than in the saline group ($p<0.05$). The cavity volume in the VPA group was significantly reduced compared with the control (saline-injected) group ($p<0.05$). The level of histone acetylation recovered in the VPA group, while it was significantly decreased in the control rats ($p<0.05$). The macrophage level was significantly decreased in the VPA group ($p<0.05$).
Conclusion : VPA influences the restoration of hyperacetylation and reduction of the inflammatory reaction resulting from SCI, and is effective for histology and motor function recovery.

Key Words : Valproic acid · Spinal cord injury · Clip compression model · Acetylation · HDAC inhibitor.

INTRODUCTION

Traumatic spinal cord injury (SCI) is a serious and complex medical condition that bestows significant and catastrophic dysfunction and disability[35,40,41,51]. Therapy has aimed at treatment and even recovery from SCI has been a research goal for a long time. Recent advancements in the knowledge of the pathophysiology, including the potential of stem cell therapy, prepare to take off for recovery from SCI.

SCI pathophysiology consists of two temporally-related events. Initial mechanical trauma results in the direct injury of the neural elements, the primary injury. Secondary injury is based largely on the primary event and a subsequent series of secondary degenerative processes that lead to apoptosis[1,2,4,47]. Although secondary injury should, in principle, be preventable, no innovative and effective treatment options exist[41]. To date, only steroids have been clinically approved for the drug-related treatment of SCI. However, at high-doses, steroids may produce complications which can be detriment to their long-term use[8,9,18,23,39]. Efforts to develop alternative treatments such as minocycline, erythropoietin, and statins, have focused on the reduction of secondary degeneration and recovery of neurological function. While these alternate compounds have been at least partially effective, questions remain concerning their benefits versus risk[11,13,21,31,50,53].

Valproic acid (VPA; 2-propylpentanoic acid) is a well-established drug in the long-term treatment of epilepsy[5,22,43]. VPA can directly inhibit histone deacetylase (HDAC), which is crucial in histone acetylation regulation, chromatin remodeling, and gene expression[22,31,43]. VPA-mediated enhanced acetylation of histones H3 and H4 and altered gene transcription[19] can alleviate neuron death induced by lipopolysaccharide, excitotoxicity, or aging[12,27-29]. In addition, VPA-mediated neuroprotection has been demonstrated in various neurodegenerative diseases such as amyotrophic lateral sclerosis, spinal muscular atrophy, middle cerebral artery occlusion, intracerebral hemorrhage, traumatic brain injury, and sciatic nerve axotomy[10,14,15,17,24,28,41,45,49].

This amply-demonstrated neuroprotection has spurred interest in VPA as the basis of a novel therapy for neurodegenerative diseases, including SCI[36]. However, little is known regarding the therapeutic potential of VPA in SCI. The present study employed a rat model of SCI to investigate 1) how the treatment of VPA has effects on the various histological changes including cavitation volumes, histon acetylation, and inflammatory reaction, and 2) whether it also helps the functional recovery after SCI.

MATERIALS AND METHODS

Animal model and drug administration

All animal experiments were performed in accordance with the National Institute of Health guidelines on animal care, and were approved by the Institutional Animal Care Committee. All efforts were made to minimize the number of animals used and animal suffering. Adult male Sprague-Dawley rats weighing 290-310 grams (Samtako Bio, Osan, Korea) were randomly and blindly allocated into three groups (n=12 per group). In group 1 (sham), laminectomy was performed. In group 2 (SCI-VPA), the animals received single doses of VPA (Sigma-Aldrich, St. Louis, MO, USA). In group 3 (SCI-saline), animals received 1.0 mL of the saline vehicle solution. Initially, rats were anesthetized intraperitoneally with a mixture of xylazine (10 mg/kg) and ketamine (60 mg/kg). After laminectomy at T9, the extradural plane between the dura and adjacent vertebrae was carefully dissected. A modified aneurysm clip with a closing force of 30 grams (Aesculap, Tuttlingen, Germany) was held in an applicator in the open position. The clip was rapidly released from the applicator and applied vertically onto the exposed spinal cord for a 2-minute compression. For the sham controls the same surgical procedure was followed, but clip compression was not applied. After surgery, the muscle, fascia, and skin were sutured using a 4-0 silk suture. The rectal temperature was maintained at 37.0±0.5°C by a thermostatically-regulated heating pad during surgery, and during recovery, animals were placed overnight in a temperature- and humidity-controlled chamber. To reduce post-surgery isolation-induced stress, rats were housed in pairs at an ambient temperature of 22-25°C in an alternating 12-hour light/dark cycle. Bladders were manually emptied twice daily until spontaneous voiding occurred (usually within 7-10 days). A dose of 200 mg/kg of VPA or normal saline as a vehicle control was intraperitoneally injected twice daily at 12 hours intervals for 7 days. The total daily VPA dose of 400 mg/kg/day was similar to doses used in previous studies[15,52]. To evaluate histological changes, the animals were sacrificed and the spinal cords were collected 2 weeks after SCI.

Locomotor and behavioral analyses

The rats were tested for functional deficits each week for 2 weeks after the surgery using the open field locomotor rating scale developed by Basso, Beattie, and Bresnahan (the BBB score)[3]. Two evaluators who were unaware of the group allocations and previous functional scores observed each animal for 1 minute. Functional scores for each hind limb were recorded and averaged.

Histopathological examination

Six rats in three groups were deeply anesthetized by intraperitoneal injection of ketamine prior to decapitation 14 days after SCI. Following decapitation, a 1.5 cm segment of the spinal cord centered at the injury site was immediately harvested from the vertebral canal and postfixed in 10% formalin overnight. The portion of the spinal cord was divided into seven segments at 2-mm intervals from the lesion epicenter, and seven segments were embedded in paraffin. The segments were (6 mm, 4 mm, and 2 mm rostral to the lesion; lesion epicenter; and 2 mm, 4 mm, and 6 mm caudal to the lesion). Seven spinal cords from each of the two injury groups and the sham group were randomly selected. Representative sections were sliced into 5 μm-thick sections on the horizontal plane and stained with hematoxylin-eosin. For quantitative evaluation of spared tissue and cavity areas, 20 sequential slides of the serial sections were obtained from representative segments. The tissues were examined and photographed using a Zeiss Axioplan microscope (Carl Zeiss Meditec Incorporation, Jena, Germany) with high power differential interference contrast (DIC) optics. The images were viewed on a computer monitor using a Zeiss Plan-Apochromat 5x objective and a Zeiss AxioCam HRc digital camera (Carl Zeiss). The area of cavitation and total spared tissue area of each section were traced and measured using Axio vision 4 software (Carl Zeiss). Due to variable shrinkage of the lesion cavity, we chose to measure the area of remaining white and gray matter, in addition to measuring the area of the lesion itself. Any necrotic tissue within the cavities was counted as part of the lesion. The total cavity volume was calculated by a summation of the measured cavity area of each section multiplied by the intersection distance.

Immunohistochemistry (IHC) analysis

Six rats in three groups were deeply anesthetized by an intraperitoneal injection of ketamin and were perfused intracardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH=7.4). The thoracic spinal cord was excised, postfixed for 24 hours, and maintained overnight in 30% sucrose in 0.1 M PB. Spinal cord tissues were sectioned at a thickness of 30 μm on a cryostat, and sections were floated on the surface of 0.1 M PB. To detect ED-1 (marker for activated macrophages) and histone acetylation, spinal cord sections were blocked with 4% normal serum in 0.5% Triton X-100 for 1 hour at room temperature and incubated overnight at 4°C with a 1 : 500 dilution of mouse monoclonal anti-rat ED-1 (Serotec, Oxford, UK) and a 1 : 1000 dilution of polyclonal anti-rat acetyl-histone H3 (K9, Ac-H3/K9; Cell Signaling Technology, Danvers, MA, USA) and a 1 : 1000 dilution of polyclonal anti-rat acetyl-histone H3 (K18, Ac-H3/K18; Cell Signaling Technology), and rinsed for 3×10

min in 0.1 M PB. Sections were then incubated in 0.1 M PB containing 4% normal serum and 0.5% Triton X-100 for 2 hours at 25°C on a shaker, and then in primary antiserum in 0.1 M PB containing 4% normal serum and 0.5% Triton X-100 for 12 hours at 25°C. After rinsing (3×10 min) in 0.1 M PB, sections were incubated in a 1 : 200 dilution of biotinylated anti-mouse IgG (Sigma-Aldrich) and a 1 : 200 dilution of anti-rabbit IgG (Vector Laboratory, Burlingame, CA, USA) in 0.1 M PB containing 4% normal serum and 0.5% Triton X-100 at 25°C for 2 hours. The sections were then incubated in a 1 : 50 dilution of avidin-biotinylated horseradish peroxidase (Vector Laboratory) in 0.1 M PB for 2 hours and rinsed (3×10 min) in 0.25 M Tris. Finally, staining was visualized by reaction with 3, 3'-di-aminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide in 0.25 M Tris for 3-10 min using a DAB reagent set (Kirkegaard & Perry, Gaithersburg, MD, USA). All the sections were then rinsed in 0.1 M PB and mounted on Superfrost Plus slides (Fisher, Pittsburgh, PA, USA) and dried overnight at 37°C. The mounted sections were then dehydrated with alcohol, cleared with xylene, and coverslipped with Permount mounting medium (Fisher).

The labeled cells were identified and counted with separation of each antibody from two sites at four tissues in six different animals. The labeled tissues were photographed using a Zeiss Axiopan microscope with high power DIC optics (Carl Zeiss). The images were viewed on a computer monitor using a Zeiss Plan-Apochromat 40x objective (Carl Zeiss) and a Zeiss Axio-Cam HRc digital camera (Carl Zeiss). For comparison, labeled cells were respectively counted in 48 sampled areas in both the gray and white matter (each 250×250 μm field). Enumeration of immune-positive cells used a Labworks, version 4.5, computer-assisted image analyzer (UVP, Upland, CA, USA).

Statistical analysis

All statistical comparisons were computed using SPSS 17.0 (SPSS, Chicago, IL, USA). Data are expressed as mean±standard error of the mean (SEM). Repeated measure ANOVA was used to compare groups. Significance was accepted for p-values <0.05.

RESULTS

Locomotor and behavioral analysis

SCI in rats was followed by an injection regimen of VPA or saline (n=12/group). The injured rats were assessed for 2 weeks after surgery according to the open field motor testing using the BBB locomotor rating scale (Fig. 1). While all rats exhibited severe functional impairment the week following SCI, the motor function of the VPA-injected rats was markedly better than their saline-injected counterparts. The average BBB scores (mean±SEM) of the saline-injected rats were 4.33±0.27 on day 1, 5.06±0.21 on day 3, 6.22±0.54 on day 7, 8.44±0.53 on day 10, and 9.83±0.56 on day 4. The corresponding BBB scores of the VPA-injected rats were 4.9±0.19, 5.45±0.19, 8.20±0.47,

10.80±0.42, and 12.10±0.34. A difference in BBB scores at day 7 between the two groups was significant (p<0.05).

Lesion cavity

Two weeks following SCI, histological examination of the injured spinal cords revealed a central cavity with severe necrosis at the lesion epicenter. The lesions extended to over 4 mm rostrally and caudally, tapering gradually to cavities affecting the central and dorsal areas of the spinal cord gray and white matter (Fig. 2). In VPA-treated rats, the area of the preserved tissue was significantly increased compared to that of the rats that received saline (Fig. 3A). The spared area (μm²) of the spinal cord was 3.64±0.13 at 6 mm rostral section from epicenter, 3.65±0.10 at rostral 4 mm, 3.65±0.07 at rostral 2 mm, 3.57±0.08 at epicenter, 3.71±0.08 at caudal 2 mm, 4.09±0.19 at caudal 4 mm, and 4.43±0.15 at caudal 6 mm in the control rats. In VPA-treated rats, the corresponding values were 3.37±0.05, 3.40±0.70, 3.35±0.08, 2.84±0.16, 3.56±0.19, 3.81±0.11, and 4.19±0.11 μm². In case of the saline-injected groups, the corresponding values were 3.37±0.09, 3.23±0.11, 3.05±0.11, 2.39±0.16, 3.08±0.21, 3.53±0.13, and 4.09±0.13 μm². The cavitation volume was 3.17±0.28 μm³ and 1.83±0.27 μm³ in the saline- and VPA-treated group, respectively. The difference was significant (p<0.05) (Fig. 3B)

IHC analysis

SCI causes significant hyperacetylation of histones. However, VPA injection significantly alleviated reduction of Ac-H3/K9, and Ac-H3/K18 at the 6 mm rostral and caudal segments from the lesion sites (Fig. 4, 5). Separate analysis of the gray and white matter revealed a quantitatively similar level of histone acetylation in the same group, but a significantly dissimilarity between the treated and control rats (p<0.05). In addition, decreased immunoreactivity of the ED-1 macrophage marker was evident in the VPA-injected group, while immunoreactivity was pro-
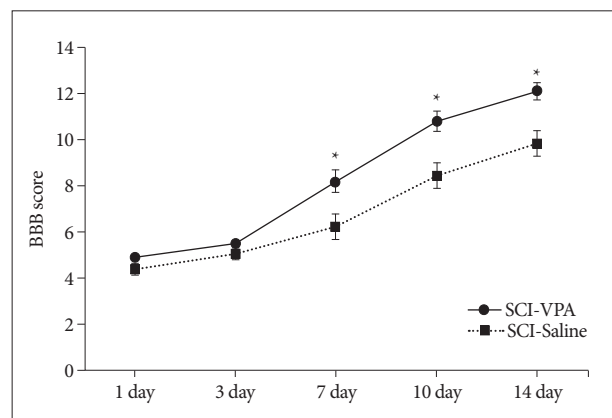

Fig. 1. Neurological function of rats after SCI between VPA- and saline-injected groups, assessed by the BBB locomotor rating scale. VPA improved functional recovery after SCI. The error bars indicate the SEM. *p<0.05 on 7, 10, and 14 day (n=12/group). SCI : spinal cord injury, VPA : valproic acid, SEM : standard error of the mean, BBB : Basso, Beattie, and Bresnahan.
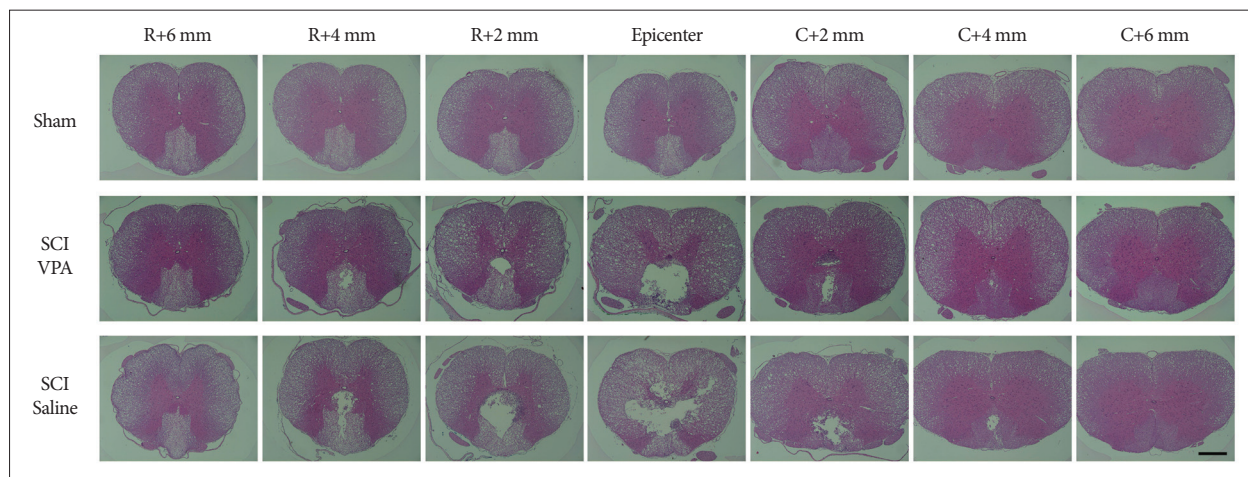
Fig. 2. Representative spinal cord sections stained with hematoxylin and eosin showing the cavities at 2 weeks after T9 clip compression injury taken at the epicenter and at 2 mm increments rostral and caudal in a rat receiving saline vehicle and a rat treated five times with valproic acid (VPA). Larger cavitation is evident in saline-injected group than the VPA-injected group, a progressive diminishment with distance from the epicenter of the lesion. Scale bar=500 μm; 5× magnification. SCI : spinal cord injury.
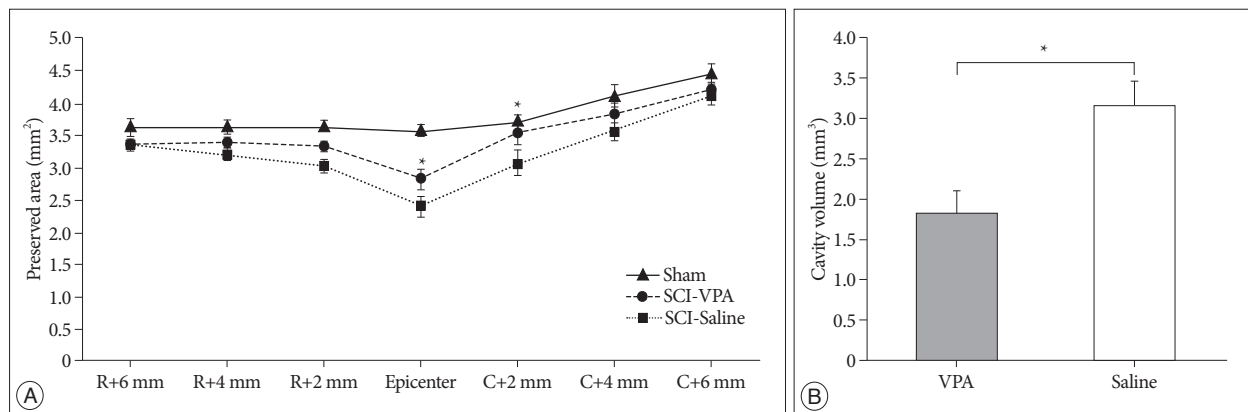

Fig. 3. VPA improves spinal cord tissue sparing after SCI. A : Measurements of the average area of preserved cord tissues at the injury epicenter and adjacent sections at an interval of 2 mm up to 6 mm rostrally and caudally. B : Histogram showing the cavitation volume of the spinal cord lesion in both groups. There was a considerable reduction of the cavity volumes in the VPA-treated group compared to the saline-injected group. The error bars indicate SEM. *p<0.05 for VPA-injected groups vs. saline-injected groups after SCI. R : rostral, C : caudal (n=6/group), SCI : spinal cord injury, VPA : valproic acid, SEM : standard error of the mean.

nounced in the control group (Fig. 6, 7). Furthermore, the separate analyses of the gray and white matter revealed no disparity of immunoreactivity, although the number of ED-1 immunoreactive cells was considerably dissimilar in VPA-treated and control rats (p<0.05). Within 4 mm rostral and caudal from the injury site, expression of histone acetylation was too low and ED-1 expression was too great to allow comparison of the two groups. Besides, injured spinal cords had injury-induced cavities <4 mm from each epicenter. Thus, we compared IHC staining at distances further removed from the injury sites (6 mm).

DISCUSSION

Traumatic SCI results in durable or permanent neurological deficiencies in motor and sensory systems[35,51]. In addition, patients with traumatic SCI are at great risk of substantial morbidity and mortality. Alleviation of SCI has been one of the grand challenges in modern medical science. A variety of morphologic changes occurs after acute SCI, including petechial hemorrhage in the gray matter, small ruptures in the venules, increased size of the extracellular spaces in the gray and white matter, and an enlarged periaxonal space[47]. To overcome permanent damage after SCI, it is of paramount importance to cease the successive secondary injuries and restore the damaged spinal cord neural networks. Even stem cell therapy progresses from the theoretical to the possible, the restoration of an impaired neural network could remain technically elaborate and difficult. Thus, minimizing the secondary injury that drives rampant apoptosis is crucially important to overcome SCI. Drugs such as minocycline, erythropoietin, and statins are involved with neuroprotection in animal models of SCI[11,21,34,50,53]. The drug's effects relate mainly to apoptosis signaling, the core of the secondary injury after SCI. However, until recently, the capability of these drugs to diminish secondary injury has been
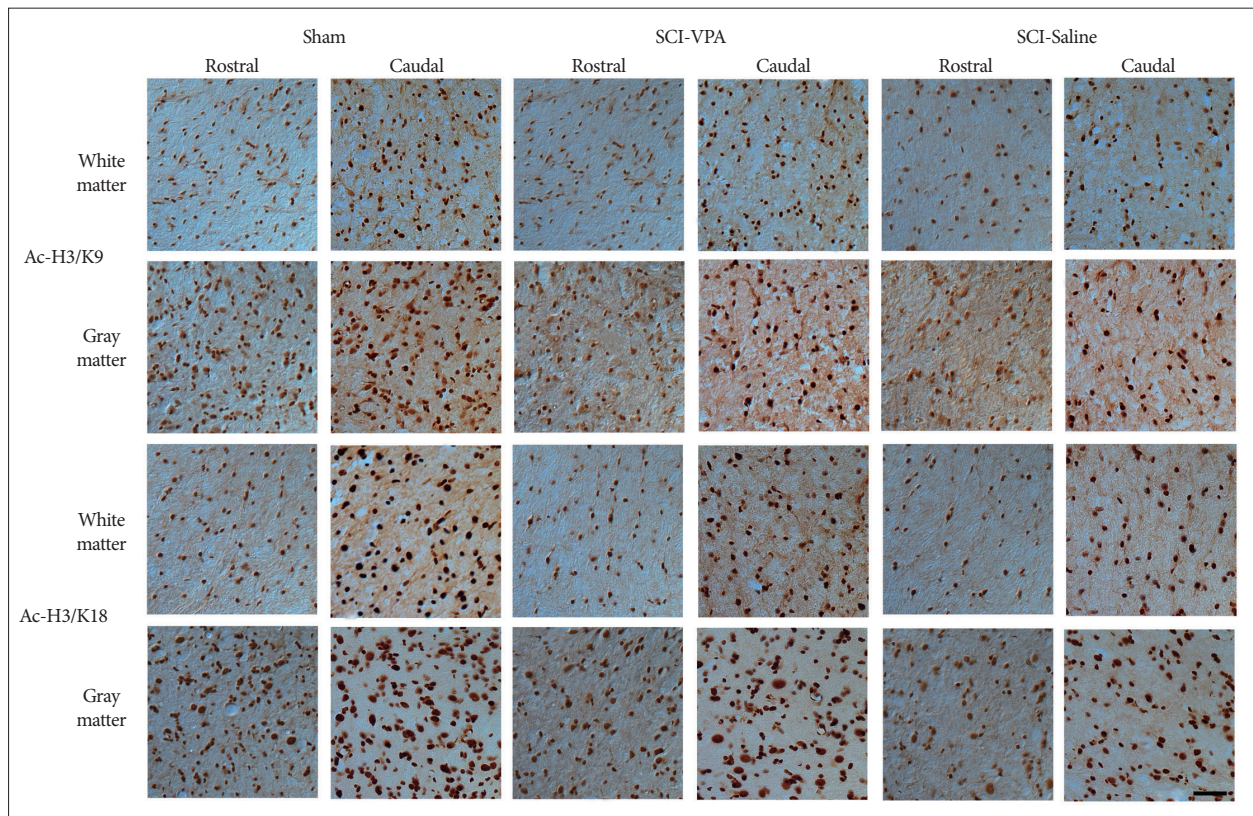
Fig. 4. Representative photographs of histone acetylation immunoreactive cells from SCI to sham animals at 6 mm both rostral and caudal to the lesion epicenter, 40×. There is a significantly restoration of the immunoreactivity of histone acetylation at both white and gray matter in VPA-injected group while in saline-injected groups, there are hyperacetylation at both white and gray matter. Scale bar=50 μm; 40× magnification. SCI : spinal cord injury, VPA : valproic acid.
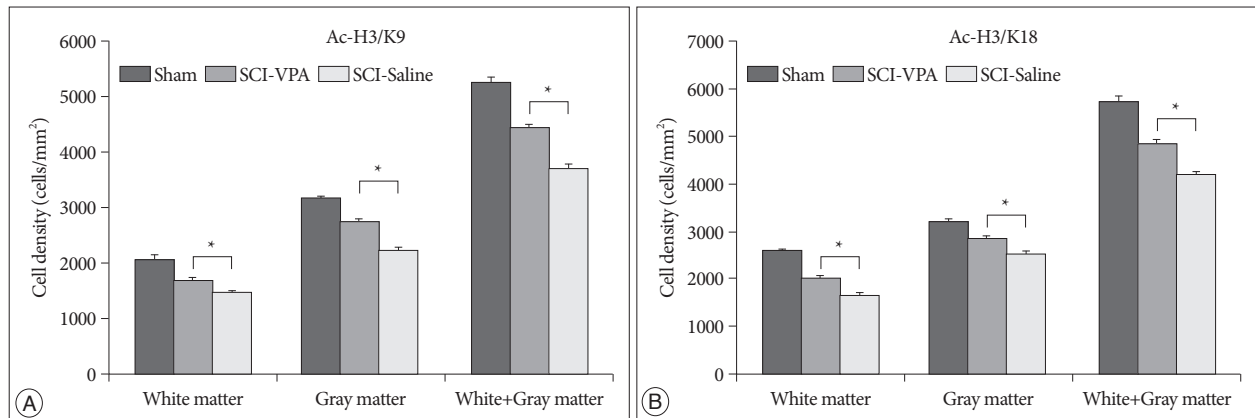

Fig. 5. Histogram of the quantification of the histone acetylation immunoreactive cells showing that VPA treatment increases the number of histone acetylation immunoreactive cells at 6 mm both rostral and caudal to the lesion epicenter. A : Acetyl-histon H3/K9. B : Acetyl-histon H3/K18. The error bars indicate SEM. *$p<0.05$ (n=6/group). SCI : spinal cord injury, VPA : valproic acid, SEM : standard error of the mean.

unknown.

The present study, VPA considerably promoted functional recovery after SCI. Minutes to hours after SCI, the lesion is thought to spread centripetally, initially by the induction of necrotic cell death, with cavitation occurring. These events likely influence the serious dysfunction that results from SCI[4,47]. Also, it is reported that the amount of spared spinal cord tissue has been shown to be closely relevant to functional recovery after SCI[6,55]. Various

trials have sought to diminish the cavitation caused by SCI. Erythropoietin, Nogo-66 receptor antagonist, and minocycline produce less scar tissue and tissue cavitation after SCI[21,32,33,36,53]. In present study, VPA also significantly diminished the cavitation volume resulting from SCI. Cavitation volume of VPA-injected groups was decreased approximate 42.27% compared to saline-injected groups. In particular, the cavitation volume was markedly decreased within both rostral and caudal 2 mm from the
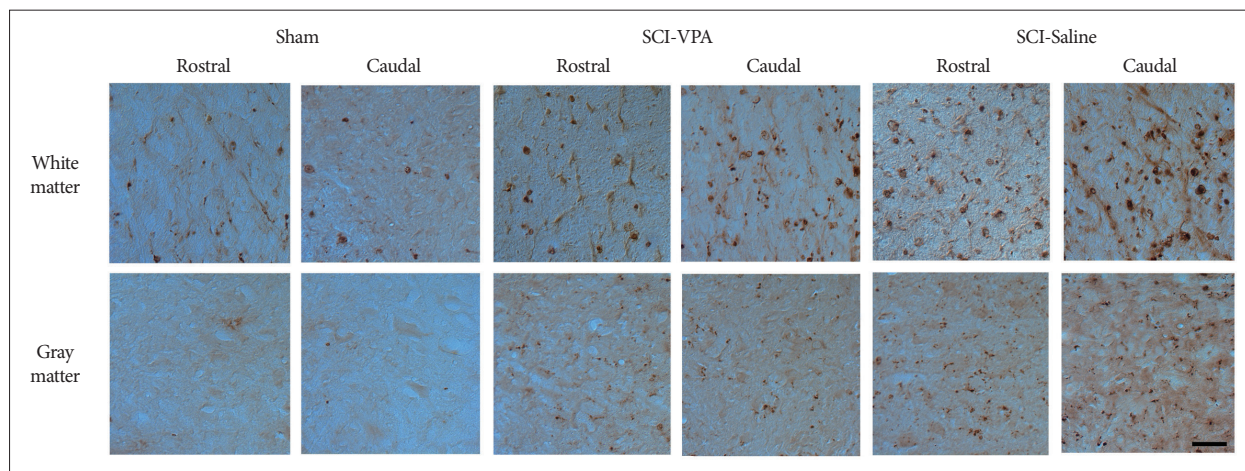
Fig. 6. Representative photographs of ED-1 immunoreactive cells from SCI to sham animals at 6 mm both rostral and caudal to the lesion epicenter, 40×. Considerable decline of the immunoreactivity of ED-1 is evident in both white and gray matter in VPA-injected groups, while in saline-injected groups high immunoreactivity of ED-1 is evident. Scale bar=50 μm; 40× magnification. SCI : spinal cord injury, VPA : valproic acid.
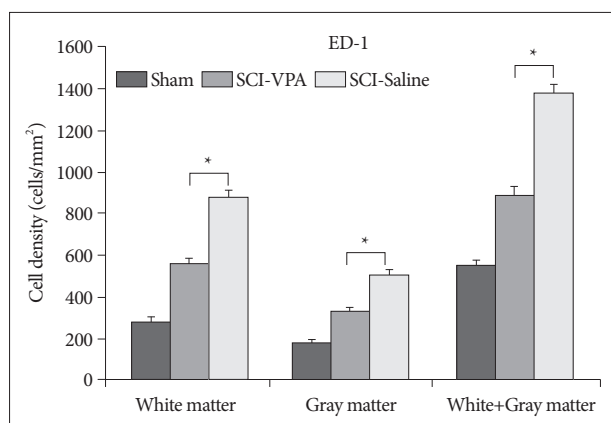

Fig. 7. Histogram of the quantification of the ED-1 immunoreactive cells showing that VPA treatment decreases the number of ED-1 immunoreactive cells at 6 mm both rostral and caudal to the lesion epicenter. The error bars indicate SEM. *$p<0.05$ (n=6/group). SCI : spinal cord injury, VPA : valproic acid, SEM : standard error of the mean.

epicenter. We also confirmed that VPA considerably promotes functional recovery after SCI.

VPA is a well-established treatment for epilepsy and bipolar disorder[43,46]. Remodeling of chromatin is crucial in gene expression regulation, and results from the alteration of the acetylation and deacetylation of histone N-terminal tails, which interact between histones and DNA molecules in chromatin[26,31]. In general, hyperacetylation is intimately linked with repression. HDAC inhibitors such as VPA may modulate the expression of downstream target genes by regulating the activities of hyperacetylated transcription factors, which are non-histone substrates of HDAC[7], providing the basis for VPA-mediated neuroprotection. *In vitro*, VPA can preserve rat cortical neurons form glutamate-induced excitotoxicity[24] and hippocampal neurons from oxygen-glucose deprivation injury[44]. In addition, VPA has been linked with the prolonged life span of cultured cortical neurons[27]. Neuronal protection afforded by VPA treat-

ment might be mediated through the extracellular signal-regulated kinase pathway and via the inhibition of proapoptotic molecules[42]. VPA involvement in neuroprotective genes such as Hsp70 and Bcl-2 has been described[16,45,48,54]. Since over-expression of the latter genes is associated with protection from cerebral ischemia[20,30,37,56,57] and SCI[49,57], the cellular neuroprotective mechanism of VPA is likely due to the upregulation of Hsp70 and Bcl-2 gene activity. Hsp70 over-expression ameliorates neurological deficits induced by transient focal ischemia[45], which may be affiliated with the inhibition of the cytochrome c-dependent activation of caspase-3[38] and the attenuation of inflammation[25].

The present results corroborate the findings of previous studies. The complex mechanisms related to VPA influence the reduction of secondary injury after SCI. Presently, VPA-treated rats displayed restored levels of histone acetylation compared to the control rats. In the case of Ac-H3/K9, this group showed a decline of only 15.4% from the sham group, while the saline-injected rats displayed a decline of 29.52% from the sham group. In the case of Ac-H3/K18, a decline of 15.72% was evident, while the saline-injected rats displayed a decline of 27.04%. VPA-treated rats also displayed decreased quantity of macrophages. This group showed an increase of 63.41% from the sham group, while rats treated only with saline displayed a far higher increase of 154.86%. The results indicate that VPA mitigates hyperacetylation and inflamatory reaction after SCI.

The use of various bioactive agents, neurotrophic factors, transplanted neuro-cellular, and other tissues for efficacy to limit the amount of secondary damage or promote healing and regeneration of the injured spinal cord have been studied. However, the complex mechanisms of healing and regeneration have proved very challenging to overcome. In the present study, VPA-treated rats displayed recovered cavitation volume and motor function. In addition, a high level of histone acetylation and decreased macrophage level was evident in VPA-treated rats, compared to

saline-treated rats.

In conclusion, we investigated the effects of VPA, a HDAC inhibitor involved with secondary damage, to minimize secondary injury and promote the damaged motor functions after SCI. The results demonstrate the potential of VPA, which should spur further study.

CONCLUSION

VPA treatment enhances functional recovery after SCI by diminishing cavitation volume inflammatory reactions, and restoring the histon acetylations in injured spinal cords. VPA should be evaluated for use for clinical trials of SCI.

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