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The Neuroprotective Effect of Treatment with Curcumin in Acute Spinal Cord Injury: Laboratory Investigation

Kyoung-Tae KIM,[1] Myoung-Jin KIM,[1] Dae-Chul CHO,[1] Seong-Hyun PARK,[1] Jeong-Hyun HWANG,[1] Joo-Kyung SUNG,[1] Hee-Jung CHO,[2] and Younghoon JEON[3]

Departments of [1]Neurosurgery and [2]Anatomy, School of Medicine, and [3]Department of Anesthesiology and Pain Medicine, School of Dentistry, Kyungpook National University, Daegu, Republic of Korea

Abstract

The purpose of this study was investigating the effects of curcumin on the histological changes and functional recovery following spinal cord injury (SCI) in a rat model. Following either sham operation or SCI, 36 male Sprague–Dawley rats were distributed into three groups: sham group, curcumin-treated group, and vehicle-injected group. Locomotor function was assessed according to the Basso, Beattie, and Bresnahan (BBB) scale in rats who had received daily intraperitoneal injections of 200 mg/kg curcumin or an equivalent volume of vehicle for 7 days following SCI. The injured spinal cord was then examined histologically, including quantification of cavitation. BBB scores were significantly higher in rats receiving curcumin than receiving vehicle ($P < 0.05$). The cavity volume was significantly reduced in the curcumin group as compared to the control group ($P = 0.039$). Superoxide dismutase (SOD) activity was significantly elevated in the curcumin group as compared to the vehicle group but was not significantly different from the sham group ($P < 0.05$, $P > 0.05$, respectively) at one and two weeks after SCI. Malondialdehyde (MDA) levels were significantly elevated in the vehicle group as compared to the sham group ($P < 0.05$ at 1 and 2 weeks). MDA activity was significantly reduced in the curcumin group at 2 weeks after SCI when compared to the vehicle group ($P = 0.004$). The numbers of macrophage were significantly decreased in the curcumin group ($P = 0.001$). This study demonstrated that curcumin enhances early functional recovery after SCI by diminishing cavitation volume, anti-inflammatory reactions, and antioxidant activity.

Key words: curcumin, spinal cord injury, neuroprotective effect

Introduction

Traumatic spinal cord injury (SCI) causes long-lasting or permanent neurological deficits that affect both motor and sensory systems.[1,2] The pathophysiology of acute SCI is complex and occurs via two mechanisms, involving a primary and secondary injury. The primary injury results from the structural damage caused by the initial mechanical trauma. The secondary injury after the primary impact is a subsequent series of deleterious processes that lead to apoptosis.[1–4] Oxygen free radicals and inflammation play an important role in the pathogenesis of secondary injury after SCI.[5,6] Primary injury is immediate and irreversible, but the secondary injury evolves over time and provides a window of opportunity for therapeutic intervention. Thus, early neuroprotective intervention soon after SCI, intended to limit early deleterious effects leading to neuronal cell death, has been the goal of numerous research studies.[2,5]

There have been many studies investigating pharmacological agents that protects or reduces secondary injury after experimental SCI, such as methylprednisolone (MPSS), minocycline, erythropoietin, and valproic acid. However, these drugs are only modestly effective and their use in the clinical treatment of SCI is debated.[2,7–13]

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a polyphenol extracted from the rhizomes of the turmeric plant (*Curcumin longa L.*), which has been used for centuries as a dietary spice and as a traditional Indian medicine. Curcumin is well known as a multifunctional drug with a wide range of pharmacological activities, including anti-inflammatory, antioxidant, and anticancerous

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effects.[14–21] Experimental cortical ischemic studies in rats have demonstrated that curcumin inhibits xanthine oxidase and glutathione peroxidases, and promotes superoxide dismutase (SOD), which results in decreased malondialdehyde (MDA) and superoxide anions and thus preserves cerebral capacity while decreasing neuronal damage.[6,22–24]

However, little is known regarding the therapeutic potential of curcumin in SCI. A recent experimental SCI study showed that curcumin inhibited apoptosis and neuron loss, and significantly improved neurological deficit seven days after spinal cord hemisection.[25] Another study demonstrated the neuroprotective effect of curcumin by increasing tissue levels of SOD and catalase.[26]

To date, the histological, biochemical, and functional recovery effects of curcumin treatment after SCI have not been fully evaluated. Thus, the aim of the present study is to investigate (1) the effects of curcumin treatment on various histological changes following SCI including cavitation volume, inflammatory response, and plasma levels of MDA and SOD and (2) whether treatment with curcumin aids in the functional recovery after SCI in a rat model.

Materials and Methods

I. 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 of our institute. Thirty-six 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 group), laminectomy was only performed without SCI. Group 2 animals (SCI-curcumin group) were given a SCI and received 200 mg/kg curcumin (Sigma-Aldrich, St. Louis, Missouri, USA) once a day by intraperitoneal injection from the day of injury to 7th day after injury. Group 3 animals (SCI-vehicle group) were treated with vehicle only by intraperitoneal injection for 7 days following SCI.

The surgical technique used for SCI in rats has been previously described by the authors.[13] Rats were anesthetized via intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (60 mg/kg). After laminectomy at T9, a modified aneurysm clip with a closing force of 30 grams (Aesculap, Tuttlingen, Germany) was applied vertically onto the exposed spinal cord for a 2-minute compression. For the sham controls the same surgical procedure was followed without clip compression. 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). The curcumin dose of 200 mg/kg/day was similar to doses used in previous studies[26] and it dissolved in 1.0 mL dimethyl sulfoxide (DMSO) before intraperitoneal injection.

On 7th day after injury, we collected blood samples after 12 hours of curcumin injection. Blood samples were collected by cardiac puncture into heparin-containing vials for plasma isolation. Plasma was separated by centrifugation (at 1,500×g) for the measurement of plasma levels of MDA and SOD. To evaluate histological changes, the animals were sacrificed and the spinal cords were collected 2 weeks after SCI.

II. Locomotor and behavior 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).[27] 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.

III. Histopathological examination

Fourteen days after SCI, six rats from each of the three groups were sacrificed. 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 (6, 4, and 2 mm rostral to the lesion; lesion epicenter; and 2, 4, and 6 mm caudal to the lesion) at 2 mm intervals from the lesion epicenter. Representative sections were sliced into 5 μm-thick sections on the transverse 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 area of cavitation and total spared tissue area of each section were traced and measured using Axio Vision 4 software (Carl Zeiss). The total cavity volume was calculated by summation of the measured cavity area of each section multiplied by the intersection distance.

IV. Immunohistochemistry (IHC) analysis

Six rats from each of the three groups were anesthetized and were intracardially perfused

with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH = 7.4). 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), spinal cord sections were blocked with 4% normal serum in 0.5% Triton X-100 for 1 hour at room temperature, incubated overnight at 4°C with a 1:500 dilution of mouse monoclonal anti-rat ED-1 (Serotec, Oxford, UK) and a 1:250 dilution of mouse monoclonal anti-rat ED-2 (Serotec). The 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, California, 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. Finally, staining was visualized through reaction with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide in 0.25 M Tris for 3–10 minutes using a DAB reagent set (Kirkegaard & Perry, Gaithersburg, Maryland, USA).

The labeled cells were identified and counted using the IHC staining slices from 6 mm rostral and caudal to the lesion epicenter. The labeled tissues were photographed using a Zeiss Axiopan microscope with high power DIC optics (Carl Zeiss). For comparison, labeled cells were counted in 48 sampled areas in both the gray and white matter, respectively (each 250 × 250 μm field). Labworks, version 4.5, computer-assisted image analyzer (UVP, Upland, California, USA) was used for the enumeration of immune-positive cells.

V. Plasma biochemical markers for antioxidation

Plasma MDA levels, the sensitive biomarkers for lipid peroxidation (LP), were estimated by the NWLSS NWKMDA01 assay (Northwest Life Science Specialties), and activity was expressed as micromoles per gram of protein. SOD activity was determined using a superoxide dismutase assay kit (catalog no.: 706002-Cayman Chemical, Ann Arbor, Michigan, USA). SOD activity was expressed as units per gram of protein.

VI. Statistical analysis

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

Results

I. Locomotor and behavioral analysis

The injured rats were assessed for 2 weeks after surgically-induced SCI according to the open field motor testing using the BBB locomotor rating scale (Fig. 1). While all rats exhibited severe functional impairment following SCI, the motor function of the curcumin-injected rats was markedly better than the vehicle-injected rats at 7 days after surgery, which was statistically significant ($P < 0.05$). This significance was maintained throughout the experimental period.

II. Lesion cavities

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 over 2 mm rostrally and 4 mm caudally, tapering gradually to cavities affecting the central and dorsal areas of the spinal cord gray and white matter (Fig. 2). In curcumin-treated rats, the area of the preserved tissue at the lesion epicenter was significantly increased compared to that of the rats that received vehicle solution only ($P = 0.006$; Fig. 3A). The cavitation volumes were 2.21 ± 0.31 mm^3 and 1.18 ± 0.20 mm^3 in the vehicle- and curcumin-treated group, respectively. The difference was significant ($P = 0.039$; Fig. 3B).

III. Immunohistochemistry (IHC) analysis

Decreased immunoreactivity of the ED-1 macrophage marker was evident in the curcumin-injected group, while immunoreactivity was pronounced in the vehicle-injected group (Figs. 4, 5). The separate

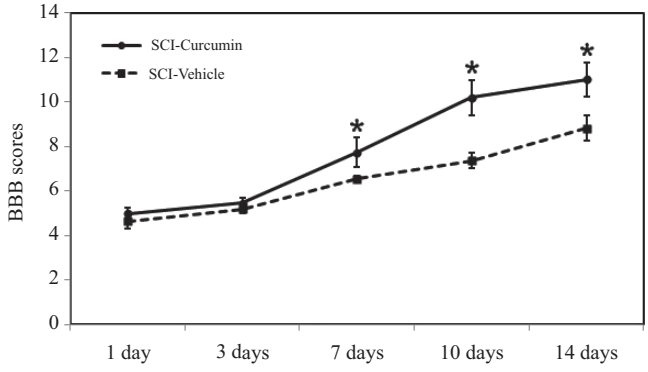

Fig. 1 Neurological function of rats after SCI between curcumin- and vehicle-injected groups, assessed by the BBB locomotor rating scale. Curcumin improved functional recovery after SCI. The error bars indicate the standard error of the mean. * indicates $P < 0.05$ on Days 7, 10, and 14 (n = 8/group). BBB: Basso, Beattie, and Bresnahan, SCI: spinal cord injury.

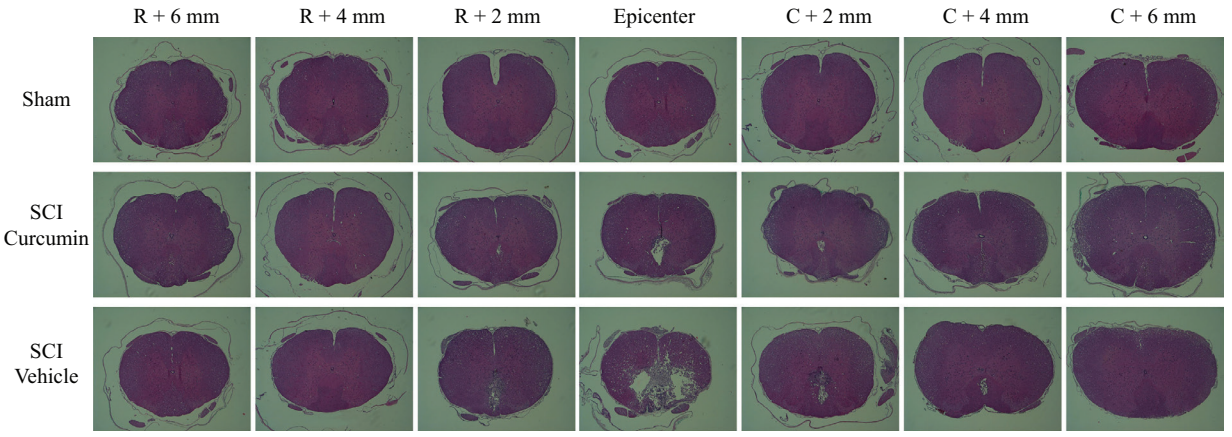

Fig. 2 Representative spinal cord sections stained with hematoxylin and eosin showing the cavities at 2 weeks after T9 clip compression injury taken at the epicenter and at 2 mm increments rostral and caudal in a rat receiving vehicle and a rat treated with curcumin. Larger cavitation was evident in vehicle-injected group than the curcumin-injected group, a progressive diminishment with distance from the epicenter of the lesion. SCI: spinal cord injury.

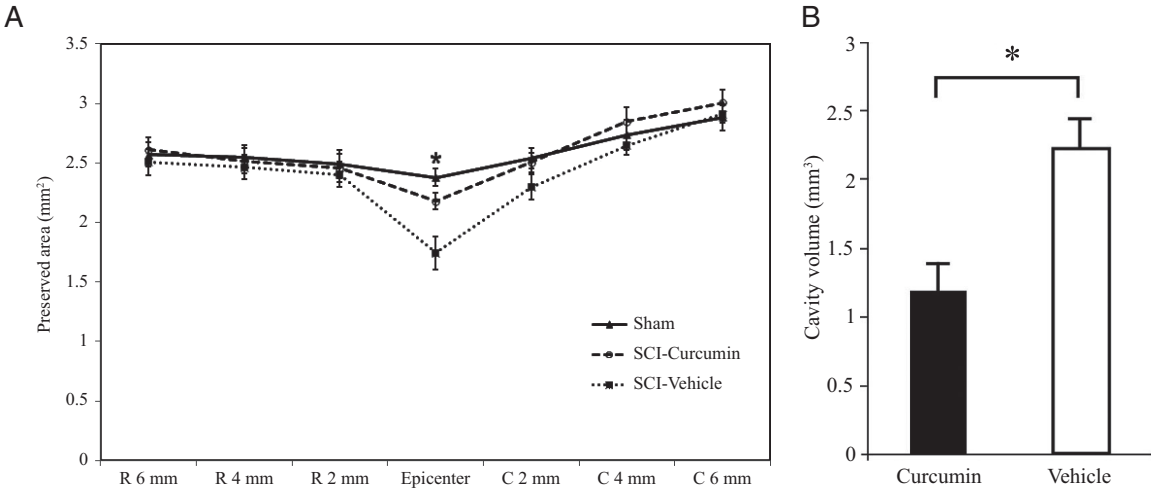

Fig. 3 Curcumin improves spinal cord tissue sparing after SCI. A: Measurements of the average area of preserved cord tissues at the injury epicenter and adjacent sections at an interval of 2 mm up to 6 mm rostrally and caudally. B: Histogram showing the cavitation volume of the spinal cord lesion in both groups. There was a considerable reduction of the cavity volumes in the curcumin-treated group compared to the vehicle-injected group. The error bars indicate standard error of the mean. * indicates $P < 0.05$ for curcumin-injected groups vs. vehicle-injected groups after SCI. R: rostral, C: caudal (n = 4/group), SCI: spinal cord injury.

analysis of the gray and white matter revealed a quantitatively similar level of immunoreactivity of ED-1 in the same group, but the number of ED-1 immunoreactive cells was considerably dissimilar between curcumin-treated and control rats ($P = 0.001$). Within 4 mm rostral and caudal from the injury site, ED-1 expression was too great to allow comparison of the two groups. In addition, injury-induced cavities were present < 4 mm from each epicenter in the injured spinal cords. Thus, we compared IHC staining at distances further removed from the injury sites (6 mm).

IV. Plasma biochemical markers for antioxidation

SOD activity in the vehicle-injected group was significantly reduced as compared to the sham group ($P < 0.05$ at both 1 and 2 weeks after SCI). SOD activity in the curcumin-treated group was significantly elevated when compared to the vehicle-treated group but was not significantly different from the sham group ($P < 0.05$, $P \geq 0.05$, respectively) at 1 and 2 weeks after SCI.

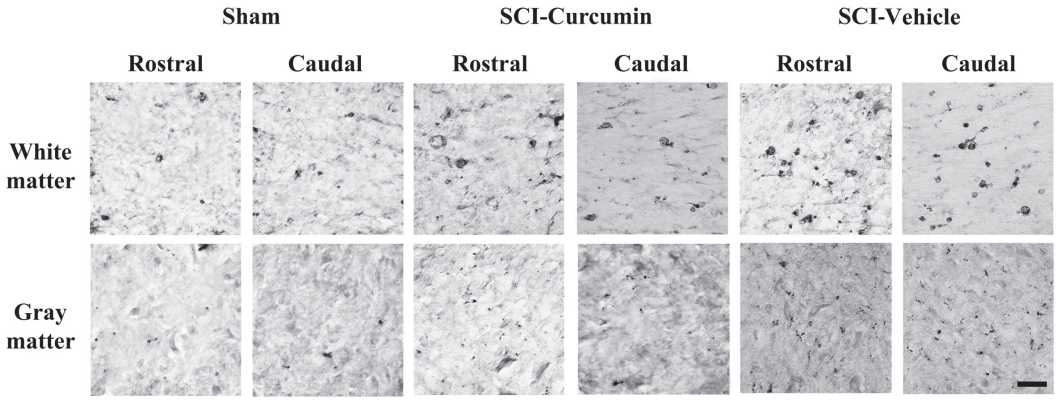

Fig. 4 Representative photographs of ED-1 immunoreactive cells from SCI to sham animals at 6 mm both rostral and caudal to the lesion epicenter, 40×. Considerable decline of the immunoreactivity of ED-1 was evident in both white and gray matter in curcumin-injected groups, while in vehicle-injected group high immunoreactivity of ED-1 was evident. Scale bar = 50 μm; 40× magnification. SCI: spinal cord injury.

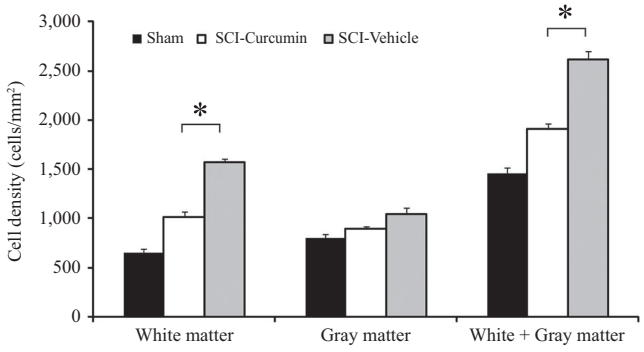

Fig. 5 Histogram of the quantification of ED-1 immunoreactive cells showing that curcumin treatment decreases the number of ED-1 immunoreactive cells at 6 mm both rostral and caudal to the lesion epicenter. The error bars indicate standard error of the mean. * indicates P < 0.05 (n = 8/group). SCI: spinal cord injury, SOD: superoxide dismutase.

MDA levels were significantly elevated in the vehicle-injected group as compared to the sham group ($P < 0.05$ at 1 and 2 weeks). MDA activity in the curcumin-injected group was significantly reduced when compared to the vehicle-injected group ($P = 0.004$) at 2 weeks after SCI. The results are shown in Fig. 6.

Discussion

Traumatic SCI typically leads to permanent neurological deficits in motor and sensory systems.[1,2,25] Many of the pathological changes after a SCI are secondary to the initial impact, and are active biological processes, including local inflammation, production of free radicals, and hyperoxidation.[25]

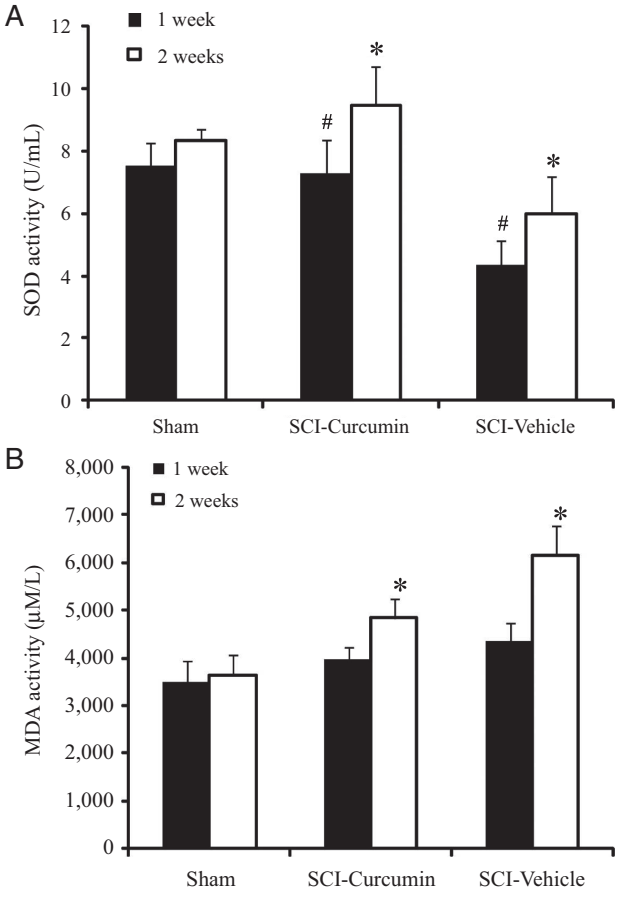

Fig. 6 The antioxidant effect of curcumin treatment on levels of SOD and MDA at one and two weeks after SCI. A: Histogram of SOD activity showing that curcumin treatment increases the level of SOD. B: Histogram of MDA activity showing that curcumin treatment decreases the level of MDA. The error bars indicate standard error of the mean. * and # indicates P < 0.05 (n = 8/group). MDA: malondialdehyde, SCI: spinal cord injury, SOD: superoxide dismutase.

Many investigators suggested that the extent of damage following SCI might be reduced by early neuroprotective intervention soon after SCI and thus recently has been the subject of much research.

In experimental animal SCI models, many drugs targeting diverse pathophysiological mechanisms, such as methylprednisolone, minocycline, erythropoietin, valproic acid and statins have been shown to have neuroprotective properties.[9,13,28–32] However, these drugs are only modestly effective, and their use in the clinical treatment of SCI is debated.[2,7–13] Although methylprednisolone has been clinically approved for the treatment of SCI, high-dose steroids may produce complications which can be detrimental in their long-term use.[2,7,8] Therefore, there is an increasing interest in the discovery of natural substances that may limit the successive secondary injuries following SCI and restore the damaged spinal cord. These natural substances have promising potential for the treatment of SCI.

Curcumin, which has been used for centuries as a dietary spice and as a traditional Indian medicine, is well known as a multifunctional drug with a wide range of pharmacological properties, including anti-inflammatory, antioxidant, and anticancerous effects.[14–24] In a recent study by Al-Omar et al., treatment with curcumin (200 mg/kg/day, intraperitoneal) at three different times (immediately, 3 hours, and 24 hours after ischemia) significantly reduced neuronal damage 7 days after transient forebrain ischemia in a rat model.[22] Thiyagarajan and Sharma also demonstrated the neuroprotective potential of curcumin in cerebral ischemia and showed that this effect is mediated through its antioxidant activity.[24] However, there are few studies regarding the therapeutic potential of curcumin in SCI.

According to one recent study by Lin et al.,[25] curcumin significantly protected neurons after SCI as compared to the treatment with a vehicle. Furthermore, 7 days after SCI, apoptosis, as detected by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), was reduced in a curcumin-treated group in comparison to a vehicle-treated group. Thus, these researchers concluded that the neuroprotection observed with curcumin treatment after spinal cord hemisection may be due to an anti-apoptotic effect and the prevention of neuronal damage. In the present study, we achieved similar results to previous studies. The area of preserved tissue at the lesion epicenter in curcumin-treated rats in our model was significantly increased compared to that of the rats that received only vehicle ($P = 0.006$; Fig. 3A). Also, the cavitation volumes were 2.21 ± 0.31 mm^3 and 1.18 ± 0.20 mm^3 in the vehicle- and curcumin-treated group, respectively ($P = 0.039$; Fig. 3B). Theses histopathologic results have relevance to the functional outcomes. The motor function of the curcumin-injected rats was markedly better than the vehicle-injected rats at 7 days after surgery, which was statistically significant ($P < 0.05$).

Major secondary deleterious mechanisms after traumatic injury include attack on the cell membrane by free radicals and LP used as an indicator of oxidative stress in cells and tissues. MDA is one of the reactive carbonyl compounds, and measurement of MDA is widely used as an indicator of lipid peroxidation.[26,33,34] SODs are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide.[35] As such, SODs are an important antioxidant defense in nearly all cells exposed to oxygen. Cemil et al. demonstrated the neuroprotective effects of curcumin in an experimental SCI model,[26] and in their study the SOD activity in the curcumin treated group was significantly increased when compared to the trauma-only group ($P < 0.05$). By increasing tissue levels of glutathione peroxidase, SOD, and catalase, curcumin seemed to reduce the effects of injury to the spinal cord, which may be beneficial for neuronal survival. Light microscopy results also showed preservation of tissue structure in the curcumin treatment group.[26]

In our study, we assessed LP induced by oxygen-free radicals. In the curcumin-treated group at 2 weeks, MDA activity was significantly reduced as compared to the vehicle-injected group. On the contrary, SOD activity in the curcumin-treated group was significantly elevated when compared to the vehicle-treated group. Our results are consistent with a previous study,[26] where they indicate that treatment with curcumin may decrease secondary deleterious cellular damage after traumatic SCI.

It is well known that, following SCI, a pronounced cellular inflammatory reaction occurs, which is characterized by the accumulation of activated microglia and macrophages.[36] As mentioned above, the turmeric plant has been used for centuries as a traditional Indian medicine with anti-inflammatory properties.[17,18] Recently, it has been demonstrated that the anti-inflammatory activity of curcumin is primarily due to the inhibition of arachidonic acid metabolism, cyclo-oxygenase, cytokines (interleukins and tumor necrosis factor), and nuclear factor-κB.[15,16,19] In our study, we compared the activity of ED-1 (a marker for activated macrophage) between the curcumin-treated group and the vehicle-injected group. Compared to the control group, the curcumin-treated group had on average 27.1% less ED-1 activity at 2 weeks after SCI, which was consistent with a previous study.[19]

Our study had some limitations. The first limitation was that the levels of MDA and SOD were only plasma, and not traumatized tissue. Plasma assay is not specific to events happening within the spinal cord. However, tissue assay could not show time-dependent changes of MDA and SOD in same tissue, and plasma assay had a similar tendency with central nervous system tissue.[37] Therefore, we checked the tissue analysis to evaluate of MDA and SOD. The second limitation was that the functional outcomes were checked in two weeks. It is not clear from this data whether these benefits would be sustained or if they were only temporary. However, we evaluated both the histological findings and functional outcome. Tissue preservation and cavitation volumes are more clear evidence, so we are able to suggest that treatment with curcumin enhances early functional recovery after SCI and decreases cavitation volume.

Conclusion

This study demonstrated that treatment with curcumin enhances early functional recovery after SCI by diminishing cavitation volume, anti-inflammatory reactions, and antioxidant activity. Further studies are needed to verify the potential benefits of curcumin treatment after SCI and to clarify the therapeutic window, dosage, and duration of treatment.

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Conflicts of Interest Disclosure

The authors have no personal, financial, or institutional interest in any of drugs, materials, or devices in the article.

Author disclosure: Authors KTK and MJK contributed equally to this work.

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Address reprint requests to: Younghoon Jeon, MD, PhD, Department of Anesthesiology and Pain Medicine, Kyungpook National University Hospital, 50 Samduk-2-ga, Jung-gu, Daegu 700-721, Republic of Korea.
e-mail: nskimkt7@knu.ac.kr