

Original Article

Arctigenin suppresses inflammation and plays a neuroprotective effect in mice with spinal cord injury

Guodong Sun^{1*}, Xiangjiang Hu^{2*}, Guimin Zhang³, Chenghong Sun³, Renwen Zhang⁴, Shujie Tang⁴, Yongxin Lin¹, Zhizhong Li^{1,5}

¹Department of Orthopedics, First Affiliated Hospital, Jinan University, Guangzhou 510632, Guangdong, China; ²Department of Orthopedics, The First affiliated Hospital of Hunan University of Medicine, Huaihua 418000, Hunan, China; ³Linyi Key Laboratory for Immunopharmacology and Immunotoxicology of Natural Medicine, Lunan Pharmaceutical Group Corporation, Linyi 276000, Shandong, China; ⁴College of Traditional Chinese Medicine, Jinan University, Guangzhou 510632, Guangdong, China; ⁵Department of Orthopedics, Heyuan People's Hospital (Heyuan Affiliated Hospital of Jinan University), Heyuan 517000, Guangdong, China. *Equal contributors.

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Abstract: Inflammation plays a key role in secondary injury after spinal cord injury (SCI) leading to spinal cord demyelination and neuronal death. Previous studies have shown that arctigenin markedly inhibits the phosphorylation of MAPKs and the activation of NF- κ B, and reduced the expression of IL-1, IL-6, and TNF- α , resulting in anti-inflammatory effects. However, it remains unclear whether arctigenin can be used to treat SCI. In the present study, SCI mice were treated with different concentrations of arctigenin, and their motor functional recovery, neuronal density, microglial activation, and inflammatory cytokine levels were investigated. We found that arctigenin improved the basso mouse scale score, regularity index, and MaxContact area, and enhanced the amplitude of motor evoked potentials, promoted functional recovery in a dose and time-dependent manner in the SCI mice. In addition, arctigenin protected the neurons in a dose-dependent manner. Furthermore, arctigenin inhibited microglial activation, down-regulated the expression of TNF- α , IFN- γ , IL-17, MCP-1, IL-6, and G-CSF, and the effect became more obvious as the concentration of arctigenin increased. In conclusion, arctigenin plays a neuroprotective role by inhibiting the activation of microglia, and reducing the expression of inflammation in the early stage of SCI in mice.

Keywords: Arctigenin, inflammatory factor, microglia, spinal cord injury

Introduction

Spinal cord injury (SCI) due to vehicular accidents is a common clinical injury, which may cause the impairment of motor function, sensory impairment, paralysis, and even death [1]. SCI adds an enormous impact functionally, financially, and emotionally on affected individuals and their families. SCI is usually divided into primary injury and secondary injury according to the pathological process [2]. By inhibiting the process of secondary injury, further deterioration of the disease can be prevented and motor functional recovery can be promoted [3]. Current treatment modalities are focused on minimizing secondary injury and maximizing residual function via rehabilitation [4]. Some treatments currently being investigated for use in SCI target neuroprotective or neuroregenerative strategies, while many cell therapies have also shown promise [5]. However, since multi-

ple factors determine the progress of the injury in SCI, a combinatorial therapeutic approach will most likely be required to establish the most effective treatment for SCI. At present, SCI remains an incurable disease without effective treatment.

In recent years, Chinese medicine treatment of SCI has become a new research focus, globally. The use of *Salvia miltiorrhiza* and *Ligusticum wallichii* has provided a new direction for the treatment of SCI [6, 7]. *Arctium*, a traditional Chinese medicine, promotes blood circulation to the skin surface, curing skin diseases, such as eczema, and treating chronic diseases, such as cancers, diabetes, and AIDS [8]. Arctigenin is the main active ingredient extracted from *Arctium*; it comprises plant-derived lignans with stronger pharmacological activity than *Arctiin*. Previous studies have shown that arctigenin has many biological activities, including anti-

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inflammatory, immunoregulatory, antiviral, anti-tumor, and neuroprotective effects [9-12]. However, whether arctigenin can be used to treat SCI is unclear.

In the present study, SCI mice were treated with different concentrations of arctigenin, and their motor functional recovery, neuronal density, microglial activation, and inflammatory cytokine levels were investigated.

Materials and methods

Establishment of the SCI model and treatment

Animal experiments were performed with the approval of the Ethics Committee at Jinan University. A total of 56 adult wildtype female C57BL/6 mice, weighing 20 ± 2 g, were provided by the Guangdong Experimental Animal Center. Arctigenin (purity > 99%) was purchased from Southern Shandong Pharmaceuticals (Linyi, Shandong, China).

The SCI model was established as described previously [13]. Under aseptic conditions, all mice were completely anesthetized with 13 μ l/g of a tribromoethanol mixture. A 2-cm longitudinal skin incision, centered over the ninth thoracic (T9) spinous process, was made along the midline. The para-spinal muscles and ligaments were laterally dissected and retracted, followed by removal of bony elements (i.e., spinous process and laminae) from the posterior spine, using a micro-rongeur. Without disrupting the dura mater, T9 spinal segment was exposed by removing the dorsal part of the vertebra under an operating microscope (Leica DM651; Wetzlar, Germany). Mice were clamped in a special card slot and placed in an NYU Impactor Model II (New York, NJ). The exposed spinal cord was hit by a 10-g weight dropped from a height of 6.25 mm. The impact velocity and compression were monitored and recorded to guarantee consistency between animals. After contusion, the injured parts of the spinal cord rapidly appeared congested and edematous; the mouse hind limb jittered and convulsed, and the mouse demonstrated tail waving; these signs indicated that the SCI model had been established successfully. The aponeurotic fascia, subcutaneous tissue, and skin were sutured, and mice were returned to their cages and routinely examined. The temperature of the cages was regulated for the follow-

ing 24 h, and mice had ad libitum access to water and food. Urine squeezing was performed twice daily until the micturition reflex was regained. After the operation, the 56 mice were randomly divided into four groups. Mice were treated with 0.25 mg/kg arctigenin, 0.5 mg/kg arctigenin, 1 mg/kg arctigenin, or phosphate-buffered saline (PBS).

Motor function evaluation

Motor function was assessed using the Basso mouse scale (BMS) scoring system [9] at 1 day, 3 days, 5 days, 7 days, 2 weeks, 3 weeks, and 4 weeks after treatment. The BMS score was recorded by the single-blind method, by two independent, blinded observers, and the mean score of the two observers was used as the BMS score of the mouse. After treatment for 8 weeks, the regularity index (the percentage between the number of normal step sequence patterns and the total number of paw placements) and MaxContact area (complete surface area contacted by the paw during a stance phase) were automatically recorded and analyzed using the CatWalk XT 9.0 software (Noldus, Netherlands).

Inflammatory cytokine analysis

At 24 h after treatment, the levels of TNF- α , IFN- γ , IL-17, MCP-1, IL-6, and G-CSF in injured spinal cords tissues of mice were detected using a 23-Plex Cytokine Array Kit (Bio-Rad, Hercules, CA) and analyzed using the Bio-Plex system (Bio-Rad).

Motor-evoked potentials

To evaluate SCI recovery, the motor evoked potentials (MEPs) were assayed by Key Point Electromyograph and Evoked Potential Equipment (Medtronic, Copenhagen, Denmark) before treatment and after 6 weeks of treatment, following previously described methods [14]. First, the mice were anesthetized using a compound anesthetic (3.0 mL/kg). Then, a stimulation electrode was applied to the rostral ends of the surgical spinal cord. A recording electrode was placed in the gastrocnemius and the reference electrode was placed in the paravertebral muscles, midway between the stimulation point and the recording point. The ground electrode was placed on the tail. A single square wave stimulus of 8.0 mA, 0.1 ms in

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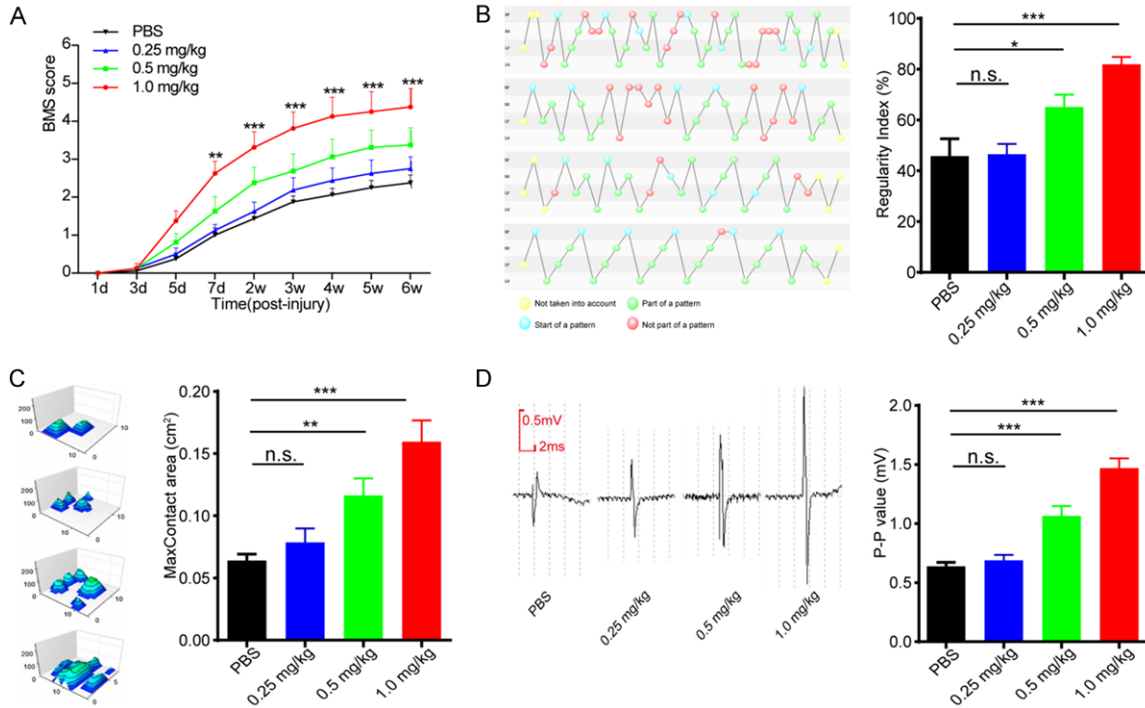


Figure 1. Arctigenin promotes functional recovery after spinal cord injury (SCI). A. Arctigenin improves basso mouse scale (BMS) in a dose- and time-dependent manner in the SCI mice. B. Arctigenin improves the regularity index (the number of normal step sequence patterns/the total number of paw placements \times 100%) in a dose-dependent manner in SCI mice. C. Arctigenin improves the MaxContact area (complete surface area contacted by the paw during a stance phase) in a dose-dependent manner in SCI mice. D. Arctigenin promotes the amplitude of motor evoked potentials (MEPs) in a dose-dependent manner in SCI mice. Data were presented as a mean \pm SD. * P < 0.05, ** P < 0.01, *** P < 0.001, vs PBS group.

duration, with a 2-ms time delay, and a frequency of 4 Hz, was used. The latency period was measured as the length of time from the stimulus to the onset of the first response wave. The amplitude was measured from the initiation point of the first response wave to its highest point. All potentials were amplified and obtained using a digital oscilloscope (Tektronix 450S; Beaverton, OR).

Nissl staining and immunofluorescence

Six weeks after surgery, specimens were fixed for 24 h with 4% neutral paraformaldehyde, embedded, and sectioned using a cryostat, with a sagittal plane thickness of 20 μ m. To measure neuronal density and structure, frozen sections were stained with Nissl reagent (Genmed, Laval, QC) strictly according to the manufacturer's instructions. To measure microglial cells, frozen sections were also probed with rabbit antibodies against ionized calcium-binding adaptor molecule 1 (Iba1, diluted 1:1000; Abcam, Cambridge, UK), labeled with

AlexaFluor488-conjugated goat anti-rabbit IgG (diluted 1:500; Abcam), and images were obtained using a fluorescence inverted microscope (Leica DM1000). Fluorescence intensity was calculated using ImageJ (National Institutes of Health, Bethesda, MD).

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (IBM SPSS, Armonk, NY). Data are presented as means \pm SD. Groups were compared using one-way analysis of variance (ANOVA) followed by a post-hoc SNK test. P -values of < 0.05 were considered statistically significant.

Results

Arctigenin promotes functional recovery in SCI mice

The BMS were analyzed at 1 day, 3 days, 5 days, 7 days, 2 weeks, 3 weeks, and 4 weeks

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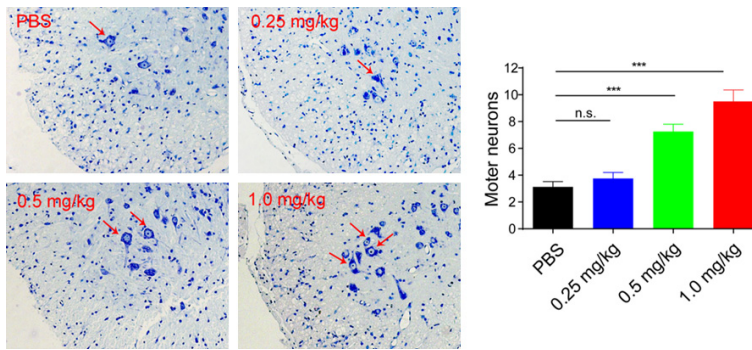


Figure 2. arctigenin has a dose-dependent neuroprotective effect on the SCI mice. The specimens of injured spinal cords tissues were stained with Nissl reagent. Representative images of Nissl staining for motor neurons are displayed ($\times 200$). Neurons were blue and purple after staining. Data were presented as a mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs PBS group.

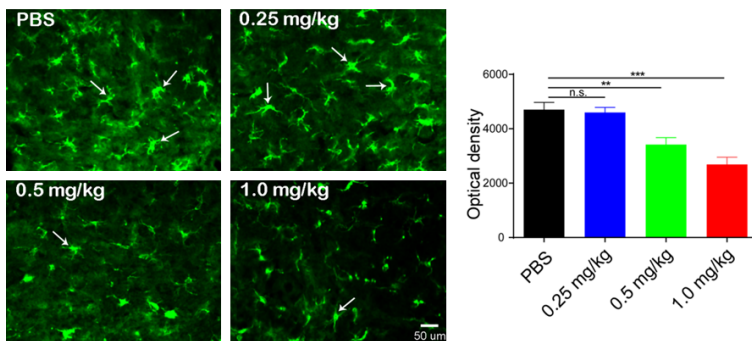


Figure 3. Arctigenin inhibits the expression of Iba1 in a dose-dependent manner in the SCI mice. The specimens of injured spinal cords tissues were analyzed by immunofluorescence. Representative immunofluorescence images for Iba1 detection are shown ($\times 200$). Microglia were observed after immunofluorescence assay. Data were presented as a mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs PBS group.

after treatment (**Figure 1A**). All mice presented complete paraplegia with a BMS score of 0 after surgery. The BMS score revealed a different degree of recovery in the various groups after 5 days of treatment. Compared to the PBS group, the BMS score in the 0.25 mg/kg arctigenin group, 0.5 mg/kg arctigenin group, and 1.0 mg/kg arctigenin group were significantly improved ($P < 0.05$). The BMS score in the 1 mg/kg arctigenin group was significantly higher than that in 0.25 mg/kg arctigenin and 0.5 mg/kg arctigenin groups ($P < 0.05$). Additionally, the regularity index and MaxContact area in the 1 mg/kg arctigenin group was significantly higher than those in the other three groups ($P < 0.05$; **Figure 1B** and **1C**). Furthermore, the amplitude of MEPs in the 1 mg/kg arctigenin group was evidently higher

than those of the other three groups ($P < 0.05$, **Figure 1D**). Thus, arctigenin promoted functional recovery in a dose-dependent manner in SCI mice.

arctigenin has a neuroprotective effect in SCI mice

Nissl staining was performed to evaluate the density and structural distribution of motor neurons after treatment with arctigenin for 6 weeks. The numbers of motor neurons was significantly higher in the 1.0 mg/kg arctigenin group than that in the other three groups ($P < 0.05$; **Figure 2**). Arctigenin therefore played a neuroprotective role, in a dose-dependent manner, in SCI mice.

Arctigenin inhibits microglial activation in SCI mice

Iba1 is a sensitive marker of activated microglia. The expression of Iba1 was detected after treatment of SCI mice with arctigenin for 6 weeks, using immunofluorescence (**Figure 3**). Iba1 expression levels were significantly lower

in the 1.0 mg/kg arctigenin group than in the other three groups ($P < 0.05$). Arctigenin inhibited the expression of Iba1 in a dose-dependent manner in the SCI mice. Therefore, arctigenin inhibited microglial activation in a dose-dependent manner in SCI mice.

Arctigenin inhibit the levels of inflammatory cytokine in injured spinal cords of SCI mice

The levels of TNF- α , IFN- γ , IL-17, MCP-1, IL-6, and G-CSF were evaluated after SCI treatment for 24 h (**Figure 4**). The levels of TNF- α , IFN- γ , IL-17, MCP-1, IL-6, and G-CSF were significantly lower in the 1.0 mg/kg arctigenin group than those in the other three groups (all $P < 0.05$). Arctigenin therefore inhibited the level of inflammatory cytokine in a dose-dependent manner in SCI mice.

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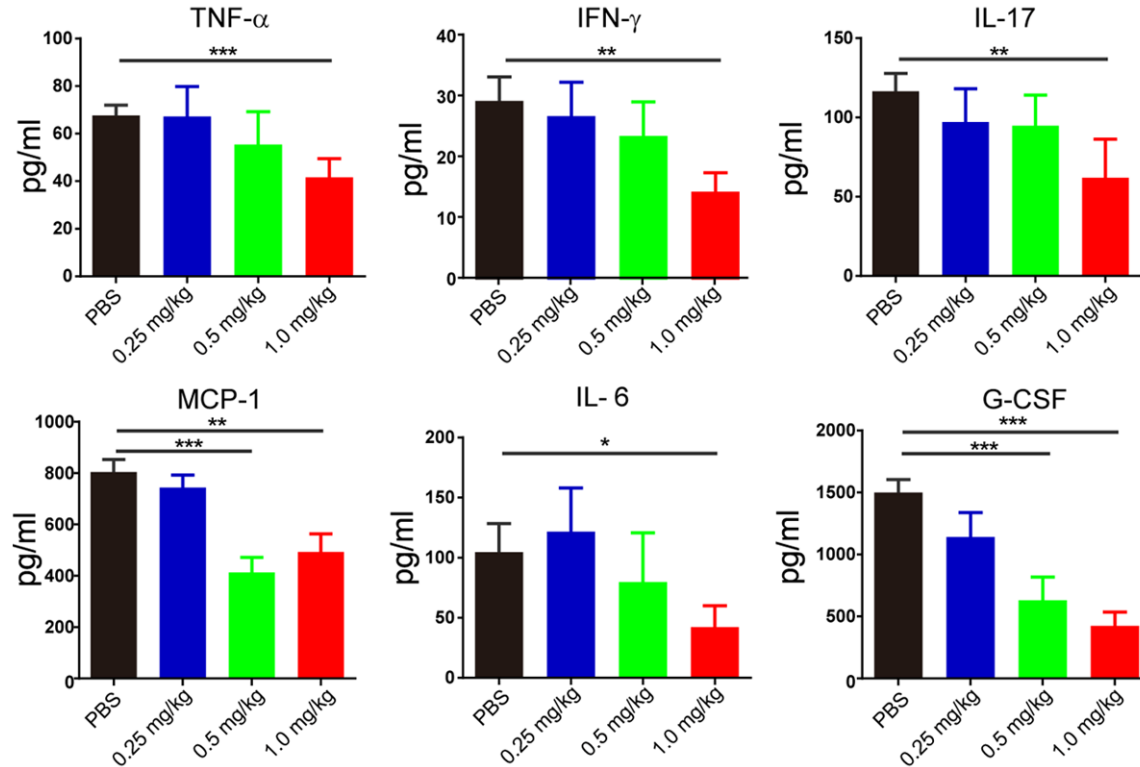


Figure 4. Arctigenin inhibits the level of inflammatory cytokines in a dose-dependent manner after SCI. The levels of TNF- α , IFN- γ , IL-17, MCP-1, IL-6, and G-CSF in injured spinal cords tissues were detected using a 23-Plex Cytokine Array Kit. Arctigenin inhibits the level of TNF- α , IFN- γ , IL-17, MCP-1, IL-6, and G-CSF in a dose-dependent manner after SCI. Data were presented as a mean \pm SD. * P < 0.05, ** P < 0.01, *** P < 0.001, vs PBS group.

Discussion

In this study, we found that arctigenin significantly increased the BMS score, regularity index, MaxContact area, MEP amplitude, and protection of neurons, and the effects of arctigenin became more marked as the concentration of arctigenin increases. The results suggested that arctigenin promoted functional recovery in a dose-dependent manner in SCI mice, and the treatment effect was most marked when used a dose of 1 mg/kg arctigenin. The effect of arctigenin promoting functional recovery in SCI mice has not been reported previously. A previous study had found that arctigenin had an anti-inflammatory and neuroprotective role in a mechanical trauma injury model using SH-SY5Y cells in vitro [15], which supported our finding that arctigenin had significant effects on SCI.

Previous studies have shown that arctigenin could markedly inhibit the phosphorylation of MAPKs and the activation of NF- κ B, and could reduce the expression of IL-1, IL-6, and TNF- α ,

with anti-inflammatory effects [16]. The levels of inflammatory factors were increased dramatically within 24 h after SCI injury [17], these factors play a key role in secondary injury after SCI, leading to spinal cord demyelination and neuronal death [18]. After SCI, the microglia were activated, promoting the secretion of inflammatory factors, such as TNF- α , IFN- γ , IL-17, MCP-1, IL-6, and G-CSF, participating in the secondary injury of SCI [17]. IL-17 upregulates the microglial production of IL-6 and nitric oxide [19], and activates the T cells and other immune cells to secrete inflammatory factor (e.g., IL-1, IL-6, TNF- α , G-CSF, etc.) [20]. Inhibition of microglial activation may represent a potential treatment approach for SCI [21]. Song et al. [15] found that, in a mechanical trauma injury model using SH-SY5Y cells in vitro, arctigenin treatment down-regulated TNF- α and IL-6 levels, up-regulated IL-10 levels, and increased the survival rate of SH-SY5Y cells. In this study, we found that arctigenin significantly inhibited microglial activation in SCI mice. We also found that arctigenin significantly inhibited microglial activation, down-regulated the expression of

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TNF- α , IFN- γ , IL-17, MCP-1, IL-6, and G-CSF, and the effect became more marked as the concentration of arctigenin increased. The treatment effect of arctigenin was most obvious when an arctigenin dose of 1 mg/kg was used to treat SCI. In this study, we found that arctigenin inhibited the activation of microglia, and reduced the expression of inflammatory factors, which were similar to the results of Song et al. [15].

In conclusion, arctigenin treatment has a neuroprotective effect, inhibiting the activation of microglia and reducing the expression of inflammatory cytokines during the early stage of SCI in mice. However, the mechanism by which arctigenin exerts these effects in response to SCI warrants investigation in future studies.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhizhong Li and Dr. Yongxin Lin, Department of Orthopedics, First Affiliated Hospital of Jinan University, 613 West Huangpu Road, Guangzhou 510632, Guangdong, China. Tel: 86-762-3185138; E-mail: zhizhongli-2014@163.com (ZZL); Tel: 86-15989114632; E-mail: 384131931@qq.com (YXL)

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