Original Investigation

Neuroprotective Effect of Genistein in Peripheral Nerve Injury

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ABSTRACT

AIM: To investigate the effects of genistein in a rat model of sciatic nerve crush injury and complete sciatic nerve transection. The effects of genistein were compared with those of gabapentin, which is widely used in clinical practice for peripheral nerve injury.

MATERIAL and METHODS: Forty-eight rats were randomly divided into six groups (8 rats in each group): group 1 (sham); group 2, sciatic nerve crush injury (control); group 3, sciatic nerve crush injury+genistein 20 mg/kg; group 4, sciatic nerve crush injury+gabapentin 90 mg/kg; group 5, sciatic nerve transection+genistein 20 mg/kg; group 6, sciatic nerve transection+gabapentin 90 mg/kg. The effects of genistein and gabapentin were assessed with immunohistochemical staining for growth associated protein-43 (GAP-43) and myelin basic protein (MBP). Interleukin-1β and tumor necrosis factor α levels in the injured nerve specimens were assessed as a measure of inflammatory response; walking track analysis and sciatic function index for neurological recovery and the paw mechanical withdrawal threshold were examined for neuropathic pain.

RESULTS: On histopathological examination, genistein use was associated with a greater immunoreactivity for GAP-43 and MBP compared with that associated with gabapentin. Genistein and gabapentin had similar effects on anti-inflammatory activity, functional recovery, and neuropathic pain.

CONCLUSION: Genistein and gabapentin exhibit positive effects on histopathology, inflammation, and clinical findings of peripheral nerve injury. When the systemic side effects of gabapentin are considered, genistein (a basic soy isoflavone that has no side effects) can be used as an alternative to medical treatment in peripheral nerve injury.

KEYWORDS: Genistein, Gabapentin, Sciatic nerve injury, Neuroprotection

■ **INTRODUCTION**

Although microsurgical techniques have been developed and positive effects of clinically and experimentally different neurotrophic drugs, steroids, hormones, and even low-dose radiation have been reported, desirable motor and sensory recovery after peripheral nerve injury is a clinical challenge (6,16,18,20,25). Methylprednisolone and gabapentin are considered as reference agents, against which the medical treatment of traumatic peripheral nerve injury is evaluated. However, their adverse effects are a major limitation associated with their clinical use (16).

Genistein was shown to promote recovery in experimental peripheral neuropathy and chronic peripheral nerve injury (23,24). However, the effect of genistein in animal models of acute crush injury or complete transection of peripheral nerve has not yet been investigated.

The purpose of this study was to investigate the effects of genistein after experimental sciatic nerve crush injury and complete sciatic nerve transection in rats and to compare its effects with those of gabapentin.

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■ MATERIAL and METHODS

The Medical and Surgical Research Center of Eskisehir Osmangazi University and Committee on Animal Experiments of the Medical Faculty approved the present experimental study (Permit No. 2014/384). All experimental procedures were performed in accordance with the National Institute of Health Principles of Laboratory Animal Care.

Animal sample preparation and surgical procedure

Forty-eight male Sprague–Dawley rats (200–250 g each) were divided into six groups of eight rats each. The rats were housed in separate cages during the test and were fed standard rodent food and water. They were fasted for 12 hours prior to the operation, but had *ad libitum* access to water. Paw mechanical withdrawal threshold, walking track analysis, and sciatic function index were measured between 9 and 15 hours of daylight.

Intraperitoneal sodium pentobarbital (50 mg/kg) was used to induce anesthesia during the surgical procedure in all groups. The right hind legs of the animals were de-epithelized with creams before the surgery, and the surgical field cleaned with povidone iodine solution. A skin incision was made on the right hip of the rats in the group 1 (sham) and the sciatic nerve within the gluteal muscle exposed. The surgical wound was closed without injury, and dimethyl sulfoxide (solvent, DMSO) was intraperitoneally administered. The same procedure was followed for rats in the group 2 (control) plus a force of 54 N was applied on the sciatic nerve for 60 seconds with an aneurysm clip placed 0.5 cm proximal to its trifurcation and the crush injury achieved. After the crush injury was inflicted in rats in group 3, 20 mg/kg genistein was administered intraperitoneally for 30 days. Rats in group 4 underwent the same procedure as in group 3. After inflicting the crush injury, 90 mg/kg gabapentin was administered intraperitoneally for 30 days. The sciatic nerve was sectioned transversely through the full-thickness in rats in group 5 and 6 and the truncated portions sutured epineurally at four sites with 9/0 nylon suture, aided by binocular loupes (Supervu loupes 2.5×42 cm). Subsequently, 20 mg/kg genistein and 90 mg/kg gabapentin was administered intraperitoneally for 30 days in the rats in groups 5 and 6, respectively.

Rats were sacrificed after the last walking track analysis and mechanical withdrawal threshold measurement at 4th week. The right hind limb was re-exposed and the sciatic nerve harvested en-bloc after careful dissection. The removed sciatic nerve was sent to the laboratory for immunohistochemical and biochemical analyses.

Immunohistochemical analysis

Tissue samples from each experimental group were fixed in neutral formalin for 72 hours and embedded in paraffin. Sections of 4-5 μm thickness were processed for polylysine microscope slides. Heat-induced antigen retrieval was performed to increase the sensitivity of immunohistochemical examination. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide. Epitopes were stabilized by application of serum-blocking solution. Slides were incubated overnight with myelin basic protein (MBP) antibody (C-16, Santa Cruz-13914) and growth-associated protein 43 (GAP-43) antibody (B-5, Santa Cruz-17790) at 4°C. After incubation, the secondary antibody was applied. AEC (3-amino-9-ethylcarbazole) was used as chromogen. Subsequently, the slides were counterstained with hematoxylin for 1 minute, dehydrated in graded ethanol, and mounted in a conventional medium. The sections were examined under a photomicroscope (BX51 Olympus, Japan).

Measurement of pro-inflammatory cytokine level in sciatic nerve

The levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in the sciatic nerve specimens were measured to evaluate the inflammatory response induced by peripheral nerve injury. The Rat TNF-α ELISA Kit (Bendermed BMS 622) and Rat IL-1 ELISA Kit (BMS Bendermed 630) were used for measurements. The sciatic nerve was resected in the whole group after 30 days and stored at −80°C. The nerve samples were homogenized in 0.25 mL of ice-cold phosphate buffered saline and centrifuged. The levels of TNF-α, IL-1β, and total protein in the supernatant were measured using the Lowry method with ELISA kits according to the manufacturer's instruction.

Walking Track analysis and sciatic function index (SFI)

Walking track analyses were performed in the animals at weeks 0 (after the surgery), 2, and 4 (before the sacrifice) using stamp and paper method (17). The lengths of the third toe to heel (PL), first to fifth toe (TS), and second toe to fourth toe (IT), on the experimental (E) and contralateral normal sides (N) were measured in all animals; sciatic function index (SFI) was calculated by using the following formula:

$$SF = -38.3 \times (EPL-NPL)/NPL + 109.5 \times (ETS-NTS)/NTS + 13.3 \times (EIT-NIT)/NIT - 8.8$$

SFI scores range from −100 (total dysfunction) to 0 (normal nerve function).

Paw mechanical withdrawal threshold measurement

Paw mechanical withdrawal threshold was measured using rat paw pressure analgesia-meter device (Ugo Basile, Italy), as described by Randall-Selitto (15). Quantitative measurement of pain was achieved by applying a mechanical force on the paws of the rats with a cone-shaped pusher with a rounded tip. The mechanical force (a maximum of 25 g) was applied with increasing weight towards the dorsal surface of the hind paws of the rats which had underwent surgery, and then the weight was measured at the time when they pulled their paws. The withdrawal threshold of paws was measured four times and the average values used in the analysis. The measurements were transferred to the computer using SeDaCom v 2.0 software program. Responses to mechanical stimuli were measured before the surgical intervention and after 7, 14, and 30 days after the surgery.

Statistical analysis

Data pertaining to all variables are presented as mean ± [Standard deviation (SD)]. SPSS version 15.0 was used to perform statistical analysis. Intergroup differences were

assessed by one-way Analysis of variance followed by Tukey's ad hoc test for multiple comparisons. p values of <0.05 were considered indicative of a statistically significant intergroup difference.

■ RESULTS

Immunohistochemical results

GAP-43 and MBP immunoreactivity were similar in the sham and control groups. GAP-43 and MBP immunoreactivity in the genistein groups were higher than those in the other groups. GAP-43 and MBP immunoreactivity were higher in the gabapentin groups compared with that in the sham and control groups, but lower than that in the genistein groups (Figures 1A-F and 2A-F). These results suggest that genistein improved nerve regeneration after traumatic nerve injury.

Pro-inflammatory cytokine levels in sciatic nerve

No significant different was observed between the genistein and gabapentin groups (crush+transection) with respect to the levels of IL-1β and TNF-α in the sciatic nerve samples ($p>0.05$). There were statistically significant differences between the control and sham groups ($p<0.001$) and among the control, genistein, and gabapentin groups ($p<0.001$) (Figures 3, 4).

Sciatic function index

A statistically significant difference in functional recovery was observed among the control group, crush injury+genistein, and crush injury+gabapentin groups at 2nd weeks ($p<0.01$). No significant difference was observed in this respect between the control and transection groups ($p>0.05$). However, at 4th weeks, a statistically significant difference was observed between the control and all treatment groups (genistein and gabapentin) (Figure 5).

Paw mechanical withdrawal threshold results

Seven days after injury, the mechanical withdrawal threshold significantly decreased in comparison to that in the sham-operated animals ($p<0.001$). On day 14, a statistically significant difference was observed between the sham and control groups ($p<0.001$) and between the sham and transection (+genistein, +gabapentin) groups ($p<0.05$). No statistically significant differences were observed between the sham and crush injury (+genistein, +gabapentin) groups in this respect ($p>0.05$). Further, no significant differences were observed between the sham and all treatment groups (genistein and gabapentin) on day 30 ($p>0.05$); however, a significant difference was observed between the sham and control groups on day 30 ($p<0.001$) (Figure 6).

■ DISCUSSION

Primary peripheral nerve injury results from compression, focal contusion, traction or transection of the nerve. This process is followed by secondary ischemic injury, which is dominated by inflammation (also referred to as degeneration).
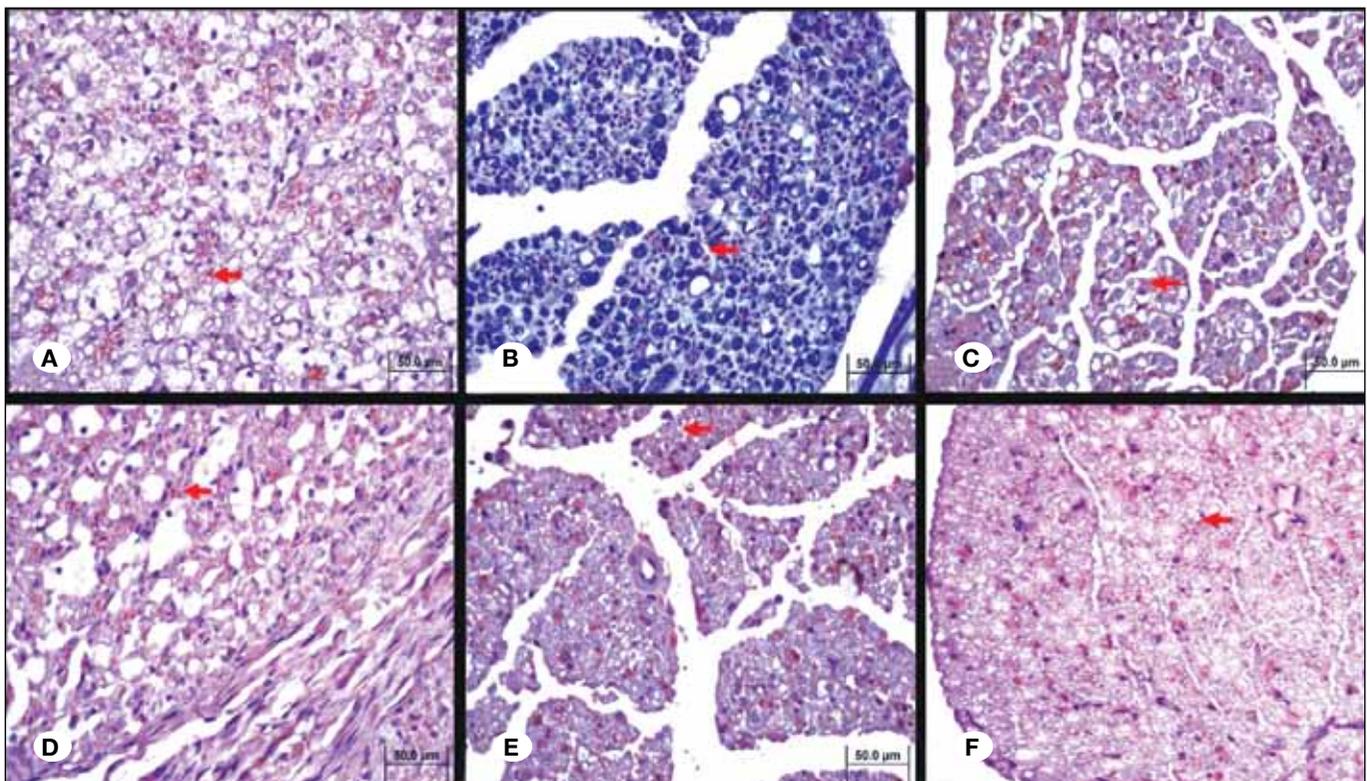

Figure 1: GAP-43 immunoreactivity. **A)** Sham, **B)** Control, **C)** Crush+Genistein, **D)** Crush+Gabapentin, **E)** Transection+Genistein, **F)**Transection+Gabapentin (GAP-43, x40).

Cytokines, and in particular, TNF-α, play a major role in this inflammatory response. The degeneration is followed by regeneration and recovery. The axon grows by forming axonal colonies in the regeneration period and a new myelin sheath is formed. The important immunohistochemical markers of regeneration are GAP-43 and MBP (1,22). Motor functional improvement and reduction in neuropathic pain are also important indicators of neurological recovery after peripheral nerve injury.

In this study, the effects of genistein and gabapentin were compared after experimental peripheral nerve injury in rats. The results were examined on immunohistochemistry (GAP-43, MBP), assessment of inflammatory cytokine expressions (IL-1β, TNF-α) and functional assessment (motor; SFI, neuropathic pain; paw withdrawal threshold). GAP-43 and MBP immunoreactivity in the genistein group was higher than that in the other groups on immunohistochemistry.
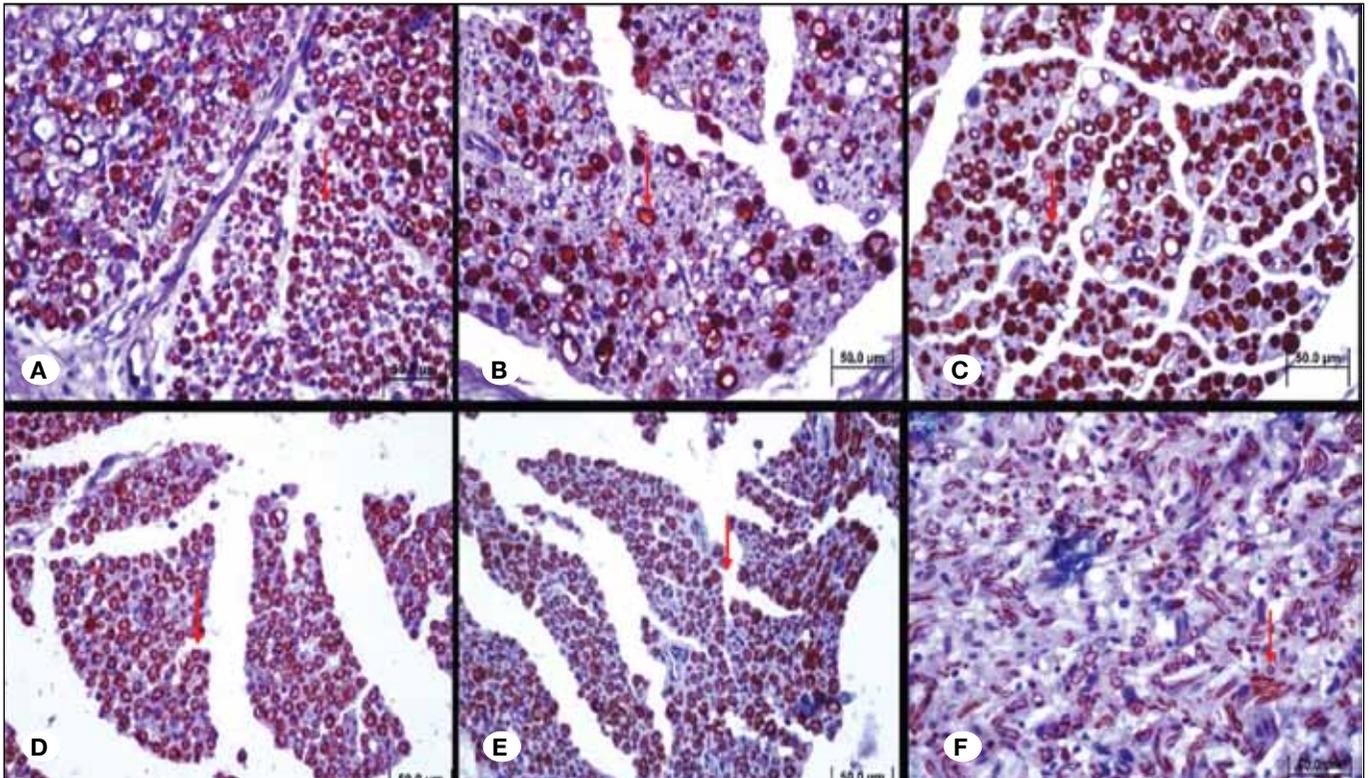
Figure 2: MBP immunoreactivity. **A)** Sham, **B)** Control, **C)** Crush+Genistein, **D)** Crush+Gabapentin, **E)** Transection+Genistein, **F)**Transection+Gabapentin (MBP, x40).
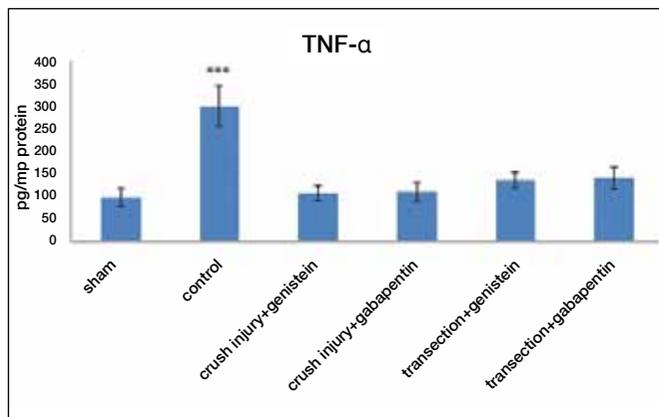
Figure 3: TNF-α levels. Bar graphs representing the TNF-α results as mean ± SD. There was no statistically significant difference between sham, genistein and gabapentin groups (p>0.05). There were statistically significant differences between the control group and the other groups (*** p<0.001).
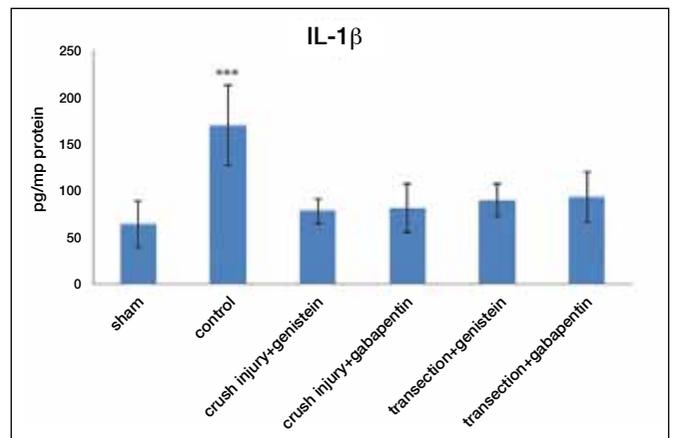
Figure 4: IL-1β levels. Bar graphs representing the IL-1β results as mean ± SD. There was no statistically significant difference between sham, genistein and gabapentin groups (p>0.05). - There were statistically significant differences between the control group and the other groups (*** p<0.001).
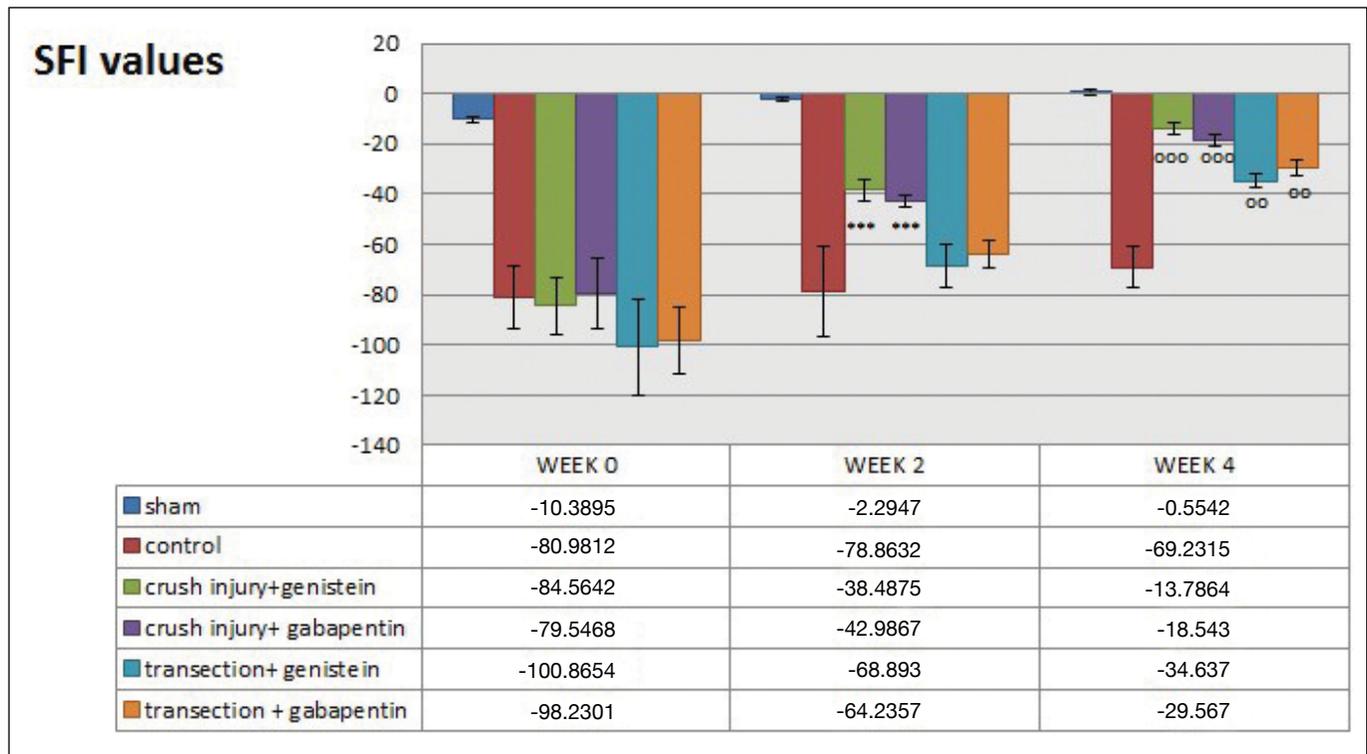
With respect to the inflammatory and functional results, the effects of genistein and gabapentin were found to be comparable.

Genistein is a natural phytoestrogen isoflavone extracted from soybean (24). Its protective effect against various cancers is well documented (19). Its neuroprotective effects have also been demonstrated in *in-vitro* models of cerebral ischemia (2,11). It was shown to remarkably reduce the neuropathic pain in diabetic peripheral neuropathy (24). A soy-rich diet was shown to attenuate hyperalgesia and allodynia after peripheral nerve injury (5), and these effects were attributed to genistein (23).

	WEEK 0	WEEK 2	WEEK 4
■ sham	-10.3895	-2.2947	-0.5542
■ control	-80.9812	-78.8632	-69.2315
■ crush injury+genistein	-84.5642	-38.4875	-13.7864
■ crush injury+ gabapentin	-79.5468	-42.9867	-18.543
■ transection+ genistein	-100.8654	-68.893	-34.637
■ transection + gabapentin	-98.2301	-64.2357	-29.567

Figure 5: SFI values. Bar graphs representing the SFI values as mean±SD. ***$p < 0.01$ versus control group at 2nd week. °°°$p<0.0001$, oop<0.001 versus control group at 4th week.
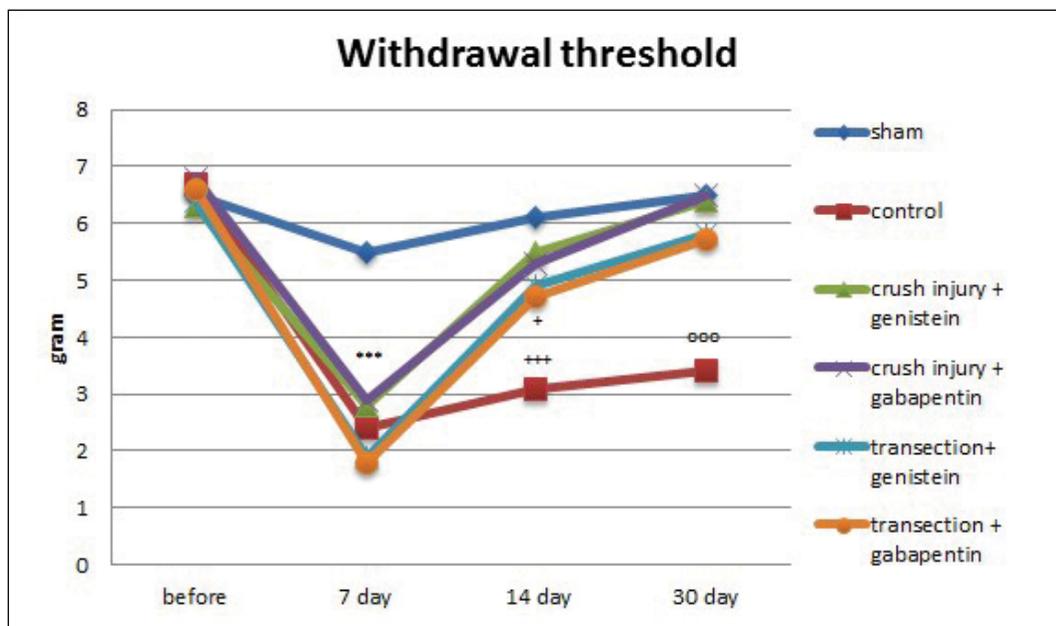
Figure 6: Mechanical withdrawal threshold results. ***$p<0.001$ versus sham group at 7th day. +++$p<0.001$, +$p<0.05$ versus sham group at 14th day. ooop<0.001 versus sham group at 30th day.

Wallerian degeneration occurring after peripheral nerve injury is known to be regulated by pro-inflammatory cytokines (12-14). The pathological cascade initiates at the site of injury and progresses to the dorsal root ganglia of the peripheral nerve. Subsequently, the glial cells and neurons participate in this cascade (14). Glial cells have been shown to activate microglial cells and astrocytes in the regional inflammation area (21). The most important cytokines of this cascade are TNF-α, IL-1β, and IL-6 (13,14). Of these, TNF-α is released by the schwann cells, mast cells, endothelial cells, and fibroblasts after injury and plays a key role in inducing upregulation of other inflammatory cytokines. Suppression of inflammatory cytokines after experimental peripheral nerve injury was shown to have a positive effect on neurological recovery (3,4,9,24). The potent anti-inflammatory effect of gabapentin after sciatic nerve injury in rats has been demonstrated (3,4,9). In our study, genistein and gabapentin showed a similar anti-inflammatory effect and reduced the level of cytokines in sciatic nerve tissue when compared with that in the control group.

In this study, the expressions of GAP-43 and MBP were measured to determine regeneration in the sciatic nerve injury. GAP-43 is a nervous tissue-specific structural protein which is present in the cytoplasm or on cell membrane in relation to the formation of axonal colonies and neuronal growth and plasticity (1,22). It has a high expression level in nerve cells during regeneration and axonal growth. MBP is one of the important proteins which play a role in the myelination in the nerve cells. In the present study, we found a significant elevation in expression levels of GAP-43 and MBP in genistein and gabapentin treatment groups and MBP compared with that in the sham and control groups.

The SFI is a standard metric for the evaluation of motor function, which reflects nerve function after peripheral nerve injury (7). In our study, a significant motor deficit was seen in the crush injury and transection groups compared with that in the sham group in the early period. While the motor improvement was more obvious in the crush injury+genistein and crush injury+gabapentin groups compared with that in the control group at 2 weeks, no significant difference was observed between the control and transection groups. A significant motor improvement was observed in all groups except the control group at 4 weeks. The motor improvement occurred later in the transection (+genistein, +gabapentin) groups compared to the crush injury (+genistein, +gabapentin) groups.

In our study, the paw mechanical withdrawal threshold test was performed to assess the effect of genistein on neuropathic pain (24). The results were compared with those of gabapentin, which is known to attenuate neuropathic pain in previous animal experiments (8,10). The pain threshold on day 7 was significantly lower in all groups compared with that in the sham group. On days 14 and 30, the pain threshold was significantly higher in the crush injury (+genistein, +gabapentin) groups as compared to that in the control group. A significant improvement occurred later (on day 30) in the transection (+genistein, +gabapentin) groups. Our findings suggest that the increase in the neuropathic pain threshold was similar in the genistein and gabapentin groups.

■ CONCLUSION

Genistein promotes the neurological recovery after peripheral nerve injury. Our study results indicate that use of genistein as an alternative and complementary treatment for nerve injury should be encouraged.

■ ACKNOWLEDGMENT

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