

# Scolopendra Pharmacopuncture Ameliorates Behavioral Despair in Mice Stressed by Chronic Restraint

Yu-Jin Choi<sup>1,2</sup>, Hwa-Young Lee<sup>2,3</sup>, Yunna Kim<sup>1,2</sup>, Seung-Hun Cho<sup>2,3\*</sup>

<sup>1</sup>Department of Clinical Korean Medicine, Graduate School, Kyung Hee University, Seoul, Korea

<sup>2</sup>Department of Neuropsychiatry, College of Korean Medicine, Kyung Hee University, Seoul, Korea

<sup>3</sup>Research Group of Neuroscience, East-West Medical Research Institute, WHO Collaborating Center, Kyung Hee University, Seoul, Korea

## Key Words

depression, glial fibrillary acidic protein, pharmacopuncture, Scolopendra, Scolopendra pharmacopuncture

## Abstract

**Introduction:** Pharmacopuncture, which combines acupuncture with herbal medicine, is one of the newly developed acupuncture techniques that has recently been put into use. The possible mechanisms of scolopendra pharmacopuncture, as well as its potential effects on depressive symptoms, were investigated in this study by using a mouse model of chronic immobilization stress (CIS).

**Methods:** C57BL/6 male mice were randomly assigned into three groups: mice not stressed with restraint and injected with distilled water, mice stressed with restraint and injected with distilled water, and mice stressed with restraint injected with scolopendra pharmacopuncture at a cervical site. Behavioral tests (an open field test, tail suspension test, and forced swimming test) were carried out after two weeks of CIS and injection treatments. The expression levels of glial fibrillary acidic protein (GFAP) in the hippocampus were determined by using western blot and immunohistochemistry analyses.

**Results:** Mice exposed to CIS showed decreased be-

havioral activity, while scolopendra pharmacopuncture treatment significantly protected against the depressive-like behaviors induced by CIS. Moreover, scolopendra pharmacopuncture treatment increased GFAP protein levels in the hippocampi of the mice stressed by chronic immobilization.

**Conclusion:** Scolopendra pharmacopuncture has an ameliorating effect on depressive behavior, which is partially mediated through protection against glial loss in the hippocampus.

## 1. Introduction

Major depressive disorder is a common [1] and is characterized by various symptoms, such as depressed or irritable mood, decreased interest in activities, inability to experience pleasure, loss of appetite, sleep disturbances, and inappropriate feelings of guilt and worthlessness [2]. Despite the significant progress in treating depression, novel treatments for depression with greater efficacy and fewer adverse effects are needed. Acupuncture, one of the most popular complementary and alternative treatments, has been reported to have a potential effect in the management of depressive symptoms [3, 4]. Additionally, many herbs have shown anti-depressant-like effects [5, 6]. Based on traditional Oriental treatments like acupuncture and herbal medicine, innovative therapies, including electroacupuncture, pharmacopuncture, and laser acupuncture, have been developed [7, 8].

Pharmacopuncture is a newly developed acupuncture technique that combines acupuncture with herb-

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\*Corresponding Author

Seung-Hun Cho, Kyung Hee University Medical Center, Kyung Hee University, 23, Kyungheedaero-ro, Dongdaemun-gu, Seoul, 02447, Republic of Korea  
E-mail address: chosh@khmc.or.kr Tel: +82 2 958 9186 Fax: +82 2 958 9187

al medicine. Through the injection of purified herbal extracts into acupuncture points, pharmacopuncture has the potential for use in the treatment of many different kinds of diseases [9]. However, little research on the topic of pharmacopuncture as a treatment for depression can be found in the literature. Sumsu (*Bufonis venenum*) pharmacopuncture, an animal-based pharmacopuncture, was demonstrated to have a positive effect on depressive-like symptoms in both animal and clinical research [10, 11]. Scolopendra pharmacopuncture, another animal-based pharmacopuncture, which is extracted from the giant scolopendrid centipede (*scolopendra subspinipes mutilans*), has been used clinically in traditional Korean medicine to control pain [12]. Furthermore, some antidepressants have an additional effect of relieving physical pain [13]. Taking all of this into consideration, we hypothesized that scolopendra pharmacopuncture might have an effect in ameliorating depressive behavior and might potentially relieve physical pain.

To address this topic, we used a mouse model of stress caused by chronic immobilization to investigate scolopendra pharmacopuncture's ameliorative effects on depressive symptoms. Further, we examined the possible mechanism of the antidepressant-like effects of scolopendra pharmacopuncture by examining the expression of glial fibrillary acidic protein in the hippocampus of the mouse brain.

## 2. Materials and Methods

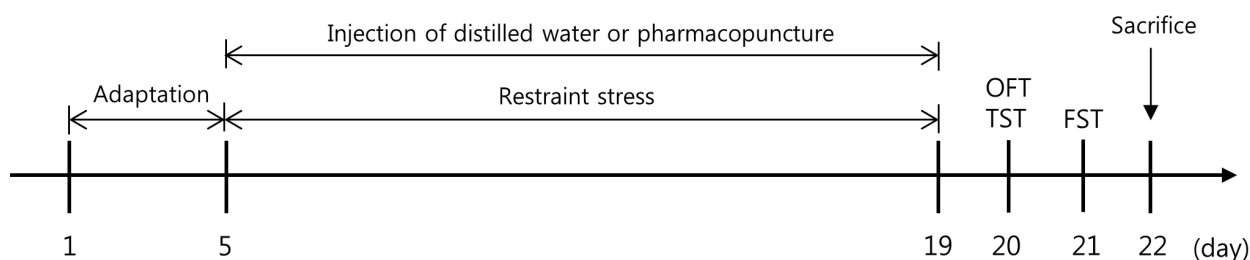
Male C57Bl/6 mice ( $25.3 \pm 1.4$  g), aged 7 weeks, were purchased from Orient Bio Inc., Korea ( $n = 30$ ). The mice were housed in acrylic cages  $2\text{ cm} \times 27\text{ cm} \times 12\text{ cm}$  under standard experimental conditions at constant temperature ( $22 \pm 2^\circ\text{C}$ ), humidity ( $60 \pm 10\%$ ), and light (12-hour light/12-hour dark cycle, with lights off at 6 pm). Animals were allowed free access to food and water and had a period of acclimation before the start of each experiment. The Kyung Hee University Medical Center Institutional Animal Care and Use Committee approved all procedures (KHMC-IACUC 16-033). All efforts were made to minimize animal suffering during the experiments. Scolopendra pharmacopuncture extract was obtained

from the Korean Pharmacopuncture Institute (Seoul, Korea) [14]. Mouse monoclonal antibodies against  $\beta$ -Actin and glial fibrillary acidic protein (GFAP) were obtained from Santa Cruz Biotechnology (CA, USA). Horseradish peroxidase-conjugated anti-mouse secondary antibody was purchased from Pierce Biotechnology (Rockford, IL, USA). Normal horse serum and biotinylated secondary antibody were purchased from Vector Laboratories (CA, USA).

After the five-day adaptation period, mice were randomly assigned to one of three parallel groups: control (no restraint + distilled water injection), negative control (restraint stress + distilled water injection), and experimental (restraint stress + scolopendra pharmacopuncture). Ten mice were assigned to each group. The negative control and the experimental groups were exposed to chronic immobilization stress (CIS) for two consecutive weeks (described in greater detail below) while the control mice remained undisturbed in their cages. During the CIS period, an injection of distilled water or scolopendra pharmacopuncture was administered daily prior to exposure to stress. The behavioral tests included the open field test, the tail suspension test, and a the forced swimming test, which were performed after two weeks of restraint stress and injection treatment (Fig. 1).

Chronic restraint stress was conducted as previously described [15-17]. Briefly, the mice were subjected to immobilization stress in a restrainer once a day for 2 hours from 10:00 am to 12:00 pm. Each mouse was placed in a restrainer (cylinder diameter of 30 mm x height of 95 mm), which restricted their movement completely. Immobilization stress was repeated once daily for 14 consecutive days. While the negative control and experimental groups were receiving restraint stress, the control group was left undisturbed and stayed in their cage without access to food and water.

Pharmacopuncture treatment was performed at the GV14 (Dazhui) acupoint, which is located on the posterior midline in the depression below the spinal process of the 7th cervical vertebra [18], by using a 1-mL disposable syringe with a 30-gauge needle to slowly inject 200  $\mu\text{L}$  of scolopendra extract into the mice in the experimental group. The depth of needle insertion was about 10 mm. Similarly, the control and the negative control groups also had



**Figure 1** Experimental schedule. Mice were randomly divided into three groups: control (no restraint stress + distilled water injection), negative control (restraint stress + distilled water injection), and experimental (restraint stress + scolopendra pharmacopuncture injection). After two weeks of restraint stress and injection of water or pharmacopuncture, behavior tests, including the open field test (OFT), tail suspension test (TST), and forced swimming test (FST), were performed.

200  $\mu$ L of distilled water injected in an identical fashion. Administration of scolopendra pharmacopuncture or distilled water was performed between 9:40 am to 10:00 am, prior to a CIS session.

The open field test (OFT) was carried out to assess locomotor activity [19]. Briefly, mice were placed in the center of a white, acrylic, plastic square box (50 cm  $\times$  50 cm) and allowed to freely explore the apparatus for 10 min. A video camera installed above the apparatus recorded the activity of the mice. The total distance traveled during the 10 min was evaluated using a computer-aided control system (SMART 3.0, Panlab Harvard Apparatus). The tail suspension test (TST) and the forced swimming test (FST) were conducted to study the effects of scolopendra pharmacopuncture on depression. For the TST, mice were suspended for 6 minutes 50 cm above the floor by adhesive tape placed 1 cm from the tip of the tail. The immobility time was defined after the 2-minute mark as the duration of time the animal hung passively and completely motionless during the remaining 4-minute period [20]. In the FST, mice were placed in an inescapable open cylindrical container (diameter of 20 cm, height of 35 cm) with 15 cm of water maintained at 25°C for a total of 6 minutes. The immobility time was defined as the time the mouse ceased struggling and floated motionless during the last 4 minutes of the 6-minute test, following an initial 2 minutes of activity [21].

After the behavioral testes had been completed, the hippocampus of each mouse was removed for a western blot analysis and stored in a freezer at -80°C. Samples were homogenized with RIPA buffer, and the supernatant was collected after centrifugation at 12,000 rpm. The concentration of protein in the supernatant was analyzed using the BCA protein assay kit (Pierce Biotechnology, IL, US). Extracts of 30  $\mu$ g of protein were resolved on a 12% SDS-polyacrylamide gel electrophoresis system and subsequently transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The primary antibodies used included mouse monoclonal anti-GFAP (1:2000) and mouse monoclonal anti- $\beta$ -actin (1:2000). The membranes were incubated overnight with specific primary antibodies, followed by incubation with horseradish peroxidase-conjugated anti-mouse secondary antibody. The signals were visualized using a chemiluminescent kit (Pierce Biotechnology, IL, US), and the immunoreactive bands were captured and quantified using Davinch-Chemi (Celltagen, Korea).

Mice were quickly anesthetized with diethyl ether and then perfused with phosphate-buffered saline (PBS), followed by a 4% paraformaldehyde solution. The brains were fixed in 4% paraformaldehyde for 24 h, followed by PBS containing 20% sucrose for 24 h. Ten- $\mu$ m-thick coronal sections of each brain were embedded in optimal cutting temperature (OCT) compound and cut with a cryostat. After the sections had been washed in PBS, they were incubated for 1 h at room temperature with 1% normal horse serum in PBS and incubated overnight at 4°C with primary antibody against the glial fibrillary acidic protein (GFAP) at 1:500 dilution in PBS, containing 2.5% normal horse serum. After the sections had been washed in PBS, they were incubated for 1 h at room temperature

with biotinylated secondary antibody (1:50) in PBS containing 2% normal horse serum and then subsequently incubated with ABC reagents (Vector Laboratories, CA, USA) in PBS. After the sections had been washed again in PBS, they were incubated in 3,3'-diaminobenzidine tetra hydrochloride (DAB; Dako, CA) and mounted in permount (Fisher Scientific International, USA). GFAP-positive cells were detected using a microscope (Nuance 2.10). All result values are expressed as means  $\pm$  standard errors of the mean (SEMs). Statistical differences in the means were analyzed using the one-way analysis of variance (ANOVA), followed by Dunnett's test for intergroup comparisons. For all the analyses, a P-value less than 0.05 was considered statistically significant. The analyses were conducted using SPSS 22.0 (IBM Inc., Armonk, NY, USA).

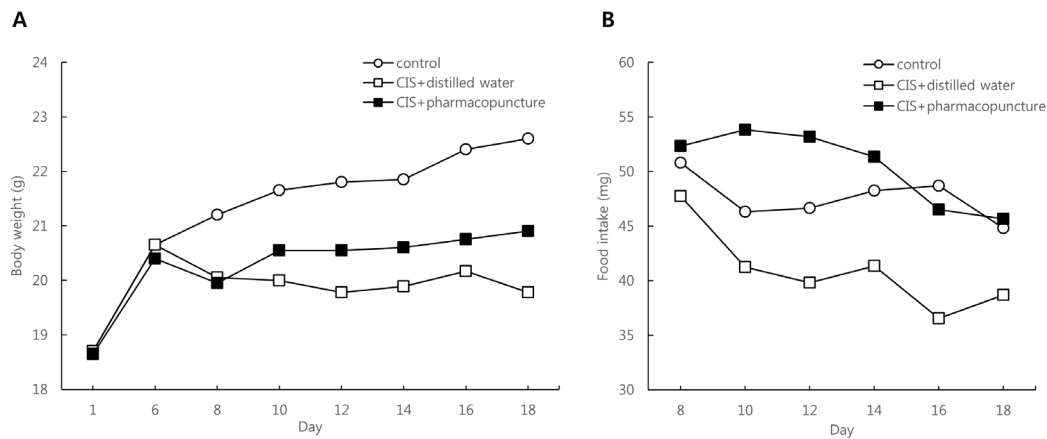
### 3. Results

The changes in body weight and food intake during the experimental period are shown in Fig. 2. Mice subjected to stress due to chronic restraint showed comparatively lower body weights than those in the control group did. Additionally, scolopendra pharmacopuncture treatment partially protected against the loss in weight caused by stress due to restraint. Moreover, stress due to restraint induced a decrease in the food intakes of mice injected with distilled water, but the food intakes of mice treated with scolopendra pharmacopuncture were higher than those of the mice in the two other groups.

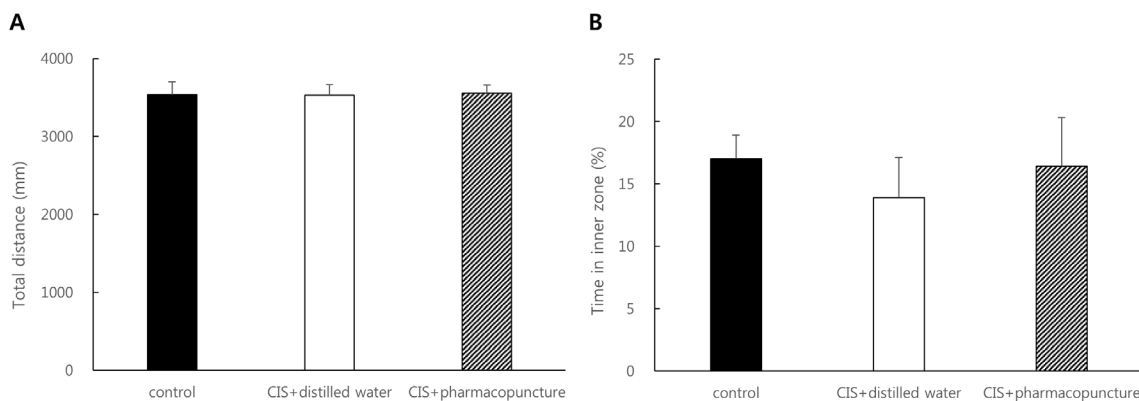
The locomotor activities of the mice were measured using open field tests (Fig. 3). No significant differences were observed among the groups as to the total distance moved ( $P = 0.993$ ). Thus, stress due to chronic restraint did not affect the locomotor activities of the mice injected with scolopendra pharmacopuncture. Figure 4 shows the effects of scolopendra pharmacopuncture on the immobility times of mice on the TST (Fig. 4A) and the FST (Fig. 4B). Compared to the control mice, mice stressed by restraint displayed significantly increased immobility times on the TST ( $P = 0.010$ ) and the FST ( $P = 0.033$ ). Scolopendra pharmacopuncture treatment effectively decreased those immobility times to the values for the control mice, indicating that scolopendra pharmacopuncture reversed or inhibited the behavioral despair induced by the stress of restraint ( $P = 0.001$ ,  $P = 0.043$ ).

The results of the western blot analysis are shown in Fig. 5. The protein levels of GFAP in the hippocampus in the brains of mice in the two groups stressed by chronic restraint were significantly lower than they were in the brains of the control mice that had been left undisturbed ( $P < 0.001$ ). In contrast, the protein levels of GFAP in the hippocampus in the brains of mice that had been injected with scolopendra pharmacopuncture were significantly higher than they were in the brains of the mice that had been stressed by chronic restraint and had been administered distilled water ( $P < 0.001$ ).

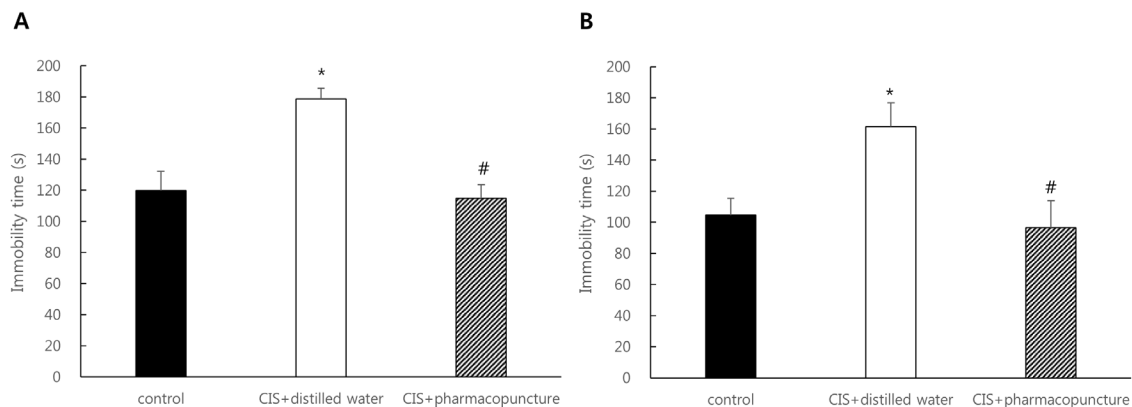
The immunoreactivity of GFAP in the CA3 region of the hippocampus is shown in Fig. 6. Slides from the control mice displayed moderate GFAP immunostaining in the CA3 region of the hippocampus (Figs. 6A and 6D) where-



**Figure 2** Effects of scolopendra pharmacopuncture on (A) body weight and (B) food intake in a mouse model of chronic immobilization stress. The body weights of the mice stressed by restraint were decreased compared to those of the control mice. The food intakes of the mice treated with scolopendra pharmacopuncture were higher than those of the mice treated with distilled water.

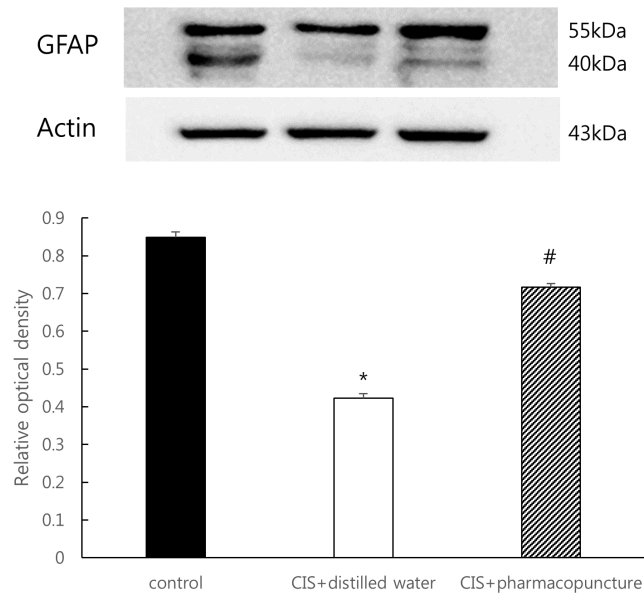


**Figure 3** Effects of scolopendra pharmacopuncture and chronic immobilization stress on (A) the total distance moved and (B) the time spent in the inner zone on the open field test (OFT). No significant differences were observed among the three groups.

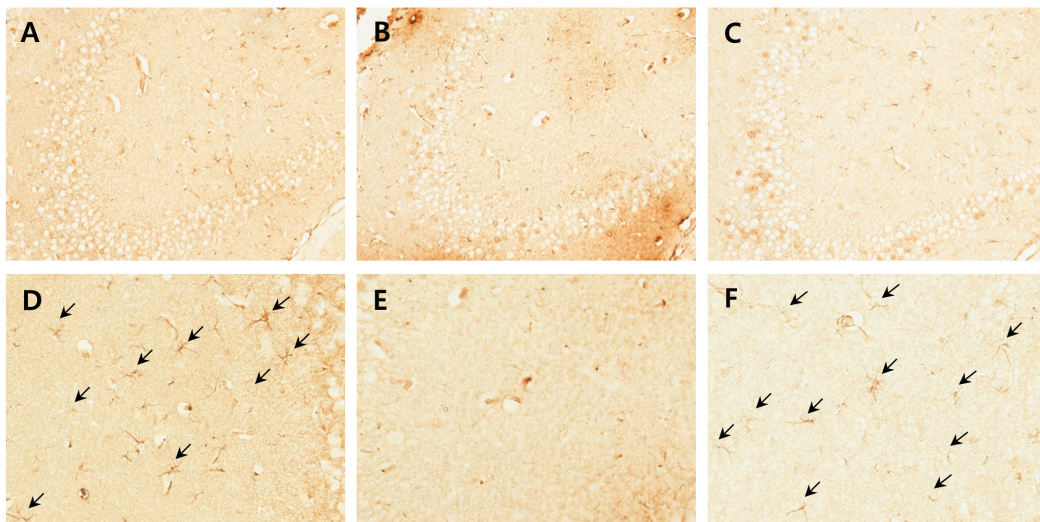


**Figure 4** Effects of scolopendra pharmacopuncture on the immobility times of mice on (A) the tail suspension test and (B) the forced swimming test. Each column represents the mean  $\pm$  the standard error of the mean (SEM). \*P < 0.05 as compared with the control group; #P < 0.05 as compared with the CIS + distilled water group.





**Figure 5** Effects of scolopendra pharmacopuncture on the immobility times of mice on (A) the tail suspension test and (B) the forced swimming test. Each column represents the mean  $\pm$  the standard error of the mean (SEM). \*P < 0.05 as compared with the control group; #P < 0.05 as compared with the CIS + distilled water group.



**Figure 6** Effects of scolopendra pharmacopuncture on glial fibrillary acidic protein (GFAP) expression in the hippocampus of the mouse brain. Images are shown for GFAP immunostaining in the CA3 region of the hippocampus of a mouse in (A and D) the control group, (B and E) the CIS + distilled water group, and (C and F) the scolopendra pharmacopuncture group.

as slides from the stressed mice treated only with distilled water revealed lowered immunostaining (Figs. 6B and 6E). Notably, slides from stressed mice treated with scolopendra pharmacopuncture also displayed a moderate level of GFAP immunostaining, resembling that seen in the slides of the control mice, in the CA3 region of the hippocampus (Figs. 6C and 6F).

#### 4. Discussion

The main finding of our study is that in a mouse model, scolopendra pharmacopuncture can alleviate the depressive symptoms induced by stress due to chronic immobilization. Importantly, scolopendra pharmacopuncture blocked weight loss and decreased food intake in mice stressed by chronic immobilization. Stressed mice treated with scolopendra pharmacopuncture showed increased activities on both the FSTs and the TSTs compared to stressed mice injected with distilled water. Scolopendra pharmacopuncture appeared to protect against the decrease in GFAP expression induced by stress due to chronic immobilization. Therefore, the results indicate that scolopendra pharmacopuncture may offer a potential alternative treatment for depressive disorders.

Loss of appetite and weight loss are neurovegetative symptoms of depressive disorders. Increased immobile times on the TST and the FST reflect a state of despair as is often observed in cases of depression. In the present study, scolopendra pharmacopuncture was able to maintain the level of food intake and to decrease immobile times on the FST and the TST without affecting the mouse's locomotor activity. Thus, these results suggest that scolopendra pharmacopuncture attenuates the depressive-like symptoms produced by the mouse model of stress due to chronic immobilization.

GFAP is a marker of astrocytes, whose pathology (i.e., reductions in astrocytes) contributes significantly to major depressive disorder [22, 23]. For example, a significant decrease in the density of GFAP-immunoreactive astrocytes was reported in the left hippocampus of patients with untreated major depressive disorder [24]. However, that decreased expression of astrocytes in the hippocampus was reversed by using clomipramine, fluoxetine, magnolol extract, and electroacupuncture treatments [25-27]. In this study, we demonstrated that stress due to chronic immobilization induced a decreased expression of GFAP in the hippocampus, which was ameliorated with scolopendra pharmacopuncture treatment; i.e., scolopendra pharmacopuncture was able to protect against the reduction in GFAP expression induced by stress due to chronic immobilization.

Pharmacopuncture is a newly developed acupuncture technique that combines acupuncture with herbal medicine. This technique, which involves injecting a purified extract into specific points on the body, might be both effective and economical. To date, a few attempts have been made to develop pharmacopuncture treatments for mental health [28]. Previous animal studies and clinical research have demonstrated that Sumsu (*Bufonis venenum*) pharmacopuncture [10, 11], *Hominis Placenta*

pharmacopuncture [29], *Hwangryunhaedok-tang* pharmacopuncture [30], and *Poria Cocos* pharmacopuncture [31] may have positive effects on depressive behavior. The results of our study suggest that scolopendra pharmacopuncture may be a potential alternative therapy for patients suffering from depression.

An acupuncture point of injection is not only important for the purposes of administering herbal extract, but is also an important therapeutic element of pharmacopuncture. Hence, we chose the GV14 (Dazhui) acupuncture point, which is located in the cervical region. Previous studies have reported that acupuncture, electro-acupuncture, and moxibustion at GV14 (Dazhui) ameliorate the symptoms of depression [32-35]. In this study, we injected purified scolopendra extract into the GV14 (Dazhui) site and found that treatment to be effective in treating depressive behavior in a mouse model of depression caused by stress due to chronic immobilization.

Scolopendra pharmacopuncture extract contains various amino acids, such as aspartate, arginine, alanine, leucine, and lysine [14]. The composition of centipede venom includes serotonin, histamine, lipids, polysaccharides, and polypeptides [36]. Scolopendra pharmacopuncture is usually used to treat entrapment neuropathy and inflammatory disease; it also has anti-convulsive, analgesic, anti-inflammatory, and anti-tumor effects and can lower blood pressure [12]. However, no evidence that scolopendra pharmacopuncture can effectively treat depressive disorders has been found.

An in-vivo study reported that scolopendra pharmacopuncture alleviated neuropathic pain induced by L5 spinal nerve ligation in rats. Li et al. showed that scolopendra pharmacopuncture induced deactivations of microglia and astroglia in the spinal cords of rats [37]. Those findings are consistent with our result that scolopendra pharmacopuncture induces activation of astroglia in the hippocampus of the mouse brain. In the model of neuropathic pain, acupoint KI1, which is located in the metatarsal area, was selected; this differs from GV14, which is located in the cervical region and was used in our study. Some suggest that scolopendra pharmacopuncture might work differently on astroglial activation, depending on the target disease and the points of injection. Therefore, further research on the effects of scolopendra pharmacopuncture on pain control and depression management is warranted. Overall, this study suggests that scolopendra pharmacopuncture might be an effective alternative treatment for patients with depression, as well as for patients suffering with pain.

#### 5. Conclusions

The present study demonstrates the potential effects of scolopendra pharmacopuncture on depressive symptoms. Here, we show that scolopendra pharmacopuncture has the ability to protect the mouse brain from stress-induced glial loss that can occur in the hippocampi of chronically-immobilized mice. The results suggest that scolopendra pharmacopuncture might serve as another potential therapy for patients with depression. However, further

research and clinical trials are required to understand the effects of scolopendra pharmacopuncture on depressive behavior.

## Conflict of interest

All authors declared that they have no conflicts of interests.

## Acknowledgements

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