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Scorpion ethanol extract and valproic acid effects on hippocampal glial fibrillary acidic protein expression in a rat model of chronic-kindling epilepsy induced by lithium chloride-pilocarpine[★]

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Abstract

The present study analyzed the effects of ethanol extracts of scorpion on epilepsy prevention and hippocampal expression of glial fibrillary acidic protein in a lithium chloride-pilocarpine epileptic rat model. Results were subsequently compared with valproic acid. Results showed gradually-increased hippocampal glial fibrillary acidic protein expression following model establishment; glial fibrillary acidic protein mRNA expression was significantly increased at 3 days, reached a peak at 7 days, and then gradually decreased thereafter. Ethanol extracts of scorpion doses of 580 and 1 160 mg/kg, as well as 120 mg/kg valproic acid, led to a decreased number of glial fibrillary acidic protein-positive cells and glial fibrillary acidic protein mRNA expression, as well as decreased seizure grades and frequency of spontaneously recurrent seizures. The effects of 1 160 mg/kg ethanol extracts of scorpion were equal to those of 120 mg/kg valproic acid. These results suggested that the anti-epileptic effect of ethanol extracts of scorpion were associated with decreased hippocampal glial fibrillary acidic protein expression in a rat model of lithium chloride-pilocarpine induced epilepsy.

Key Words: Chinese herbs; epilepsy; glial fibrillary acidic protein; lithium chloride-pilocarpine; scorpion ethanol extraction; valproic acid

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INTRODUCTION

The brain undergoes a variety of pathological changes in response to seizure, such as neuronal lesions, as well as significant abnormalities in astrocyte morphology and function^[1]. Importantly, astrocytic functional changes could affect neuronal functions; astrocyte proliferation is a reliable and sensitive indicator for neuronal damage^[2]. The astrocyte activation pathways and underlying mechanisms have been the focus in recent studies, although the mechanisms of actions remain unclear. Glial fibrillary acidic protein (GFAP) is a specific marker for astrocytes, and a small number of GFAP-positive cells are visible in the hippocampus and cerebral cortex under normal circumstances. However, GFAP expression significantly increases following status epilepticus induced by a variety of chemical stimulations and electrical stimulations^[3]. Therefore, it is possible cerebral injury could be reduced by preventing the proliferation of hippocampal astrocytes following epilepsy. *Buthus martensii* Karsch, a widely

distributed scorpion species in Asia, it has been used in Chinese traditional medicine for a long time to treat epilepsy. However, the antiepileptic mechanisms remains poorly understood and the scorpion components remain difficult to determine. It has been speculated that the anti-epileptic effects of scorpion are associated with inhibition of hippocampal astrocyte activity.

To verify this hypothesis, the present study analyzed the anti-epileptic effects of ethanol extracts of scorpion (EES) and the influences on hippocampal GFAP protein and mRNA expression in rat models of chronic epilepsy induced by lithium chloride-pilocarpine. Results were then compared with valproic acid, a typical anti-epileptic drug used in the clinic.

RESULTS

Quantitative analysis of experimental animals

A total of 186 Sprague-Dawley rats were included in the present study. With exception of the control group ($n = 6$), chronic epilepsy was induced by lithium chloride-pilocarpine injection in the

remaining 180 rats and model failure was appropriately supplemented. The 180 successful model rats were randomly assigned to five groups: model (intragastrical injection of normal saline after modeling), valproic acid (intragastrical injection of valproic acid after modeling), low-, medium-, and high-dose EES (intragastrical injection of 290, 580, and 1 160 mg/kg EES after modeling). Six rats were selected from each time point at 6 hours, 1, 3, 7, 14, and 30 days after emergence of epileptic symptoms. All 186 rats were included in the final analysis.

Behavioral changes of epileptic rats

Following lithium chloride injection, the rats did not exhibit behavioral abnormalities and were freely active. However, following pilocarpine injection, some rats exhibited peripheral cholinergic activation (M-like effect) within 5 minutes, which were characterized by miosis and erected hair, as well as limbic epileptic behaviors, such as erection, running, and wet dog-like trembling. Grade I-II seizure occurred 5–15 minutes later, characterized by convulsive seizure in the forelimbs and standing at a half-upright position. Continuous seizure grading of IV or greater was observed 17–27 minutes later; the entire body and limbs experienced muscular contraction and loss of postural control, and each attack lasted for 30–120 seconds at intervals of 2–15 minutes. Status epilepticus was determined by the appearance of persistent seizure for 10 minutes; models of status epilepticus were successfully induced in 85.65% of the rats. The surviving rats entered a quiet period (7–14 days), exhibiting the following: crouching, no appetite, and extreme irritability. During this period, no

seizures were observed. Normal behaviors gradually recovered and some rats exhibited spontaneous recurrent seizure (SRS) after the quiet period. SRS emerged as an appearance of localized seizure, such as facial convulsion or unilateral forelimb clonus, which developed into global stiffness and seizures, usually lasting for < 1 minute. However, all attacks were self-terminated. The control rats exhibited no abnormal behaviors during the observation period.

Effects of varying doses of EES on hippocampal GFAP expression in epileptic rats

Immunohistochemical staining and light microscopy analysis revealed a brown-stained cytoplasm in GFAP-positive cells. Staining in the nuclei was absent, and cells were rounded or irregular in shape and were surrounded by radial processes. A small number of GFAP-positive cells was observed in the hippocampus of control rats. Some hippocampal GFAP-positive cells were visible at 6 hours after status epilepticus in the model group, and the cells were primarily located in the CA region and dentate gyrus. By 30 days, the number of positive cells significantly increased and reached a peak; the cells were darkly stained and large, with many branches and processes. The cells appeared thickened with a distorted alignment and clear boundaries. The cells were typically star-shaped, especially in the dentate gyrus. Changes in the number of GFAP-positive cells in other treatment groups were consistent with the model group, although the number of GFAP-positive cells was significantly less than the model group. Following treatment, the GFAP-positive cells were smaller and extended thin processes (Figure 1).

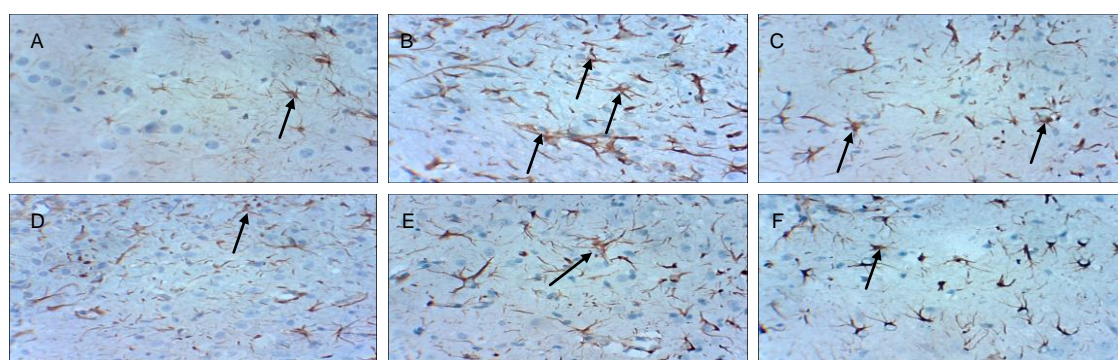


Figure 1 Glial fibrillary acidic protein (GFAP; arrows) expression in rat dentate gyrus at 30 days after model establishment (streptavidin biotin method; $\times 400$). EES: Ethanol extracts of scorpion.

(A) Control group: GFAP-positive cells exhibit distinct boundaries and brown cytoplasm.

(B) Model group: GFAP-positive cells are shaped like a spider, surrounded by radial protrusions, and the number of GFAP-positive cells is significantly increased.

(C) Valproic acid group: GFAP-positive cells exhibit distinct boundaries, typical star-shape appearance, and the number of cells is significantly less than the model group.

(D) EES low-dose group: GFAP-positive cells are shaped like a spider and surrounded by radial protrusions, but expression is lighter and the number of protrusions is less than the model group.

(E) EES moderate-dose group: GFAP-positive cells are shaped like a spider and surrounded by radial processes, but expression is significantly less and the number of protrusions is significantly less than the model group.

(F) EES high-dose group: GFAP-positive cells exhibit clear boundaries and a typical star shape; the number of cells is less than the model group.

There was no significant difference in the number of GFAP-positive cells between the low-dose EES group and model group, but the difference was statistically significant between other treatment groups and the model group ($P < 0.05$). The most significant difference was in the high-dose EES group and valproic acid group, with no significant difference between them (Tables 1–3).

Effects of varying EES doses on hippocampal GFAP mRNA expression in epileptic rats

Reverse transcription-PCR analysis showed significantly increased GFAP mRNA levels in the hippocampus of

model rats at 3 days, which reached a peak at 7 days. GFAP mRNA expression gradually decreased, but did not reach normal levels, by 30 days (Figure 2). EES treatment at varying doses, as well as valproic acid treatment, resulted in significantly decreased GFAP mRNA levels compared with the model group. There were significant differences in EES high- and moderate-dose groups and valproic acid group compared with the model group ($P < 0.05$). The GFAP mRNA expressions returned to normal levels at 30 days in the EES high-dose group and valproic acid group (Table 4).

Table 1 Quantification of cells (cells/400-fold field of view) expressing glial fibrillary acidic protein in the hippocampal CA1 area at different time points

| Group | Status epilepticus time | | | | | |
|-------|-------------------------|--------------------------|--------------------------|-------------------------|-------------------------|-------------------------|
| | 6 hours | 1 day | 3 days | 7 days | 14 days | 30 days |
| A | | | | 10.00±2.83 | | |
| B | 11.50±1.87 | 23.83±1.47 | 23.83±3.06 | 24.50±1.87 | 26.00±4.05 | 26.83±3.82 |
| C | 12.83±2.40 | 16.50±3.62 ^a | 17.33±2.66 ^a | 20.00±2.76 ^a | 21.00±3.03 ^a | 21.50±1.87 ^a |
| D | 13.17±2.14 | 21.17±2.56 ^b | 22.00±4.05 ^b | 23.67±2.16 ^b | 24.00±3.63 | 25.33±3.98 ^b |
| E | 13.00±3.29 | 16.00±4.47 ^{ac} | 18.50±4.14 ^a | 21.00±2.19 ^a | 21.83±2.32 ^a | 22.67±2.16 ^a |
| F | 12.33±3.01 | 16.83±5.12 ^{ac} | 17.67±3.27 ^{ac} | 20.83±3.25 ^a | 21.67±3.98 ^a | 22.17±2.32 ^a |

Data are expressed as mean ± SD from six rats at each time point; ^a $P < 0.05$, vs. group B; ^b $P < 0.05$, vs. group C; ^c $P < 0.05$, vs. group D (analysis of variance, pairwise comparison was tested using least significant difference test or q -test). (A) Control group; (B) model group; (C) valproic acid group; (D–F) low-, medium-, and high-dose ethanol extracts of scorpion groups.

Table 2 Quantification of cells (cells/400-fold field of view) expressing glial fibrillary acidic protein in the hippocampal CA3 area at different time points

| Group | Status epilepticus time | | | | | |
|-------|-------------------------|--------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| | 6 hours | 1 day | 3 days | 7 days | 14 days | 30 days |
| A | | | | 11.50±2.66 | | |
| B | 9.50±1.38 | 19.67±4.13 | 23.17±3.66 | 25.33±3.39 | 27.50±4.85 | 28.50±5.24 |
| C | 10.50±3.08 | 15.33±1.75 ^a | 17.33±2.88 ^a | 21.00±3.41 ^a | 22.33±4.63 ^a | 21.50±3.83 ^a |
| D | 12.67±2.66 | 19.17±3.87 ^b | 20.17±3.76 | 24.50±3.51 ^b | 26.33±4.41 | 27.33±4.08 ^b |
| E | 13.17±3.66 ^b | 17.33±2.58 | 18.83±2.93 ^a | 21.83±2.14 ^a | 22.67±3.33 | 23.83±3.19 ^a |
| F | 12.83±3.92 | 15.50±2.17 ^{ac} | 18.33±2.16 ^a | 21.33±1.86 ^a | 22.33±4.93 ^a | 23.67±2.94 ^a |

Data are expressed as mean ± SD of six rats at each time point; ^a $P < 0.05$, vs. group B; ^b $P < 0.05$, vs. group C; ^c $P < 0.05$, vs. group D (analysis of variance, pairwise comparison was tested using the least significant difference test or q -test). (A) Control group; (B) model group; (C) valproic acid group; (D–F) low-, medium-, and high-dose ethanol extracts of scorpion groups.

Table 3 Quantification of cells (cells/400-fold field of view) expressing glial fibrillary acidic protein in the hippocampal dentate gyrus at different time points

| Group | Status epilepticus time | | | | | |
|-------|-------------------------|-------------------------|-------------------------|--------------------------|---------------------------|--------------------------|
| | 6 hours | 1 day | 3 days | 7 days | 14 days | 30 days |
| A | | | | 10.50± 1.97 | | |
| B | 14.83±3.06 | 26.17±5.49 | 29.33±5.50 | 30.50± 3.89 | 33.83±3.76 | 35.00±8.65 |
| C | 13.00±2.37 | 19.67±3.61 ^a | 22.33±3.08 ^a | 25.17±4.17 ^a | 26.83±4.26 ^a | 28.67±3.20 ^a |
| D | 14.33±1.21 | 25.67±4.13 ^b | 27.17±3.54 | 29.00±6.48 | 34.00±2.83 ^b | 34.33±6.71 |
| E | 14.33±1.51 | 21.83±2.56 ^a | 24.17±6.05 | 24.33±4.37 ^a | 28.67±4.97 ^{abc} | 29.50±3.73 |
| F | 13.33±2.25 | 21.67±3.01 ^a | 23.33±6.92 ^a | 23.50±4.37 ^{ac} | 27.17±5.27 ^{ac} | 27.50±3.94 ^{ac} |

Data are expressed as mean ± SD from six rats at each time point; ^a $P < 0.05$, vs. group B; ^b $P < 0.05$, vs. group C; ^c $P < 0.05$, vs. group D (analysis of variance, pairwise comparison was tested using least significant difference test or q -test). (A) Control group; (B) model group; (C) valproic acid group; (D–F) low-, medium-, and high-dose ethanol extracts of scorpion groups.

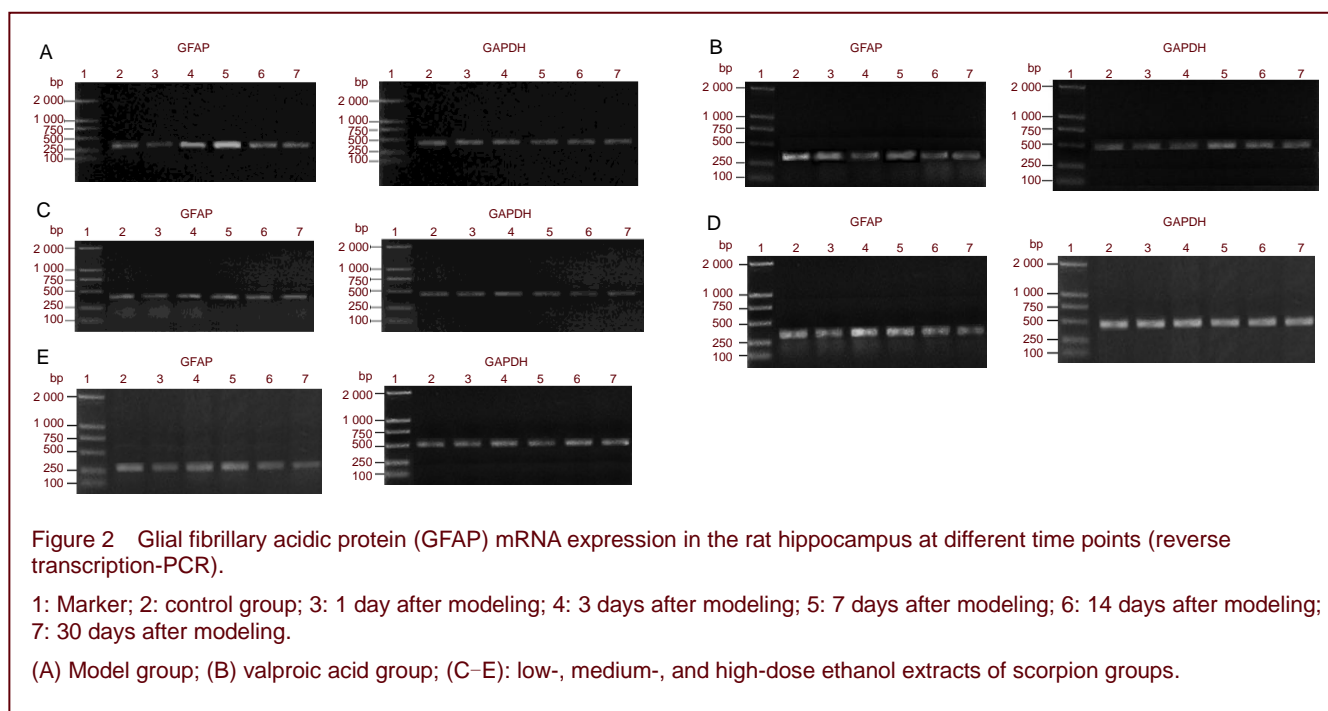


Figure 2 Glial fibrillary acidic protein (GFAP) mRNA expression in the rat hippocampus at different time points (reverse transcription-PCR).

1: Marker; 2: control group; 3: 1 day after modeling; 4: 3 days after modeling; 5: 7 days after modeling; 6: 14 days after modeling; 7: 30 days after modeling.

(A) Model group; (B) valproic acid group; (C-E): low-, medium-, and high-dose ethanol extracts of scorpion groups.

Table 4 Glial fibrillary acidic protein (GFAP) mRNA expression (absorbance ratio to GAPDH) in the rat hippocampus at different time points

| Group | Status epilepticus time | | | | |
|-------|-------------------------|-------------------------------|------------------------------|------------------------------|-------------------------------|
| | 1 day | 3 days | 7 days | 14 days | 30 days |
| A | | | 0.691 0±0.127 8 | | |
| B | 0.648 3±0.262 3 | 1.140 8±0.091 2 | 1.555 2±0.388 0 | 1.205 4±0.282 8 | 0.926 1±0.182 3 |
| C | 0.788 5±0.230 4 | 0.901 0±0.150 8 ^a | 0.942 8±0.141 3 ^a | 0.852 8±0.160 5 | 0.868 7±0.239 7 |
| D | 0.652 3±0.089 4 | 1.140 5±0.077 9 ^b | 1.136 3±0.113 9 | 1.297 5±0.088 0 ^b | 1.405 9±0.164 4 ^{ab} |
| E | 0.689 3±0.269 9 | 1.088 5±0.060 8 | 1.031 9±0.371 9 ^a | 1.043 9±0.141 3 | 1.088 5±0.024 9 ^c |
| F | 0.781 9±0.223 8 | 0.927 7±0.137 5 ^{ac} | 0.941 2±0.149 0 ^a | 0.886 5±0.148 1 ^c | 0.872 0±0.228 7 ^c |

In preliminary experiments, reverse transcription-PCR experiment was repeated at 6 hours after seizure, but amplification was not successful, so GFAP mRNA expression was not quantitatively analyzed at 6 hours. Data are expressed as mean ± SD from six rats at each time point. ^a*P* < 0.05, vs. group B; ^b*P* < 0.05, vs. group C; ^c*P* < 0.05, vs. group D (analysis of variance, pairwise comparison was tested using least significant difference test or *q*-test).

(A) Control group; (B) model group; (C) valproic acid group; (D-F): low-, medium-, and high-dose ethanol extracts of scorpion groups.

Assessment of antiepileptic efficacy of varying doses of EES and valproic acid treatment

Epileptic rats exhibiting shivering convulsions or status epilepticus lasting for > 1 hour exhibited collapse and limb clonus within 4 minutes after diazepam and atropine injection. Status epilepticus disappeared at 30–80 minutes, but seizures with grade IV and V repeatedly occurred. In the control group, no seizures or SRS were observed. Model rats were treated with corresponding treatments at 15 minutes after seizure. Treatment of EES at varying doses significantly alleviated epileptic seizure and significantly reduced the frequency of epileptic seizures with grade IV or greater and SRS in rats. There were significant differences in these observed effects in the EES moderate- and high-dose groups and valproic acid group compared with the model group (*P* < 0.05; Tables 5, 6).

Table 5 Quantification of rats with different levels of seizure grades

| Group | <i>n</i> | Seizure grade (levels) | |
|-----------------|----------|------------------------|------------------|
| | | ≤ III | IV–V |
| Control | 6 | 0 | 0 |
| Model | 36 | 6 | 30 |
| Valproic acid | 36 | 24 | 12 ^{ab} |
| Low-dose EES | 36 | 11 | 25 |
| Medium-dose EES | 36 | 24 | 12 ^{ab} |
| High-dose EES | 36 | 25 | 11 ^{ab} |

There are 36 rats in each group, except six rats in the control group. ^a*P* < 0.05, vs. model group; ^b*P* < 0.05, vs. low-dose EES group (chi-square test and rank sum test). EES: Ethanol extract of scorpion.

Table 6 Quantification of total number of spontaneous recurrent seizures in rats

| Group | n | Seizure grade (levels) | | Total number |
|-------------------|----|------------------------|------|-------------------|
| | | ≤ III | IV-V | |
| Control | 6 | 0 | 0 | 0 |
| Model | 36 | 126 | 198 | 324 |
| Valproic acid | 36 | 84 | 48 | 132 ^{ab} |
| Low-dose EES | 36 | 96 | 162 | 258 |
| Moderate-dose EES | 36 | 120 | 84 | 204 ^a |
| High-dose EES | 36 | 102 | 60 | 162 ^a |

There are 36 rats in each group, except six rats in the control group. ^a $P < 0.05$, vs. model group; ^b $P < 0.05$, vs. low-dose EES group (chi-square test and rank sum test). EES: Ethanol extract of scorpion.

DISCUSSION

Temporal lobe epilepsy is the main type of refractory epilepsy in adults, and typical pathological changes include hippocampal sclerosis and atrophy that involves hippocampal and parahippocampal structures^[4]. Seizure results in the loss of hippocampal neurons, which is thought to contribute to latency (epilepsy) gliosis and loop reconstruction-induced recurrent seizures, thereby leading to lasting brain damage, cognitive impairment^[5], and increased risk of recurrent seizures^[6]. The rat model of lithium chloride/pilocarpine-induced epilepsy is internationally recognized as an ideal animal model for studying temporal lobe epilepsy in humans^[7]. The present study established a rat model of lithium chloride/pilocarpine-induced chronic epilepsy to analyze the anti-epileptic effect of EES in a dose-dependent manner. The role of glial cells in epilepsy pathogenesis has been associated with the regulation of glutamate concentrations and suppressed neuronal hyperexcitability. Neurons are more prone to damage by diffuse excitation waves if normal functions of glial cells are absent^[8]. Injured local tissue and neuronal loss are often accompanied by astrocyte activation and increased GFAP expression^[9]. Following pilocarpine-induced epilepsy, hippocampal astrocytes become activated, the number of GFAP-positive cells increases, and astrocytes proliferate^[10]. These changes are crucial for the formation and maintenance of seizures. Increased GFAP expression is a hallmark for active protein synthesis and transportation in astrocytes, as well as indicator for astrocyte activation; expression levels have been associated with reactive gliosis following neuronal injury^[11-12].

Results from the present study demonstrated that GFAP protein and mRNA expression changes were not associated with time in a rat model of lithium chloride-pilocarpine-induced epilepsy, although expression levels were dynamic. The number of GFAP-positive cells increased at 1 day after epilepsy, and GFAP mRNA levels significantly increased at 3 days,

reached a peak at 7 days, then gradually decreased. Steward *et al*^[13] performed dot blot assays, showing that GFAP mRNA expression in the hippocampus is biphasic following brain injury; the first peak occurs at 1–2 days (10 times the control group) with a second peak at 6–8 days, whereas GFAP expression gradually increases. The human GFAP gene promoter contains a cAMP-responsive binding protein, which correspond to transcription factors, such as Sp1, NF1, Ap1, and Ap2^[14]. Wang *et al*^[15] suggested that fos protein is expressed in astrocytes of pentylenetetrazol-induced epileptic rats. Fos protein binds with jun proteins, forming a transcription factor, and binding with Ap1 sites might lead to increased GFAP expression. Besnard *et al*^[14] demonstrated that the *gfa* gene modulates GFAP expression, but the mechanisms underlying c-fos/c-jun modulation of the *gfa* gene and increased GFAP mRNA transcription remain poorly understood.

We speculated that early astrocyte responses and repair mechanisms were different from the pathophysiological mechanisms occurring during the late period of damage. Early astrocyte activation is likely achieved *via* signal transduction pathways. During the process of scar formation, GFAP mRNA transcription levels in brain tissue are down-regulated *via* inherent negative feedback mechanisms, thereby controlling the formation of the glial scar.

Currently used anti-epileptic drugs exhibit efficacy *via* ion channels, metabolic enzymes, and neurotransmitter transporters, thereby altering the discharge nature of neurons, inhibiting diffusion of epileptic discharge, reducing synchronous discharge^[16], and modulating learning, memory, and emotional behaviors^[17]. These results suggest that the use of anti-epileptic drugs for preventing seizures are also likely to induce damages to brain function and development^[17], resulting in learning, memory, and other cognitive functional deficits in patients, in particular the fetus and newborn. Epilepsy treatment remains challenging in the clinic, and many treatments for epilepsy are still in the exploration stage. Scorpion is a dried arachnid from the *Mesobuthus martensii* Karsch family and has been widely utilized as a Chinese herb for treating epilepsy^[18]. Scorpion exhibits strong anti-epileptic effects, and the anti-epileptic peptide isolated from the scorpion is more potent^[19]. Previous studies have not been performed to compare doses of EES and classic anti-epileptic drugs. It remains difficult to distinguish between EES or monomers extracted from the scorpion and classical anti-epileptic drugs for use in humans. In the present study, lithium chloride/pilocarpine-induced models of chronic epilepsy were treated with varying doses of EES, and results were compared to the anti-epileptic effects of valproic acid. Results showed that EES was efficient to treat epilepsy in lithium chloride/pilocarpine-induced models, indicating that the anti-epilepsy peptide present in scorpion is sufficient for preventing recurrent epilepsy. In addition, it

does not result in drug-dependence, making it an ideal anti-epileptic drug^[12, 20-22]. We speculate that the anti-epileptic effects of EES are equal to scorpion monomer (venom); it resulted in decreased numbers of hippocampal GFAP-positive cells and decreased GFAP mRNA expression in epileptic rats. In addition, 1 160 mg/kg of EES resulted in effects similar to 120 mg/kg of valproic acid.

Previous studies have shown that scorpion extract plays a neuroprotective role and inhibits neuronal apoptosis following seizure^[16, 18, 23], which is consistent with the present study^[24]. In addition, scorpion extract has been shown to prevent glial cell scar formation in epileptic rats^[12, 25], and scorpion toxins in EES downregulate transcription factors associated with GFAP gene expression^[12], thereby inhibiting gene transcription. The present study confirmed the inhibition effect of EES on hippocampal astrocyte proliferation. Results suggested that EES prevented seizure-induced brain injury and cognitive impairment, and reduced the risk for seizure recurrence.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

Experiments were conducted from May 2007 to April 2008 at the Drug Research & Development Center, Kanghong Pharmaceutical, China and Department of Pathology, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, China.

Materials

Animals

A total of 186 clean, healthy, male, Sprague-Dawley rats, aged 3 months and weighing 200 ± 20 g, were provided by the Experimental Animal Center of Zhengzhou University, China (license No. SCXK (Yu) 2005-0001). All experimental use of animals were in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China^[26].

Scorpion source and extraction

Scorpions are present in Shandong Province of China and are harvested from April to September each year. Chengdu Huasun Group (Chengdu City, Sichuan province, China) provided the scorpions for the present study^[27]. According to the Chinese Pharmacopoeia 2005 version^[28], scorpion has been confirmed and qualified. Scorpion extract is utilized with the hot-dip method (Appendix XA), according to the alcohol-soluble extract assay in *Pharmacopoeia of the People's Republic of China* 2005 edition. In brief, scorpions were crushed and weighed, and 3.016 6 g samples were placed in 250-mL Erlenmeyer flask, and were mixed with 100 mL diluted ethanol for 1 hour. Samples were extracted by heat reflux for 1 hour, then cooled and weighed. Sample weight loss was supplemented with diluted ethanol. Samples were

then filtrated and 25 mL was dried to a constant weight and evaporated, followed by dehydration again in a water bath in the oven at 105°C for 3 hours. The samples were then cooled in a dry dish for 30 minutes and precisely weighed.

Evaporating dish constant weight: 49.746 6 g.

Evaporating dish + sample constant weight: 49.906 7 g.

Extract content = (evaporating dish + sample constant weight – evaporating dish constant weight)/25 mL × 100 mL/tested sample weight × 100% = 21.23%.

Determinations of extract content complied with required standards.

Extraction steps: scorpions were crushed, a 2-kg sample was weighed and stored in 5 000-mL round-bottom flasks, heat refluxed with 4 L 60% ethanol for 1 hour, and then filtered. The filtrates were heat-refluxed with 3 L 60% ethanol for 30 minutes and filtered. The two filtrates were mixed and ethanol was vacuum-recovered (–0.08 mPa, 70°C). Finally, a 2 000-mL extraction solution was harvested and stored in the cold for further use.

Quality standards (total nitrogen quality): 0.131 g ammonium chloride was mixed with water to form a 1.31 mg/mL ammonium chloride solution. Harvested solution (0.05, 0.1, 0.2, 0.3, and 0.4 mL) was added to 20 mL polyvinyl alcohol solution (0.1 g/L), 1 mL naphthalene reagent, and polyvinyl alcohol solution in a 25-mL volumetric flask, which served as the control solution. A 25-mL flask containing 20 mL polyvinyl alcohol solution (0.1 g/L), 1 mL naphthalene reagent, and polyvinyl alcohol solution was considered the blank solution. The absorbance values of control and blank solutions were read at 460 nm using colorimetric determination. A standard curve was plotted to calculate the regression equation as follows: $Y = 0.542 4 X + 0.021 0$, $r = 0.999 2$ (supplementary Table 1 online). According to the standard curve, total nitrogen quality in the scorpion extract measured 8.83% and total nitrogen content was 4.49 mg/mL.

Methods

Medication preparations

According to *Chinese Pharmacopoeia* 2005 edition, the scorpion clinical dosage is 5–12 g, and the conversion factor of equal effect between various animals and human was equivalent to 7 (W). The daily dose in the present rats was defined as follows: 120 mg/kg valproic acid, 290, 580, and 1 160 mg/kg for the low-, medium-, and high-dose EES (supplementary Table 2 online).

Preparation of epileptic models

Epileptic models were established in rats as previously described^[29-30]. Briefly, rats were intraperitoneally injected with 127 mg/kg lithium chloride (Sigma, St. Louis, MO, USA) on the first day, followed by 1 mg/kg atropine sulfate (Tianjin Jinyao Amino Acid Co. Ltd., China) 18–24 hours later, and pilocarpine (30 mg/kg; Sigma) 30 minutes later. Seizures were graded according to Racine classification criteria^[31]: 0: no seizure; I level: facial spasm and isolated myoclonus; II level: global

clonic convulsions; III level: global clonic convulsions and standing; IV level: global stiffness-clonic convulsions, standing and falling; V level: IV-level performance recurred, showing status epilepticus or seizures to death. The models were considered successful upon appearance of level IV global seizures, normal behavior did not return between the two episodes^[32], and status epilepticus lasted for > 15 minutes. If the rats exhibited no grade IV seizures or greater by 30 minutes, and status epilepticus occurred within 15 minutes after intraperitoneal injection of 10 mg/kg pilocarpine, then the injection was terminated. If status epilepticus was not observed within 15 minutes after injection, the rats were treated with additional pilocarpine (10 mg/kg) every 15 minutes until status epilepticus was induced.

Experimental intervention and clinical efficacy

Rats were orally administered the corresponding drugs at 15 minutes after seizures. Valproic acid group: valproic acid (120 mg/kg; 200 mg/tablet; Hunan Xiangzhong Pharmaceutical, China); EES groups: 290, 580, and 1 160 mg/kg EES; control group and model group: equal dose of saline. If status epilepticus lasted > 1 hour, or rats were endangered by convulsions, the rats were intraperitoneally injected with atropine sulfate (1 mg/kg) and diazepam (10 mg/kg; Tianjin Pharmaceutical, China) to terminate the seizure and improve survival rates. If the seizures were not alleviated, diazepam was repeatedly administered until the seizures were controlled. At 8: 00 a.m. daily, the rats were treated with the same drug intervention to observe the seizures. Samples were collected at 4 hours after drug administration.

Observations were performed over a course of 30 days.

Preparation of rat hippocampal tissue

Rats were conventionally anesthetized and perfused with normal saline. The left cerebral hemisphere was removed and fixed with 4% paraformaldehyde solution, and the fixed paraffin sections were utilized for immunohistochemical staining. Hippocampal tissues were separated from the right hemisphere and frozen in liquid nitrogen for reverse transcription-PCR quantitative analysis.

GFAP immunohistochemistry streptavidin biotin method in the rat hippocampus

Sections were conventionally de-waxed and hydrated; endogenous peroxidase activity was eliminated by incubating the sections in 3% H₂O₂. The sections were microwaved in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval, and then the sections were incubated in rabbit anti-rat GFAP monoclonal antibody (1: 100; Beijing Biosynthesis Biotechnology, China; bs-0199R) at 4°C overnight, followed by biotinylated goat anti-rabbit IgG (1: 500; Beijing Biosynthesis Biotechnology) at 37°C for 30 minutes and horseradish peroxidase-labeled avidin (Beijing Biosynthesis Biotechnology) at 37°C for 30 minutes. DAB (DAB kit; Beijing Zhongshan Golden Bridge Company, China; ZLI-9031/9032/9033) was used as a chromogen for the staining, and hematoxylin counterstaining was

performed. The sections were then mounted onto glass slides. For the negative controls, phosphate buffer saline was used instead of primary antibody. Samples were observed under a light microscope (Leica, Solms, Germany). Within each slice, eight fields of view were randomly selected under 400 × magnification. The number of positively stained cells was calculated using Image-pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA) in accordance with stereological principles^[33]. The average number of GFAP-positive cells per unit in the CA1, CA3, and dentate gyrus of each slice was analyzed for statistical analysis.

Reverse transcription-PCR detection of GFAP mRNA expression in rat hippocampus

Total RNA was extracted from the rat hippocampus according to instructions from the Trizol kit (Invitrogen, Carlsbad, CA, USA; 15596-018) and primers were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). Primer sequencing was synthesized at Shanghai GeneCore BioTechnologies, China. Target gene GFAP upstream primer: 5'-CGA CCT TGA GTC CTT GCG-3', downstream primer: 5'-CCG TCT TTA CCA CGA TGT T-3', amplified fragment length was 416 bp. Internal reference GAPDH upstream primer: 5'-ACC ACA GTC CAT GCC ATC AC-3', downstream primer: 5'-TCC ACC ACC CTG TTG CTG TA-3', amplified fragment length was 485 bp. PCR conditions: 20 cycles total of 94°C for 2 minutes; 94°C for 15 seconds; 55.5°C for 20 seconds; and 72°C for 20 seconds. The absorbance value of PCR products was calculated using TotalLab v2.01 software (TotalLab, Newcastle, UK) and results represented the ratio of target gene product/GAPDH.

Statistical analysis

Data were analyzed using SPSS 16.0 statistical package (SPSS, Chicago, IL, USA). Grouped data were compared using the chi-square test and rank sum test, and measurement data were expressed as mean ± SD for analysis of unequal distance and repeated measurement data. Pairwise comparisons were conducted using the least significant difference test or *q*-test. *P* < 0.05 was considered statistically significant.

Author contributions: Hongbin Sun had full access to the study design and revised the manuscript. All authors participated in experiment implementation and data collection. Yi Liang drafted the manuscript and performed data interpretation and analysis in strict accordance with statistical principles and methods.

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