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# Chaihu Longgu Muli Decoction relieving temporal lobe epilepsy in rats by inhibiting TLR4 signaling pathway through miR-146a-3p and miR-146a-5p

MAO Yizhi[a, b†], LI Liang[a, b†], LUO Zhihong[c], HUANG Yahui[d], WU Huaying[e], YANG Ping[c], PENG Qinghua[a, b*]

*a. Provincial Key Laboratory of TCM Diagnostics, Hunan University of Chinese Medicine, Changsha, Hunan 410208, China*

*b. Key Laboratory of TCM Heart and Lung Syndrome Differentiation & Medicated Diet and Dietotherapy, Hunan University of Chinese Medicine, Changsha, Hunan 410208, China*

*c. Department of Psychiatry, Hunan Brain Hospital, Changsha, Hunan 410007, China*

*d. Department of Neurosurgery, Hunan Brain Hospital, Changsha, Hunan 410007, China*

*e. Medicine School, Hunan Normal University, Changsha, Hunan 410013, China*

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ABSTRACT

**Objective** To explore the effect and mechanism of Chaihu Longgu Muli Decoction (柴胡龙骨牡蛎汤，CHLGMLD) in rats with temporal lobe epilepsy (TLE).

**Methods** A total of 80 Sprague-Dawley (SD) male rats were randomized into control (CON), model (MOD), carbamazepine (CBZ, 0.1 g/kg), CHLGMLD low dose (CHLGMLD-L, 12.5 g/kg), and high dose (CHLGMLD-H, 25 g/kg) groups, with 16 rats in each group. TLE rat models were established in the four groups with the use of lithium-pilocarpine except for the CON group. After the successful establishment of TLE models, all drugs were administered through gavage, and distilled water was given to rats in the CON and MOD groups for four weeks. The frequency and duration of seizures before and after treatment were recorded for the evaluation of the alleviation degree. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression levels of miR-146a-3p and miR-146a-5p. The expression levels of toll-like receptor 4 (TLR4), interleukin-1 receptor-associated kinase 1 (IRAK1), tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), TAK1-binding protein (TAB), nuclear factor-kappa B (NF-$\kappa$B), and interleukin-1 beta (IL-1$\beta$) in hippocampus were tested by immunofluorescence assay. Correlation analysis between the above factors and expressions of miR-146a-3p and miR-146a-5p were performed separately.

**Results** CHLGMLD decreased the frequency ($P < 0.05$) and duration ($P < 0.01$) of seizures in rats. CHLGMLD down-regulated the expression levels of miR-146a-5p and miR-146a-3p ($P < 0.05$), and inhibited the expression levels of TLR4, IRAK1, TRAF6, TAB, NF-$\kappa$B, and IL-1$\beta$ ($P < 0.01$). The correlation analysis revealed that the expression levels of TLR4, IRAK1, TRAF6, TAB, NF-$\kappa$B, and IL-1$\beta$ were positively correlated with the expression levels of miR-146a-3p and miR-146a-5p detected by qRT-PCR, respectively ($P < 0.01$).

**Conclusion** CHLGMLD can inhibite the TLR4 signaling pathway by lowering the expression levels of miR-146a-3p and miR-146a-5p to alleviate hippocampal dentate gyrus inflammation in TLE rats, thus relieving seizures.

†These authors contributed equally to this work.

*Corresponding author: PENG Qinghua, Professor, E-mail: pqh410007@126.com.

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## 1 Introduction

Temporal lobe epilepsy (TLE) is a common neurological disorder but is hard to deal with clinically. It's often accompanied by pathologies like hippocampal sclerosis, degeneration, predominant neuronal cell loss, and gliosis in temporal lobe [1]. Although most of the symptoms could be relieved with the use of antiepileptic drugs (AEDs), approximately 30% patients would still have seizure attacks after medication [2]. It has been reported that inflammatory pathways are closely associated with seizure attacks or epilepsy, of which the etiopathogenesis might be the up-regulation of inflammatory gene expression [3-5]. Epilepsy is a tricky disease that would lead to complications such as agenesis and cognitive disorder, with psychosocial impacts on patients. Therefore, terminating seizures timely is pivotal in the clinical care for patients with epilepsy.

Toll-like receptor 4 (TLR4) signaling pathway plays an important role in neurodegeneration diseases and releases inflammatory factors such as nuclear factor-kappa B (NF-$\kappa$B), interleukin (IL)-1$\beta$, IL-8, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), and IL-6, which would result in inflammations. Some proteins in the signaling pathway were also reported to play an inflammatory role [6-8]. TLR4/MyD88/NF-$\kappa$B signaling pathways were found to be crucial in the treatment of osteoarthritis with Duhuo Jisheng Decoction (独活寄生汤, DHJSD), a traditional Chinese medicine (TCM) prescription [9].

MicroRNA (miRNA) involve with a pyramid of biological activities in human brains. miRNAs could be used as a biomarker and therapeutic entry point for epilepsy when some important gene expressions are regulated. As the seizure attacks, the levels of some miRNAs including transcription factors and neurotransmitter signaling components in the blood of rats would change [10, 11]. The quantity of miRNAs in blood differs significantly, which suggests a possibility for them to be diagnostic biomarkers in an eplieptic seizure [12, 13]. Previous study has shown that miR-146a-3p expression is a main cause of inflammation [14]. And miR-146a-5p contributes to degenerative neural diseases such as epilepsy, which is closely related to the NF-$\kappa$B pathways in TLE [15, 16]. Furthermore, miR-146a-3p and miR-146a-5p both have found to be able to produce cytokine responses and TLR4 signals that lead to inflammation [17-19]. Therefore, miR-146a might be an effective treatment approach for neural disorders such as TLE in which inflammatory pathways are crucial.

*Treatise on Febrile Diseases* (*Shan Han Lun*, 《伤寒论》), a classic herbal work published in Han dynasty of ancient China, explained Chaihu Longgu Muli Decoction (柴胡龙骨牡蛎汤, CHLGMLD) for the first time and has good relieving effect in clinical application of epilepsy. miRNA plays an important role in the occurrence and development of epilepsy and other neurological diseases, which has been widely concerned. Our preliminary study confirmed that CHLGMLD could alleviate epilepsy [20]. To further study how miR-146a-3p and miR-146a-5p were regulated in TLE patients after administration of CHLGMLD, the expression levels of miR-146a-3p and miR-146a-5p during the development of epilepsy in TLE rat models and the expression levels of proteins in TLR4 signaling pathway were investigated, and the mechanisms of CHLGMLD was explored.

## 2 Materials and methods

### 2.1 Drugs, reagents, and instruments

The CHLGMLD consists of 12 g Chaihu (Bupleuri Radix); 6 g Banxia (Pinelliae Rhizoma), 6 g Dahuang (Rhei Radix et Rhizoma), 6 g Dazao (Jujubae Fructus), 4.5 g Longgu (Fossilia Ossis Mastodi), 4.5 g Muli (Ostreae Concha), 4.5 g Huangqin (Scutellariae Radix), Renshen (Ginseng Radix et Rhizoma), 4.5 g Guizhi (Cinnamomi Ramulus), 4.5 g Fulin (Poria), 4.5 g Shengjiang (Zingiberis Rhizoma Recens). The CHLGMLD was purchased from Hunan Brain Hospital (China).

The drugs and reagents used in this study mainly include: lithium chloride (Sigma-Aldrich, USA), pilocarpine (ABCR GmbH & Co. KG, Germany), 4% paraformaldehyde tissue fixation solution (Kemiou Chemical Reagent Co., Ltd., China), TLR4 antibody (Servicebio Technology, China), TNF receptor-associated factor 6 (TRAF6) antibody (Boster Biological Technology, China), interleukin-1receptor-associated kinase 1 (IRAK1) antibody (Biosynthesis biotechnology, China), transforming growth factor $\beta$-activated kinase 1 (TAK1)-binding protein (TAB) antibody (Biosynthesis biotechnology, China), NF-$\kappa$B antibody (Cell Signaling Technology, USA), and IL-1$\beta$ (Abcam, England), Alexa Fluor 488-conjugated AffiniPure donkey anti-rabbit IgG (H +L) (Jackson Immuno Research Laboratories, Inc., USA).

The instruments used in this study mainly include: biological tissue spreader (Yidijinhua, YD-A), computerised biological tissue embedding machine (Kedijinhua, KD-BM II), low temperature benchtop centrifuge (Xiangyi, TDZ4-WS), high-speed benchtop freezing centrifuge (Yingtai, TGL-16), ultrasonicator (Xinzhi, JY92-IIN), ultrapure water system (Veolia Environment S.A., ELGA Purelab Chorus 1 & Chorus 2), enzyme calibrator (Tecan Trading AG, Sunrise F50), gradient PCR amplifier (BIO-RAD, T100), quantitative real-time polymerase chain reaction (qRT-PCR) instrument (ABI, 7900HT), fluorescence microscope (Olympus, BX63), laser confocal microscope (ZEISS, LSM710), ultrasonic pulverizers (Xinzhi, JY96-IIN), MS3), sample homogeniser disperser (IKA, T25), heating magnetic stirrers (IKA, CJJ78-1).

### 2.2 Experimental animals

A total of 80 male Sprague-Dawley (SD) rats, aged from six to eight weeks and weighed about 180 to 220 g, were provided by Hunan SJA Laboratory Animal Co., Ltd. [SCXK (Xiang) 2016-0002], and raised in specified pathogens free (SPF) experimental animal center [SYXK (Xiang) 2013-0005] at room temperature (25 ± 2) °C under relative humidity (50% – 70%). The whole experiment was approved by the Institutional Animal Care and Welfare Ethics Committee of Hunan University of Chinese medicine (LLBH-20170205).

### 2.3 Experimental groups and drug administration

Eighty rats were randomly assigned to five groups: the control (CON), model (MOD), carbamazepine (CBZ), CHLGMLD low dose (CHLGMLD-L), and high dose (CHLGMLD-H) groups, with 16 rats in each group.

Other than the CON group, the rest four groups included TLE rat models. Rats in these groups were administered lithium chloride (3 mEq/kg) intraperitoneally at the beginning, then atropine (1 mg/kg) after 17.5 h to block the peripheral cholinergic effects. At last, the experimental animals were given pilocarpine (30 mg/kg) after 18 h. If status epilepticus (SE) or convulsions did not start within 30 min, 10 mg/kg pilocarpine was given for additional three times until the occurrence of grade IV SE [21]. The SE was lasted for 40 min, and terminated later with 4 mg/kg diazepam and 3 mL/kg 10% chloral hydrate. Rats who had reached Racine IV level but survived from the seizures were considered as successfully having seizure attacks. Criteria of Racine leveling are as follows [22]: (0) normal behavior; (i) immobility and facial symptoms (eye closure, facial spasm, etc.); (ii) nodding head with severer facial and mouth spasms; (iii) foreleg clonic on one side; (iv) forelegs lifted with convulsions; (v) legs raised with clonus and tic, and fell off balance. A complete motor attack and failure in postural control were referred as stage V. Successful TLE model establishment was realized in all rats.

According to our preliminary study [23], rats in the CHLGMLD-L and CHLGMLD-H groups were given 12.5 g/kg (equivalent to the clinical human dose) and 25.0 g/kg CHLGMLD (two times the clinical human dose), respectively, once a day through gavage feeding after being heated to 37 °C. The CBZ group was given 0.1 g/kg CB2 once a day through gavage. The CON and MOD groups were given distilled water of equal volume by gavage also. The above procedures were repeated for four weeks after the successful modeling and then terminated four weeks later. After being monitored for one week for their behaviors, all rats were sacrificed and samples were harvested as required for further research.

### 2.4 Behavioral test

The frequency and duration of seizure attacks within 7 d were recorded after four weeks drug intervention. The rats' behaviors were rated with Racine scale [24]. Three consecutive grade IV or V SE were considered as successful seizure attacks.

### 2.5 Detection of the expression levels of miR-146a-3p and miR-146a-5p by qRT-PCR

The primers for qRT-PCR were designed according to the GenBank, and using Primer Premier 5.0. Table 1 displays the synthesized primer sequences with the use of qRT-PCR. The total reaction system was 25 μL, including SYBR-Green Mix 10 μL, Primer F, (10 μmol/L) 0.8 μL, Primer R (10 μmol/L) 0.8 μL, target gene template 2 μL, and $ddH_2O$ 11.4 μL. Reaction conditions: a total of 40 cycles were performed at 95 °C for 2 min, 95 °C for 10 s, 57 °C for 10 s, 72 °C for 15 s. This procedure was repeated for three times. The expression levels of target genes was normalized to U6 as an internal reference using the $2^{-\triangle\triangle ct}$ method.

**Table 1** Primer sequences of target genes by qRT-PCR

| Gene symbol | Upstream primer | Downstream primer |
|---|---|---|
| miR-146a-5p | 5'-GAGAACTGAA TTCCATGGGT-3' | 5'-CAGTGCGTGT CGTGGAGT-3' |
| miR-146a-3p | 5'-CCTGTGAAGT TCAGTTCT-3' | 5'- CAGTGCGTGT CGTGGAGT-3' |
| Rat-U6 | 5'-CTTCGGCAGCAC ATATACTAAAA-3' | 5'-CGTGTCATCC TTGCGCAG-3' |

### 2.6 Immunofluorescence (IHF) detecting the expression levels of TLR4, TRAF6, IRAK1, TAB, NF-κB, and IL-1β

The brain tissues of rats were sliced to 5 μm per section. The slices were washed with PBS for three times, incubated at 37 °C in DMEM containing 2% fetal bovine serum (FBS) for 30 min with membrane seal, and were washed twice with PBS. TLR4 antibody (1 : 100), TRAF6 antibody (1 : 100), IRAK1 antibody (1 : 100), TAB antibody (1 : 100), NF-κB antibody (1 : 100), and IL-1β (1 : 100) were added in sequence and incubated overnight at 4°C. The nuclei with 4',6-diamino-2-phenylindole (DAPI 1 : 1 000) was signed overnight, and the Alexa Fluor 488-conjugated AffiniPure donkey anti-rabbit IgG (H + L) (1 : 100) was incubated in a dark wet (40% – 60%) container at 37 °C for 2 h, washed with PBS for three times for 5 min, then sealed with glycerin. Green FITC-labeled proteins (nucleus and cytoplasm) were observed under a confocal laser microscope at 400× and 630×.

### 2.7 Statistical analysis

Date analysis were performed using SPSS 25.0 software. Measures are expressed as mean ± standard deviation

(SD). The Least Significant Difference (LSD) test was used for variance homogeneity data, and Dunnett's T3 for the variance of inconformity data. The Kruskal-Wallis test used for the nonnormality enumeration data. Pearson or Spearman correlation analysis was adopted for the relationship between the frequency and duration of the epileptic seizures and the expression levels of miR-146a-3p and miR-146a-5p. $P < 0.05$ was considered statistically significant.

## 3 Results

### 3.1 Effects of CHLGMLD on the frequency and duration of epileptic seizures in TLE rats

Compared with the CON group, the frequency and duration of epileptic seizures in TLE rats were significantly increased in the MOD group ($P < 0.01$). After treatment (day 28), the frequency of seizure attacks was significantly decreased in the CBZ group ($P < 0.01$) and in the CHLGMLD-H group ($P < 0.05$) compared with the MOD group. And the duration also shortened in the CBZ and CHLGMLD-H groups compared with the MOD group ($P < 0.01$). However, the frequency and duration of seizure attacks in rats showed no significant difference between the CHLGMLD-L and MOD groups ($P > 0.05$) (Table 2 and Figure 1).

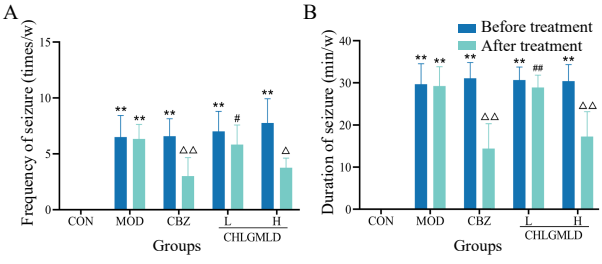


**Figure 1** Frequency and duration of epileptic seizures in rats before and after treatment

A, frequency of seizure. B, duration of seizure. Data are expressed as mean ± SD. $^{**}P < 0.01$; compared with the CON group; $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$, compared with the MOD group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the CBZ group.

### 3.2 Effects of CHLGMLD on the expression levels of TLR4, IRAK1, TRAF6, TAB, NF-κB, and IL-1β

TLR4 was found in the cytomembrane of the hippocampal dentate gyrus, while IRAK1, TRAF6, TAB, NF-κB, and IL-1β in the cytoplasm. They were distinguished by green fluorescence (Figure 2).

Compared with the CON group, the expression levels of TLR4, IRAK1, TRAF6, TAB, NF-κB, and IL-1β were significantly increased in the MOD group ($P < 0.01$). The expression level of TLR4 significantly decreased in the CBZ and CHLGMLD-H groups ($P < 0.01$), and in the CHLGMLD-L group ($P < 0.05$) compared with the MOD group. The expression levels of IRAK1, TRAF6, TAB, NF-κB, and IL-1β significantly decreased in the CBZ, CHLGMLD-L, and CHLGMLD-H groups compared with the MOD group ($P < 0.01$). The TLR4, NF-κB, and IL-1β expression levels had no difference between the CBZ and CHLGMLD-H group ($P > 0.05$). The IRAK1 and TRAF6 expression levels in the CHLGMLD-H group was lower than that in the CBZ group ($P < 0.05$), so did the TAB expression ($P < 0.01$) (Figure 2).

### 3.3 Effects of CHLGMLD on the expression levels of miR-146a-3p and miR-146a-5p

Compared with the CON group, the expression levels of miR-146a-3p and miR-146a-5p significantly incressed in the MOD group ($P < 0.01$). The expression levels of miR-146a-3p reduced in the CBZ, CHLGMLD-L, and CHLGMLD-H groups compared with the MOD group ($P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively). The expression level of miR-146a-5p was lower in the CBZ and CHLGMLD-H groups than that in the MOD group ($P < 0.01$). The CHLGMLD-L group showed no difference with the MOD group in terms of frequency and duration of epileptic seizures in rats ($P > 0.05$) (Figure 3).

**Table 2** Frequency and duration of epileptic seizures in rats before and after treatment

| Group | Frequency of epileptic eizures (times/w) | | Duration of epileptic seizures (min/w) | |
|---|---|---|---|---|
| | Before treatment | After treatment | Before treatment | After treatment |
| CON | 0 | 0 | 0 | 0 |
| MOD | 6.50 ± 1.93$^{**}$ | 6.33 ± 1.30$^{**}$ | 29.67 ± 4.87$^{**}$ | 29.25 ± 4.61$^{**}$ |
| CBZ | 6.58 ± 1.56$^{**}$ | 3.00 ± 1.65$^{\triangle\triangle}$ | 31.08 ± 3.78$^{**}$ | 14.42 ± 5.92$^{\triangle\triangle}$ |
| CHLGMLD-L | 7.00 ± 1.81$^{**}$ | 5.83 ± 1.75$^{\#}$ | 30.67 ± 3.11$^{**}$ | 28.92 ± 2.94$^{\#\#}$ |
| CHLGMLD-H | 7.75 ± 2.18$^{**}$ | 3.75 ± 0.87$^{\triangle}$ | 30.42 ± 3.92$^{**}$ | 17.25 ± 5.93$^{\triangle\triangle}$ |

$^{**}P < 0.01$, compared with the CON group; $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$, compared with the MOD group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the CBZ group.

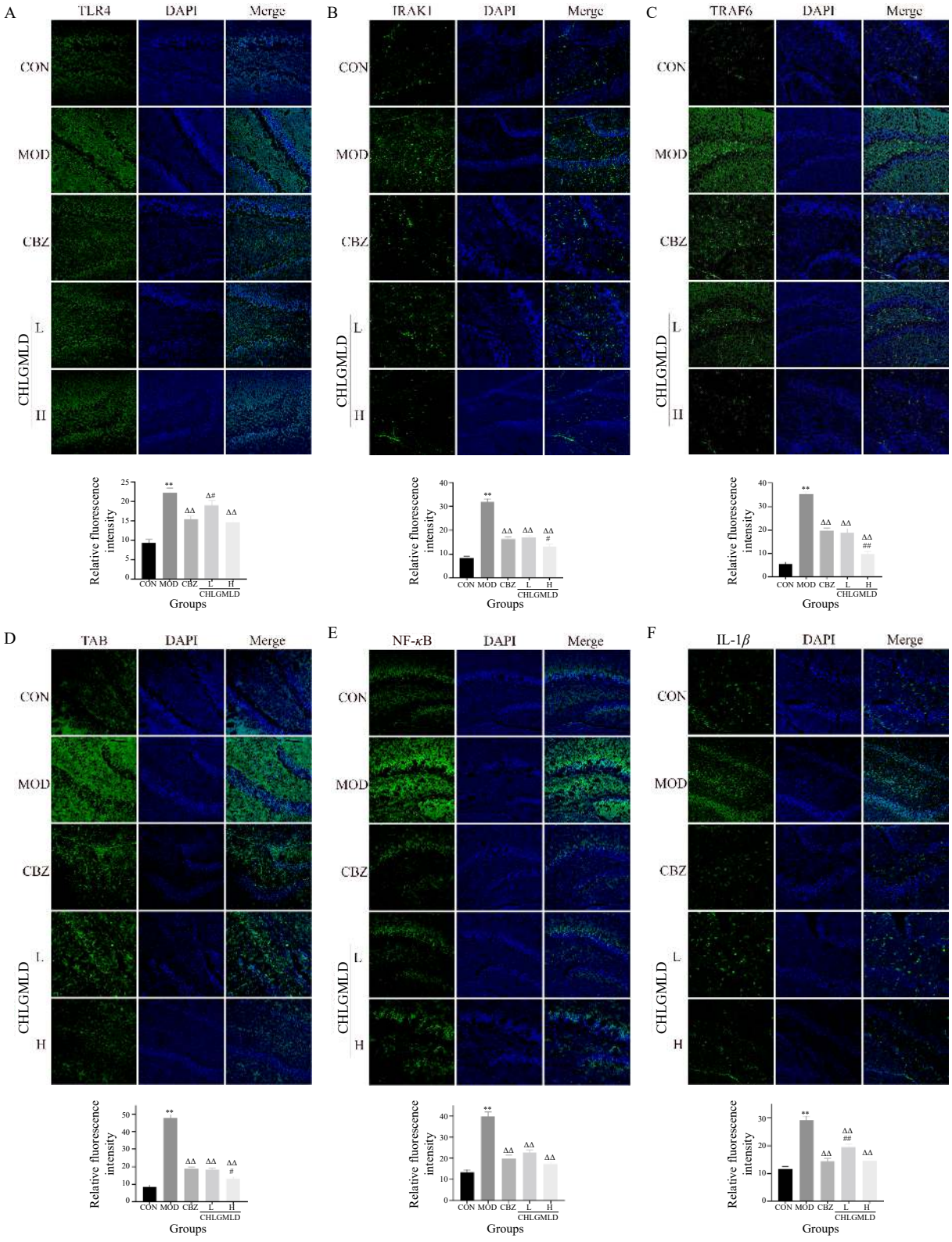


**Figure 2** The expression levels of key proteins in TLR4 signaling pathway in the hippocampal dentate gyrus (400 ×)

A, expression level of TLR4. B, expression level of IRAK1. C, expression level of TRAF6. D, expression level of TAB. E, expression level of NF-$\kappa$B. F, expression level of IL-1$\beta$. Data are expressed as mean ± SD. $^{**}P < 0.01$, compared with the CON group; $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$, compared with the MOD group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the CBZ group.

### 3.4 Correlation between TLR4, IRAK1, TRAF6, TAB, NF-κB, IL-1β and miR-146a-3p, miR-146a-5p

The correlation analysis revealed that the expression levels of TLR4, IRAK1, TRAF6, TAB, NF-κB, and IL-1β were positively correlated with the expression levels of miR-146a-3p and miR-146a-5p detected by qRT-PCR ($P < 0.01$) (Figure 4).

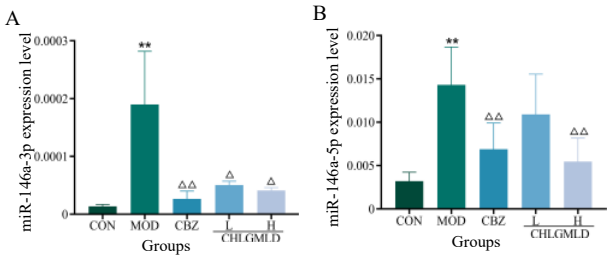


**Figure 3** The expression levels of miR-146a-3p and miR-146a-5p by qRT-PCR

A, miR-146a-3p expression level. B, miR-146a-5p expression level. Data are expressed as mean ± SD. $^{**}P < 0.01$, compared with the CON group; $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$, compared with the MOD group.

## 4 Discussion

At present, ineffective treatments of TLE still have a high proportion, and the mechanism is not clear enough [25].

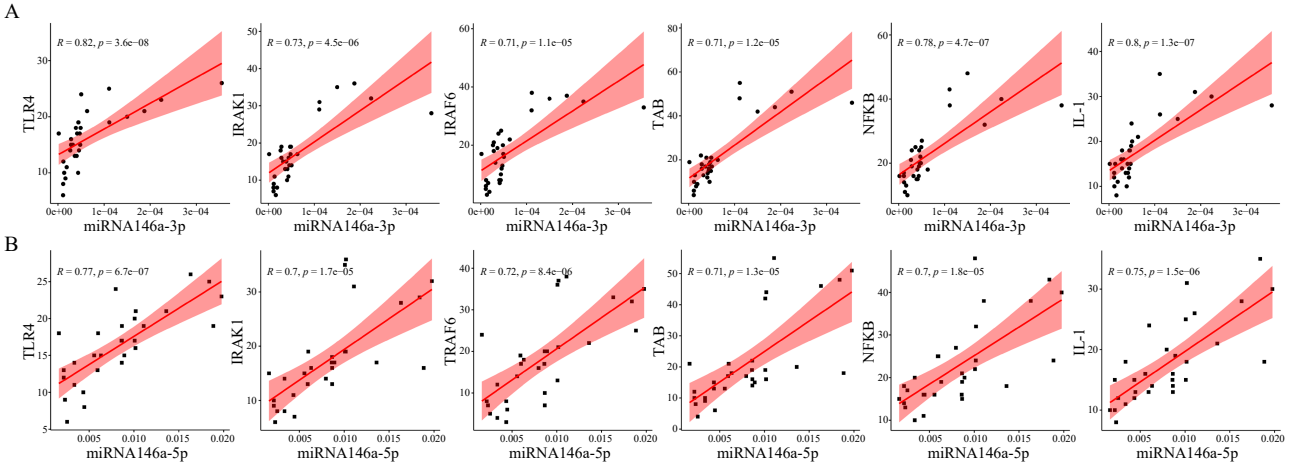


**Figure 4** Correlation between the TLR4, IRAK1, TRAF6, TAB, NF-κB, IL-1β and miR-146a-3p, miR-146a-5p

A, Correlation between the TLR4, IRAK1, TRAF6, TAB, NF-κ B, IL-1 β and miR-146a-3p. B, Correlation between the TLR4, IRAK1, TRAF6, TAB, NF-κ B, IL-1 β and miR-146a-5p.

However, TLE can be alleviated with TCM. CHLGMLD is a TCM prescription that has eleven raw herbal materials, with Chaihu (Bupleuri Radix) and Huangqin (Scutellariae Radix) as the main materials, which relieve Qi stagnancy in liver and cholecyst to dissipate sorrow. Longgu (Fossilia Ossis Mastodi) and Muli (Ostreae Concha) in combination with Banxia (Pinelliae Rhizoma) ease panic, stabilize mental health, and reduce phlegm. Guizhi (Cinnamomi Ramulus) and Fulin (Poria) prevent Qi from rushing up to decline the Qi superinverse. All these herbal medicines together are able to reconcile heat and tranquilize seizure.

According to previous studies, there are many etiologies and pathogenesis of TLE and inflammation is an important one among them. Our previous studies also showed that CHLGMLD could reduce the frequency and duration of epileptic seizures in lithium-pilocarpine-induced TLE rats, suggesting CHLGMLD has similar efficacy as CBZ [20, 23] . CHLGMLD also contains active components that have neuroprotective effects and autophagy resistance. Although low dose of CHLGMLD did not show obvious effects on epilepsy, the epileptic behaviors were significantly reduced as dose increased.

Inflammation is one of the main causes of seizures or refractory seizures. TLR4 pathway is closely tied with inflammation, with TLR4 as one of the key proteins. Proteins in the TLR4 pathway, such as TRAF6, IRAK1, TAB, NF-κB, and IL-1 participate in the pathophysiological process of TLE by altering the neuronal death course and astrocytic activation [6, 8, 26, 27]. The release of these inflammatory cytokines can increase the excitability of local neurons, thus resulting in TLE [28]. Among the downstream molecules of TLR4, TRAF6 is important for the progression of inflammation, which is associated with ubiquitin-associated proteins in nerve fiber tangles and promotes tau aggregation by catalyzing the polyubiquitylation of tau proteins connected to it. Tau protein is a key protein for neurodegenerative diseases [29]. Activation of TRAF6 and IRAK1 results in nuclear translocation of NF-κB, leading to the release of IL-6, TNF-α, and TAB over-expression, indicating a progressive inflammatory response [30, 31]. Moreover, the IL-1β promotes glutamate release, thereby increasing glutamate availability at synapses and causing neuronal hyperexcitability [32]. The results suggest that TLR4 signaling pathway plays a large role in neuron injury and neuro-inflammation leading to TLE.

miRNA-146a is an endogenous factor that regulates TLR and cytokine receptor signaling pathways, alleviating neuronal inflammation and epileptic seizures [33]. It also reduces microglia-mediated neuro-inflammation by regulating TLR4, IRAK1, TRAF6, and NF-$\kappa$B [34, 35]. WANG et al. [36] found that miR-146a-5p was related to NF-κB, both of which reduced the expression levels of IRAK1 and TRAF6 to alleviate neuropathic pain in rats. Meanwhile, miR-146a-5p can be up-regulated by NF-$\kappa$B and accelerate the inflammatory response of neuronal cells [37]. Activation of NF-$\kappa$B induces miR-146a-5p and IRAK1 to bind to cell membrane receptor and activates neuronal protein kinase C *in vivo* and *in vitro*, thereby affecting neuronal function and excitability [8]. These studies suggest that miR-146a-5p reduces the expression of inflammatory factors in TLR4 pathway of TLE rats, and alleviates the inflammatory response and nerve damage in TLE hippocampus, thereby interfering with the onset and severity of epilepsy. Currently, there are few studies of the effect of miR-146a-3p on epilepsy, but some studies have shown that miR-146a-3p is related to NF-$\kappa$B and inflammatory response in rats [38]. Up-regulation of miR-146a-3p is mediated by TLR4, causing activation of downstream IL-8 and suppressing IL-1$\beta$ in chondrocytes [39]. So, we inferred that the TLR4 pathway is connected with both miR-146a-5p and miR-146a-3p. And the effects of CHLGMLD on TLE may be mediated by downregulating the expression levels of miR-146a-5p and miR-146a-3p. Therefore that CHLGMLD may alleviate epileptic seizures by inhibiting TLR4 signaling pathway with miR-146a-5p and miR-146a-3p was concluded. Therefore, we concluded that CHLGMLD may alleviate epileptic seizures by inhibiting TLR4 signaling pathway with miR-146a-5p and miR-146a-3p.

## 5 Conclusion

TLE in rats can be caused by aggravated inflammation in rats' hippocampus. The CHLGMLD can reduce seizures by down-regulating the expression levels of miR-146a-3p and miR-146a-5p, and decreasing the expression levels of TLR4, TRAF6, IRAK1, TAB, NF-$\kappa$B, and IL-1$\beta$. CHLGMLD for the treatment of TLE attack and the inhibition of inflammation in hippocampus may be realized by regulating in TLR4 signaling pathway through miR-146a-3p and miR-146a-5p.

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## Competing interests

The authors declare no conflict of interest.

## References

[1] BLÜMCKE I, THOM M, ARONICA E, et al. International consensus classification of hippocampal sclerosis in temporal lobe epilepsy: a task force report from the ILAE commission on diagnostic methods. Epilepsia, 2013, 54(7): 1315–1329.

[2] DEVINSKY O, VEZZANI A, O'BRIEN TJ, et al. Epilepsy. Nature Reviews Disease Primers, 2018, 4: 18024.

[3] VEZZANI A, BALOSSO S, RAVIZZA T. Neuroinflammatory pathways as treatment targets and biomarkers in epilepsy. Nature Reviews Neurology, 2019, 15(8): 459–472.

[4] GOMEZ CD, ACHARJEE S, LEWIS ML, et al. Increased excitatory synaptic transmission associated with adult seizure vulnerability induced by early-life inflammation in mice. The Journal of Neuroscience, 2021, 41(20): 4367–4377.

[5] RANA A, MUSTO AE. The role of inflammation in the development of epilepsy. Journal of Neuroinflammation, 2018, 15(1): 144.

[6] YU Q, ZHAO MW, YANG P. LncRNA UCA1 suppresses the inflammation via modulating mir-203-mediated regulation of MEF2C/NF-$\kappa$B signaling pathway in epilepsy. Neurochemical Research, 2020, 45(4): 783–795.

[7] HUANG H, CUI G, TANG H, et al. Silencing of microRNA-146a alleviates the neural damage in temporal lobe epilepsy by down-regulating Notch-1. Molecular Brain, 2019, 12(1): 102.

[8] ROSETI C, VAN VLIET EA, CIFELLI P, et al. GABAA currents are decreased by IL-1$\beta$ in epileptogenic tissue of patients with temporal lobe epilepsy: implications for ictogenesis. Neurobiology of Disease, 2015, 82: 311–320.

[9] LIU L, XU L, WANG S, et al. Confirmation of inhibiting TLR4/MyD88/NF-$\kappa$B signalling pathway by Duhuo Jisheng Decoction on osteoarthritis: a network pharmacology approach-integrated experimental study. Frontiers in Pharmacology, 2021, 12: 784822.

[10] WANG W, GUO Y, HE L, et al. Overexpression of miRNA-137 in the brain suppresses seizure activity and neuronal excitability: a new potential therapeutic strategy for epilepsy. Neuropharmacology, 2018, 138: 170–181.

[11] ANTÔNIO L, FREITAS-LIMA P, PEREIRA-DA-SILVA G, et al. Expression of microRNAs miR-145, miR-181c, miR-199a and miR-1183 in the blood and hippocampus of patients with mesial temporal lobe epilepsy. Journal of Molecular Neuroscience, 2019, 69(4): 580–587.

[12] KIPNIS PA, KADAM SD. Novel concepts for the role of chloride cotransporters in refractory seizures. Aging and Disease, 2021, 12(4): 1056–1069.

[13] ZHANG H, QU Y, WANG A. Antagonist targeting microRNA-146a protects against lithium-pilocarpine-induced status epilepticus in rats by nuclear factor-$\kappa$B pathway. Molecular Medicine Reports, 2018, 17(4): 5356–5361.

[14] HE L, WANG Z, ZHOU R, et al. Dexmedetomidine exerts cardioprotective effect through miR-146a-3p targeting IRAK1 and TRAF6 via inhibition of the NF-$\kappa$B pathway. Biomedicine &

Pharmacotherapy = Biomédecine & pharmacothérapie, 2021, 133: 110993.
[15] YANG GQ, LIU DJ, CHENG BY, et al. Effects of sevoflurane preconditioning on the expression of serum NF-κB, TNF-α and IL-6 in rats model of myocardial ischemia - reperfusion injury. Journal of Clinical and Experimental Medicine, 2018, 17(17): 1801–1805.
[16] DU SX, ZHANG XH, ZHAO HY, et al. Effect of miRNA-146a-5p on the signal transduction pathway of NF-κB in hippocampal neurons of epileptic rats. Journal of Clinical and Experimental Medicine, 2019, 18(4): 341–346.
[17] WU XQ, DAI Y, YANG Y, et al. Emerging role of microRNAs in regulating macrophage activation and polarization in immune response and inflammation. Immunology, 2016, 148(3): 237–248.
[18] XO XR, WANG HB, BAI XY, et al. miR-146a-3p alleviates LPS-induced acute lung injury in mice by targeting TLR4. Chinese Journal of Respiratory and Critical Care Medicine, 2021, 20(7): 495–502.
[19] ECHAVARRIA R, MAYAKI D, NEEL JC, et al. Angiopoietin-1 inhibits toll-like receptor 4 signalling in cultured endothelial cells: role of miR-146b-5p. Cardiovascular Research, 2015, 106(3): 465–477.
[20] XIA SS, YANG P, LI F, et al. Chaihu-Longgu-Muli Decoction exerts an antiepileptic effect in rats by improving pyroptosis in hippocampal neurons. Journal of ethnopharmacology, 2021, 270: 113794.
[21] HONCHAR MP, OLNEY JW, SHERMAN WR. Systemic cholinergic agents induce seizures and brain damage in lithium-treated rats. Science, 1983, 220(4594): 323–325.
[22] RACINE RJ. Modification of seizure activity by electrical stimulation: II. Motor seizure. Electroencephalography and Clinical Neurophysiology, 1972, 32(3): 281–294.
[23] YANG P, QIN Y, ZHU Y, et al. Chaihu-Longgu-Muli Decoction relieves epileptic symptoms by improving autophagy in hippocampal neurons. Journal of Ethnopharmacology, 2020, 259: 112990.
[24] YANG P, SUN M, LI L, et al. 8-hydroxy-dipropylaminotetralin promotes neural plasticity in epileptic rats with depression. Neural Regeneration Research, 2012, 7(8): 565–571.
[25] WONG SQ, JONES A, DODD S, et al. A Caenorhabditis elegans assay of seizure-like activity optimised for identifying antiepileptic drugs and their mechanisms of action. Journal of Neuroscience Methods, 2018, 309: 132–142.
[26] BOSCHIERO MN, CAMPOREZE B, JSD S, et al. The single nucleotide variant n.60G > C in the microRNA-146a associated with susceptibility to drug-resistant epilepsy. Epilepsy Research, 2020, 162: 106305.
[27] DENG X, SHAO Y, XIE Y, et al. MicroRNA-146a-5p downregulates the expression of p-glycoprotein in rats with lithium-pilocarpine-induced status epilepticus. Biological & Pharmaceutical Bulletin, 2019, 42(5): 744–750.
[28] LIU YQ, LAI SR, MA WN, et al. CDYL suppresses epileptogenesis in mice through repression of axonal Nav1.6 sodium channel expression. Nature Communications, 2017, 8(1): 355.
[29] BOLING WW. Surgical considerations of intractable mesial temporal lobe epilepsy. Brain Sciences, 2018, 8(2): 35.
[30] ZHENG L, SU J, ZHANG Z, et al. Salidroside regulates inflammatory pathway of alveolar macrophages by influencing the secretion of miRNA-146a exosomes by lung epithelial cells. Scientific Reports, 2020, 10(1): 20750.
[31] HE Y, HARA H, NÚÑEZ G. Mechanism and regulation of NLRP3 inflammasome activation. Trends in Biochemical Sciences, 2016, 41(12): 1012–1021.
[32] SU Q, LI L, SUN Y, et al. Effects of the TLR4/Myd88/NF-κB signaling pathway on nlrp3 inflammasome in coronary microembolization-induced myocardial injury. Cellular Physiology and Biochemistry, 2018, 47(4): 1497–1508.
[33] LIU AH, WU YT, WANG YP. MicroRNA-129-5p inhibits the development of autoimmune encephalomyelitis-related epilepsy by targeting HMGB1 through the TLR4/NF-kB signaling pathway. Brain Research Bulletin, 2017, 132: 139–149.
[34] ZHANG Z, ZOU X, ZHANG R, et al. Human umbilical cord mesenchymal stem cell-derived exosomal miR-146a-5p reduces microglial-mediated neuroinflammation via suppression of the IRAK1/TRAF6 signaling pathway after ischemic stroke. Aging, 2021, 13(2): 3060–3079.
[35] YAN F, WUFUER D, DING J, et al. MicroRNA miR-146a-5p inhibits the inflammatory response and injury of airway epithelial cells via targeting TNF receptor-associated factor 6. Bioengineered, 2021, 12(1): 1916–1926.
[36] WANG Z, LIU F, WEI M, et al. Chronic constriction injury-induced microRNA-146a-5p alleviates neuropathic pain through suppression of IRAK1/TRAF6 signaling pathway. Journal of Neuroinflammation, 2018, 15(1): 179.
[37] LEI B, LIU J, YAO Z, et al. NF-κB-Induced upregulation of mir-146a-5p promoted hippocampal neuronal oxidative stress and pyroptosis via tigar in a model of alzheimer's disease. Frontiers in Cellular Neuroscience, 2021, 15: 653881.
[38] YANG Y, LI L. Depleting microRNA-146a-3p attenuates lipopolysaccharide-induced acute lung injury via up-regulating SIRT1 and mediating NF-κB pathway. Journal of Drug Targeting, 2021, 29(4): 420–429.
[39] NING F, ZHU S, GAO H, et al. NEAT1/miR-146a-3p/TrkB/ShcB axis regulates the development and function of Chondrocyte. Cell Cycle, 2021, 20(20): 2174–2194.

# 柴胡龙骨牡蛎汤通过下调 miR-146a-3p 和 miR-146a-5p 抑制 TLR4 信号通路缓解大鼠癫痫发作

毛一之[a, b†], 李亮[a, b†], 罗志红[c], 黄亚辉[d], 吴华英[e], 杨萍[c], 彭清华[a, b*]

*a. 湖南中医药大学中医诊断湖南重点实验室, 湖南 长沙 410208, 中国*
*b. 湖南中医药大学中医心肺病证辨证与药膳食疗重点研究室, 湖南 长沙 410208, 中国*
*c. 湖南省脑科医院精神科, 湖南 长沙 410007, 中国*
*d. 湖南省脑科医院神经外科, 湖南 长沙 410007, 中国*
*e. 湖南师范大学医学院, 湖南 长沙 410013, 中国*

**【摘要】目的** 探讨柴胡龙骨牡蛎汤(CHLGMLD)对颞叶癫痫大鼠的疗效及作用机制。**方法** 将 80 只雄性 SD 大鼠随机分为 5 组,分别为对照组、模型组、卡马西平(0.1 g/kg)组、CHLGMLD 低剂量(CHLGMLD-L,12.5 g/kg)组和高剂量(CHLGMLD-H,25 g/kg)组,每组各 16 只。除对照组外,其余 4 组均建立由锂-匹罗卡品诱导的颞叶癫痫大鼠模型。造模成功后,所有用药组给予相应药物,对照组和模型组给予同等体积蒸馏水治疗 4 周。记录各组大鼠的自发性癫痫反复发作的次数和持续时间以评价癫痫缓解程度。采用实时荧光定量聚合酶链反应法(qRT-PCR)检测海马齿状回 miR-146a-3p 和 miR-146a-5p 的表达。免疫荧光法检测海马齿状回中 Toll 样受体 4(TLR4)、白细胞介素-1 受体相关激酶 1(IRAK1)、肿瘤坏死因子(TNF)受体相关因子 6(TRAF6)、TAK1-结合蛋白(TAB)、核转录因子(NF-$\kappa$B)和白介素 1$\beta$(IL-1$\beta$)的表达。并对以上因子与 miR-146a-3p、miR-146a-5p 的表达分别进行相关性分析。**结果** CHLGMLD 能降低大鼠癫痫发作频率($P < 0.05$)和持续时间($P < 0.01$);CHLGMLD 能下调 miR-146a-5p 和 miR-146a-3p 的表达水平($P < 0.05$),抑制 TLR4、IRAK1、TRAF6、TAB、NF-$\kappa$B、IL-1$\beta$ 的表达($P < 0.01$)。相关性分析显示 TLR4、IRAK1、TRAF6、TAB、NF-$\kappa$B、IL-1$\beta$ 的表达分别与 qRT-PCR 检测的 miR-146a-3p、miR-146a-5p 的表达呈正相关($P <0.01$)。**结论** CHLGMLD 可通过下调 miR-146a-3p 和 miR-146a-5p 的表达水平,抑制 TLR4 通路,减轻颞叶癫痫大鼠海马齿状回炎症反应,缓解癫痫发作。

**【关键词】**柴胡龙骨牡蛎汤;颞叶癫痫;miR-146-3p;miR-146-5p;Toll 样受体 4 信号通路