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Original Research Article

Protective effect of Wuling mycelia, alone and in combination with valproic acid, on pentylenetetrazol-induced epilepsy in rats

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Abstract

Purpose: *To determine the inhibitory effects of Wuling mycelia, alone and in combination with valproic acid (VPA) on pentylenetetrazol (PTZ)-induced epileptic seizure in rats, and their protective effects on cognitive impairment.*
Methods: *Sprague-Dawley rats were randomly divided into five groups: control (sham), model, Wuling mycelia, VPA and combination groups. Rats in Wuling mycelia group were given oral Wuling mycelia alone at a dose of 594 mg/kg, while those in VPA group were given oral VPA alone at a dose of 189 mg/kg. In the combination group, rats received oral VPA at a dose of 189 mg/kg co-administered with Wuling mycelia at a dose of 594 mg/kg. One hour after the treatments, the control group was injected with physiological saline intraperitoneally, while the other four groups were injected with PTZ solution once a day for 28 days. Subsequently, seizure intensity, cognitive impairment, neuronal loss and hippocampal expressions of IL-1β, NF-κB/p65 and TLR4 were determine in all groups.*
Results: *Combined use of Wuling mycelium and VPA significantly reduced the frequency and the grade of seizures ($p < 0.01$), and also decreased the degree of cognitive impairment ($p < 0.05$). There were marked increases in neuronal damage and hippocampal expression levels of NF-κB/p65, TLR4 and IL-1β (inflammatory cytokines) in the model group ($p < 0.05$). However, these changes were reversed in the combination treatment group ($p < 0.05$).*
Conclusion: *Wuling mycelia is a potentially effective adjunct drug for the treatment of refractory epilepsy. The underlying mechanism might involve downregulations of NF-κB/p65, TLR4 and IL-1β.*

Keywords: *Wuling mycelia, Refractory epilepsy, Seizure, Traditional Chinese medicine, Hippocampal area, HMGB1/TLR4/NF-κB signalling pathway, IL-1β, NF-κB/p65, TLR4*

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INTRODUCTION

Epilepsy is a chronic disease. However, the pathogenesis of epilepsy has not yet been clearly ascertained. Many studies have shown that inflammation is closely related to the pathogenesis of epilepsy [1,2]. One study reported that the high-mobility group box-1

(HMGB1)/Toll-like receptor 4 (TLR4)/nuclear factor-kappa B (NF-κB) signaling pathway which is closely associated with the mechanism of anti-inflammatory reactions in the immune system, may have an important impact on inflammation [3]. High-mobility group box-1 (HMGB1) is a major inflammatory activator that mediates immune inflammatory responses by stimulating the pattern recognition receptor Toll Like Receptor 4 (TLR4) [4,5]. Studies have shown that TLR4 activation is important for initiation of NF-κB activation. The activation of NF-κB upregulates the expressions of pro-inflammatory cytokines and other inflammatory regulators such as interleukin (IL)-6, IL-1β and tumor necrosis factor-alpha (TNF-α) which are central to inflammatory response [6]. Extensive research has now shown that pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α play important roles in the pathogenesis of epilepsy [7].

Another important research area in epilepsy is drug resistance. Some studies have shown a high expression level of P-glycoprotein (P-gp) in the blood-brain barrier (BBB) of patients with refractory epilepsy [9,10]. This accounts for their resistance to epilepsy to drugs. Studies on the regulatory mechanism involved in multidrug resistance transporters have suggested that the HMGB1/TLR4/NF-κB signaling pathway is a key factor in the regulation of P-gp synthesis [8,11,12]. These findings indicate that the development of multidrug resistance and the expression of P-gp are closely associated with inflammatory responses.

Traditional Chinese medicine has an extensive history of use in the treatment of epilepsy. Wuling mycelia is obtained from submerged fermentation of mycelial extracts of a rare medicinal fungus called *Xylaria nigripes* (Klotzsch) Cooke, known in China as *wulingshen*. It is native to China. Clinical studies have found that Wuling mycelia have a synergistic anti-epileptic effect on refractory epilepsy when combined with AEDs, but the mechanism underlying this effect has not yet been elucidated [13-15]. The aim of this study was to investigate the inhibitory effect of combination of Wuling mycelia and valproic acid (VPA) on the HMGB1/TLR4/NF-κB signaling pathway, as well as its effects on refractory seizures and cognitive impairment.

EXPERIMENTAL

Animals

Male Sprague Dawley (SD) rats (mean weight = 240 ± 20 g) were obtained from the Animal Service Centre of Lanzhou University (Medical Experimental Animal License SCXY (G) 2018-0002). The rats were kept in colony cages under natural light-dark cycle, temperature of 22 ± 3°C and 40 % humidity. All animal experimental procedures were conducted according to the Ethical Procedures and Guidelines of the Peoples Republic of China. The study was approved by the Ethics Committee of Lanzhou University Second Hospital (approval ref ref no. 2019A-046).

Animal studies

The rats were stochastically divided into five groups: control, model, VPA, Wuling and combination groups. The Meeh–Rubner dose equation was used to determine the clinically applied maximum daily doses of VPA i.e. VPA dose of 30 mg/kg and Wuling mycelia dose of 95 mg/kg for human adults are approximately equivalent to VPA dose of 189 mg/kg and Wuling mycelia dose of 594 mg/kg for SD rats, respectively. Wuling mycelia (0.33 g/capsule) purchased from Zhejiang Zuoli Co. Ltd (#150302; Beijing, China) and VPA (0.2 g/pill) purchased from Hunan Xiangzhong Co. Ltd (#150808; Beijing, China) were each ground to a fine powder and dissolved in 0.9 % NaCl (physiological saline), alone and in combination before use.

Rats in the control and the model groups were given physiological saline only at a dose of 2.5 mL/kg body weight. For VPA, Wuling and the combination groups, each rat was given the corresponding drug at a volume of 2.5 mL/kg body weight: VPA (75.6 mg/mL in physiological saline), Wuling mycelia (237.6 mg/mL in physiological saline), and VPA + Wuling mycelia. All treatments were administered via gavage at 9 am daily for 28 days. One hour after each administration, rats in the control group were given physiological saline via intraperitoneal injection, while rats in the other groups were intraperitoneally injected with pentylenetetrazole (PTZ) solution at a dose of 35 mg/kg.

Behavioural assessment

Seizure behaviour of the rats was monitored by a researcher within 30 min after the last intraperitoneal PTZ injection. Seizure severity was assessed according to the Racine scale as follows: stage 0 = normal status; stage 1 = facial and oral palsy; stage 2 = severe facial paralysis or head nodding; stage 3 = unilateral forelimb clonus convulsion; stage 4 = rearing; and stage 5 = rolling and rearing. The phase and frequency (stage 3 and above) of seizures were also

determined and recorded by the same researcher. Rats showing two stage 4 seizures were considered kindled [16].

Evaluation of cognitive function

Cognitive function was evaluated using the MWM navigation test in which rats were assessed based on their abilities to locate a hidden platform so as to escape from water in a maze tank 100 cm in diameter and 26 cm in height [17]. The maze tank was divided into four labelled quadrants: Northwest (NW), Southwest (SW), Southeast (SE), and Northeast (NE). A hidden 10-cm diameter escape platform was located 1 cm below the liquid level in the centre of the SW quadrant. The water was rendered opaque by addition of highly dispersed titanium dioxide powder. Various kinds of fixed visible cues placed around the pool were used to make it possible for the rat to escape from the water. The two tests were spatial probe test and navigation test. All rats were trained for four consecutive days, four times a day. Care was taken not to place the rats on the platform in the southwest quadrant. The rats were placed at one of the three starting points at the same interval, facing the wall of the pool in the water. When a rat escaped to the hidden platform, the test was deemed complete, and the escape latency was noted. If a rat was not able to escape within 2 min, it was placed at a particular stage for 15 sec to determine the escape latency for 2 min. On the 5^{th} day, the probe test was performed without a platform. The rats were allowed to move around unrestricted from the NE quadrant. Then, the time spent in the NE quadrant and the platform crossover frequency were noted.

Haematoxylin and eosin (H&E) staining

Five rats were randomly picked out from the five groups on the day after the last PTZ administration, and were anaesthetised with chloral hydrate at a dose of 0.35 g/kg via abdominal injection. After inducing deep anaesthesia, the hearts of the rats were primed with physiological saline, prior to their excision and fixation with 4 % paraformaldehyde. The brains were quickly dissected out and fixed with 4 % paraformaldehyde at 4 °C for 24 h. Each brain sample was washed, fixed with 0.1 mol/L phosphate buffer, placed in the embedding cassette, and dehydrated in an alcohol gradient. Then, the tissues were cleared in xylene and embedded in paraffin wax. Subsequently, the hippocampal regions were selected and sliced with a microtome to obtain two 4-μm thick slices. The slices were dewaxed with water, cleared with xylene, rinsed with distilled water and rehydrated with an alcohol gradient. Finally, the slices were affixed to glass slides, stained with hematoxylin and 0.5 % eosin (#20160708; Beijing Suobaolai Co. Ltd., Beijing, China), and covered with coverslips. The degree of neuronal loss in the hippocampal CA3 area was determined by observing each stained section under an optical microscope.

Real-time polymerase chain reaction (PCR)

Five rats were selected from each group on the day after the last PTZ administration and anesthetised with chloral hydrate at a dose of 0.35 g/kg via abdominal injection. Their intact brains were removed, and the hippocampal region was isolated. Total RNA in the hippocampus was extracted with TRIzol reagent (#AA7104-1; Thermo Fisher Scientific Co. Ltd., MA, USA). The concentration of RNA was calculated by measuring absorbance of the extracted RNA solution at 260, 280 and 320 nm using an ultraviolet spectrophotometer. The full mRNA sequences of the desired genes were obtained from the GenBank sequence database. The corresponding PCR extension primers were designed and then synthesized by GenCopoeiaCo. (Rockville, MD). The primer sequences of TLR4 are shown in Table 1.

Table 1: Primer sequences used in the study

Primer	Sequence
TLR4 (forward primer)	5′-CTCACAACTTCAGTGGCTGGATTTA-3′
TLR4 (reverse primer)	5′-GTCTCCACAGCCACCAGATTCTC-3′
NF-κB/p65 (forward)	5′-GATGGGACGACACCTCTACACATA-3′
NF-κB/p65 (reverse)	5′-CCCAAGAGTCGTCCAGGTCA-3′
IL-1β (forward)	5′-ATGGCAACTGTTCCTGAACTC-3′
IL-1β (reverse)	5′-TTAGGAAGACACGGATTCCAT-3′
GADPH (forward)	5′-GGCACAGTCAAGGCTGAGAATG-3′
GADPH (reverse)	5′-ATGGTGGTGAAGACGCCAGTA-3′

Subsequently, the mRNA of each sample was reverse-transcribed into cDNA, and 2 μL of the cDNA solutions, along with the primers of IL-1β, NF-κB/p65, TLR4 and GAPDH genes, were subjected to real-time PCR using the 20-μL amplification system. The Ct values of the target genes were obtained as the internal reference of the Ct value of GAPDH. The data were analysed using the $2^{-\Delta\Delta Ct}$ method.

Western blot assay

On the day after the last PTZ administration, the remaining five rats from each group were anesthetised with chloral hydrate (0.35 g/kg), and their intact brains were removed, followed by isolation of the hippocampal tissues which were then subjected to total protein extraction using lysis. Then, the total protein was resolved using SDS-polyacrylamide gel electrophoresis, followed by transfer to polyvinylidene fluoride membranes (#20170206 Millipore, Billerica, MA) at 4°C with a current of 300 mA for 120 min. at room temperature, in Tris-buffered saline solution containing 0.05 % Tween 20 (TBST).

The membranes were then blocked with 5 % skimmed milk for 2 h, followed by incubation with overnight with anti-GAPDH, anti-IL-1β, anti-NF-κB/p65, and anti-TLR4 (#3253U12; Affinity Biosciences Co. Ltd., Ohio, USA), each diluted 1:1000. After washing 5 times with pre-cooled Tris-buffered saline and Tween 20 solution (TBST) (#L0113A; Amresco Commercial Finance Co. Ltd., ID, USA), the membranes were incubated with HRP-conjugated secondary antibodies (#66016; Affinity Biosciences Co. Ltd., Ohio, USA): goat anti-mouse IgG (1:5000) and goat anti-rabbit IgG (1:5000) for 2 h. Thereafter, the membranes were rinsed 4 times with TBST, followed by enhanced chemiluminescence imaging. The relative band density ratios were determined with densitometric analysis using National Institutes of Health's ImageJ software.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance was used for statistical analysis, and comparisons between two groups were done using Tukey's test. Data analysis was performed using the SPSS 17.0 software (IBM Corporation, Armonk, NY). A p value < 0.05 or 0.01 was considered as indicative of statistically significant difference.

RESULTS

Effect of Wuling mycelia and its combined application with VPA on seizure severity

The frequency and scores of seizures was assessed in each group of rats after each PTZ administration to determine the inhibitory effect of Wuling mycelia and its combined application with VPA on seizures. Rats in the saline control group did not have any episode of seizure (Figure 1). The frequencies (stage 3 and above) of seizures were lower in the combination group than in the model group (p < 0.05). The average stage of seizures in the model group was higher than those in the VPA and Wuling mycelia groups (p < 0.01). The percentages of rats kindled after 28 days in the various groups were 93.75 (model group); 48.38 (VPA group), 83.33 (Wuling mycelia group), and 45.45 (combination group). The effects of Wuling mycelia and its combination on the stages of seizures in the rat model are shown in Figure 1C. These results indicate that the Wuling mycelia and VPA combination inhibited epilepsy in rats.

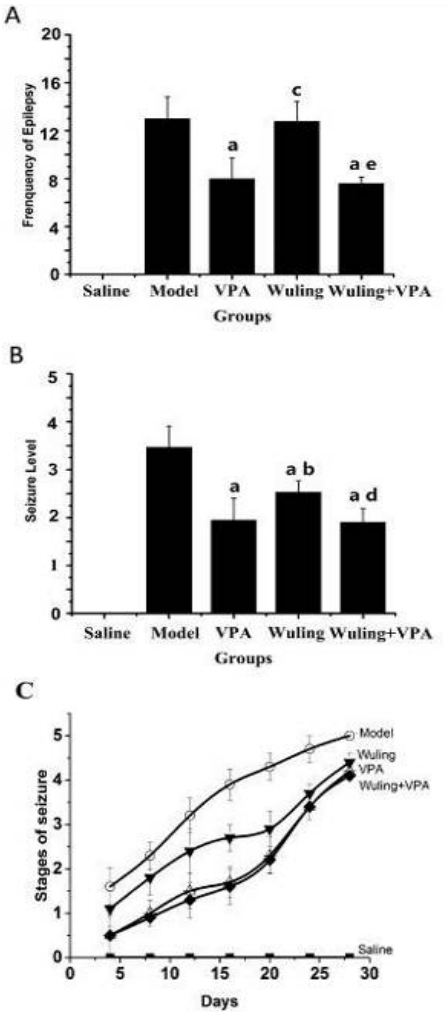

Figure 1: Effects of Wuling mycelia and its combination with VPA on severity of seizure. (A) Frequency of seizures (seizures of stage 3 or above were recorded); (B) average seizure severity score, and (C) seizure scores during development of kindling. Seizures in each group were recorded half an hour daily for 4 weeks. Results are presented as mean ± SD (n = 15). [a]P < 0.01, versus control group; [b]p < 0.05, [c]p < 0.01, versus VPA group; [d]p < 0.05, [e]p<0.05, versus Wuling group

Effect of Wuling mycelia and its combined application with VPA on cognition

As shown in Figure 2 and Table 2, cognitive impairment in the PTZ-induced chronic epilepsy rat model was effectively reduced by Wuling mycelia and its combination treatment with VPA. During the training period in the MWM navigation test, the escape latency of each group decreased over time. The durations of the escape latency in the model and the VPA groups were significantly increased, when compared to the control group on each training day ($p < 0.05$). In addition, the escape latencies of rats in Wuling mycelia and combined groups during the training sessions were significantly shorter than that of rats in model group *(*$p < 0.05$). In the MWM test, cognitive function was assessed by measuring the number of platform crossings. Furthermore, the frequencies of cross-platform swimming in the other four groups were lower than that of the control group, and there was an obvious difference between the VPA and the control group ($p <0.01$). The frequencies of crossing in Wuling mycelia group and the VPA combined group were markedly higher than that in model group ($p < 0.01$). Thus, rats in the model and VPA groups spent significantly more time in the target quadrant than rats in the control group ($p < 0.01$).

However, the time spent in the target quadrant was significantly longer in the Wuling mycelia group ($p < 0.01$) and VPA combined groups ($p < 0.05$) than in the model group. These results suggest that treatment with VPA alone was not able to significantly improve cognitive performance of the rats, while the administration of Wuling mycelia, alone and combination, produced better effects.

Effect of Wuling mycelia and its combined application with VPA on PTZ-induced neuronal death

Results from H&E staining for assessing hippocampal neuronal damage are shown in Figure 3. The control group showed normal, neatly arranged neurons in the CA3 area, with no nuclear pyknosis, dissolution or rupture. In contrast, the model group had reduced neuronal population and irregularly arranged neurons in the CA3 area, with nuclear lysis and ambiguous nucleolus.

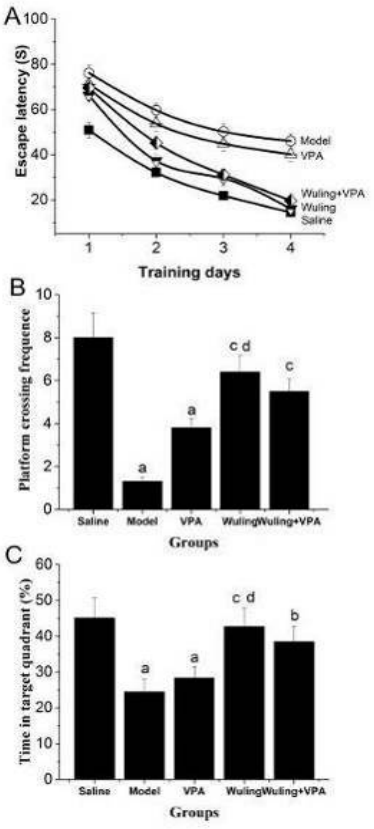

Figure 2: Effect of Wuling mycelia, alone and in combination with VPA on cognitive impairment in rats (mean ± SD, n = 6). (A) Escape latency in the training period. (B) Platform crossing frequency. (C) Percentage of time spent in the target quadrant; [a]$p <$ 0.01, versus the saline group; [b]$p < 0.05$ [c]$p< 0.0$, versus the model group; [d]$p < 0.05$, versus the VPA group

Table 2: Escape latencies on each trial day

Group	Latency (s)			
	Day 1	Day 2	Day 3	Day 4
Saline	50.8±3.4	32.1±1.8	21.8±1.4	14.4±1.3
Model	76.2±3.4[a]	59.7±3.0[a]	50.2±3.5[b]	46.0±2.9[b]
VPA	71.2±1.5[a]	53.7±3.1[a]	44.8±3.1[b]	40.1±2.9[b]
Wuling	66.2±1.8	37.2±3.0[c]	29.5±3.0[c]	16.0±2.2[d]
Wuling + VPA	69.5±2.1	45.2±2.9	31.1±2.5[c]	19.6±3.0[d]

Results are expressed as mean ± SEM (n = 6). [a]$P < 0.05$; [b]$p < 0.01$, versus saline group; [c]$p < 0.05$; [d]$p < 0.01$, versus the model group

Compared with the same area in the model group, the CA3 area of the VPA and combination groups had significantly increased levels of normal neurons, neatly arranged neurons, clearer nucleolus contours and reduced nuclear lysis. Although the neuronal damage in the Wuling mycelia group was reduced, the effect was not as significant as that in the combination group.

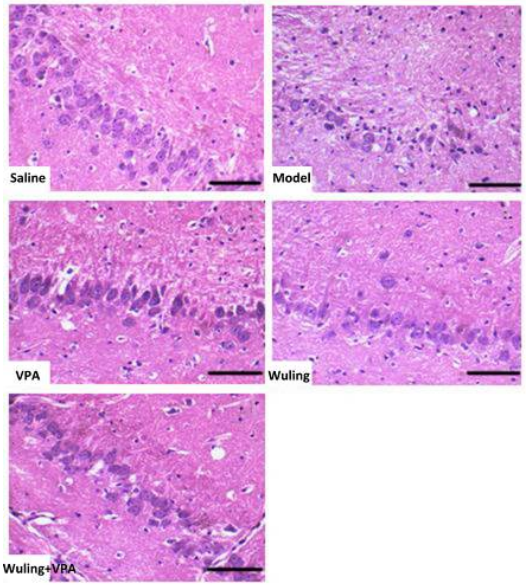

Figure 3: Photomicrographs of H & E stained hippocampal CA3 area (×200)

Effect of Wuling mycelia and its combined application with VPA on hippocampal expression levels of NF-κB/p65, TLR4 and IL-1β genes

Contrasted with the saline group, IL-1β gene expression was higher in the model, VPA, Wuling mycelia and combination groups, but the increases were significant only in the model and Wuling mycelia groups ($p < 0.01$; Figure 4). In addition, IL-1β gene expression levels in the VPA, Wuling mycelia and combination groups were lower than that in the model group, but the decrease was obvious only in the VPA and combination groups ($p < 0.01$). Moreover, tNF-κB/p65 gene expression level was higher in the model group than in the saline control group ($p < 0.05$), whereas it was markedly lower in the VPA and combination groups than in the model group ($p < 0.01$). In addition, NF-κB/p65 gene expression level significantly was lower in the combination group than in the Wuling mycelia group ($p < 0.05$). The expression levels of TLR4 gene in the VPA, Wuling mycelia and combination groups were significantly lower than that in the model group ($p < 0.01$). Results from RT-PCR showed that treatment with VPA-Wuling mycelia combination markedly reduced the gene expressions of NF-κB/p65, TLR4 and IL-1β.

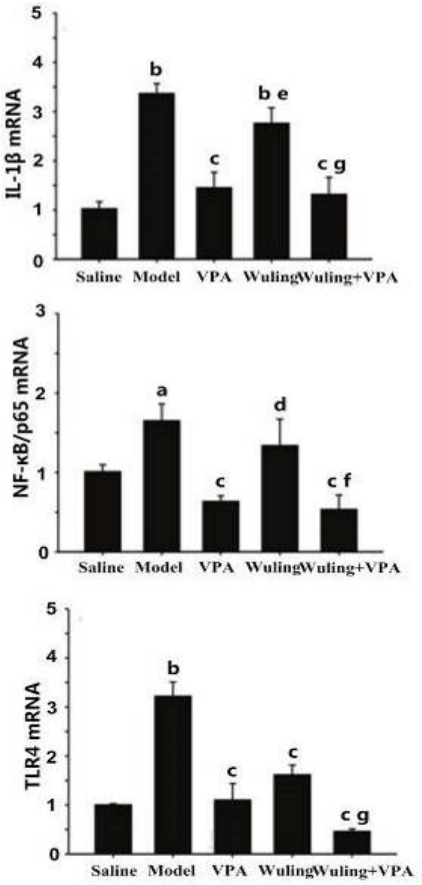

Figure 4: Effect of Wuling mycelia and its combined application with VPA on the gene expressions of NF-κB/p65, TLR4 and IL-1β in the hippocampal area, as assayed using RT-PCR (mean ± SD, n = 5). [a]$P < 0.05$; [b]$p < 0.01$, versus saline group; [c]$p < 0.01$, versus model group; [d]$p < 0.05$; [e]$p < 0.01$, versus VPA group; [f]$p < 0.05$; [g]$p < 0.01$, versus Wuling group

Effect of Wuling mycelia and its combined application with VPA on hippocampal protein expression levels of NF-κB/p65, TLR4 and IL-1β

The protein levels of NF-κB/p65, TLR4 and IL-1β in the hippocampal area were determined using western blotting on the day after the last PTZ administration. As shown in Figures 5A and 5B, the NF-κB/p65, TLR4 and IL-1β protein levels in the model and Wuling mycelia groups were markedly higher than those in the control group ($p < 0.05$). Besides, the levels of these proteins in the combination group were markedly lower than the corresponding levels in the model group ($p < 0.05$). Thus, the combination of VPA and

Wuling mycelia downregulated the protein levels of NF-κB/p65, TLR4 and IL-1β.

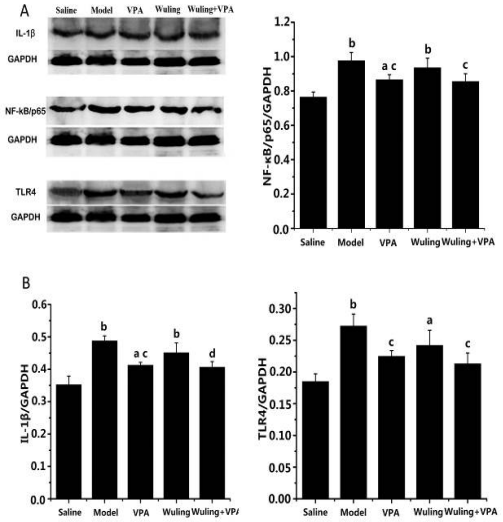

Figure 5: Effects of Wuling mycelia and its combined application with VPA on the hippocampal protein expression levels of NF-κB/p65, TLR4 and IL-1β (mean ± SD, n = 5). (A) Protein levels of NF-κB/p65, TLR4 and IL-1β. (B) Densitometric analysis with Bio-Rad Quantity One 1-D SW. The relative band densities of NF-κB/p65, TLR4 and IL-1β proteins are shown in the bar diagram. $^{a}P < 0.05$; $^{b}p < 0.01$, versus saline group; $^{c}p < 0.05$; $^{d}p < 0.01$, versus the model group

DISCUSSION

Pentylenetetrazol (PTZ) is a neuro-excitatory drug with no toxic side effects. Through repeated administration of its sub-convulsant dose (35 mg/kg), the sub-threshold chemical stimulus accumulated to a certain extent is converted to a threshold electrical distribution effect, thereby inducing epilepsy [18]. This model of epilepsy is similar to epilepsy in humans [19]. The most direct mechanism of epilepsy involves increased membrane permeability of the central neurons which results in impairment of the spontaneous release of GABA, thereby inducing an epileptic episode [20]. Traditional Chinese medicine (TCM) has a long history of use in treating epilepsy in China. When combined with AEDs, TCM produces evident synergistic effects and reduces adverse reactions. One of such traditional Chinese medicine Wuling mycelia is known to regulate immune function, inhibit inflammatory reaction, and reduce anxiety and depression [13].

It has also been reported that Wuling mycelia exerts anti-epileptic effects, with synergistic anti-epileptic properties when combined with AEDs, but the mechanism of these effects has not yet been elucidated [14,15]. Therefore, in this study, the effect of VPA combined with Wuling mycelia on the severity of seizure and frequency in rats was investigated. Significant inhibitory effects on severity of seizure and frequency were produced by VPA and VPA combined with Wuling mycelia. An obvious difference in the seizure severity was observed between the Wuling mycelia and model groups. These results indicate that Wuling mycelia is a suitable auxiliary drug for epilepsy treatment.

A previous study showed that chronic epilepsy was associated with cognitive impairment caused by damaged nerve cells [21]. A number of studies have reported that the hippocampal area is closely correlated with cognitive functions [22]. A study has shown that animals with damaged hippocampal formation had decreased memory and learning abilities, while cognitive impairment was found in the patients undergoing hippocampal resection [23].

Moreover, long-term use of AED and frequent seizures impair cognitive function [24]. In this study, results from MWM test showed that with the prolongation of seizure time, memory and learning functions decreased. The use of VPA alone for 90 days did not effectively enhance cognitive level of rats. However, the use of Wuling mycelia alone or as combined treatment with VPA significantly reduced the mean escape latency, and increased the platform crossing frequency in the target quadrant, indicating significant improvements in learning and memory abilities in the epileptic rats. Therefore, it can be concluded that Wuling mycelia mitigated neuronal damage in the hippocampus, and improved cognitive function in the brain of epileptic rats.

Hippocampal damage and neuronal loss are the pathological features of refractory epilepsy. Studies have shown that inflammation is one of the major pathophysiological mechanisms of seizures [1,2]. Inflammatory factors and their reactions are associated with neurotransmitter imbalance, abnormal synaptic transmission, and BBB disruption. Repeated episodes of epilepsy cause inflammatory reactions in the brain, leading to pathological changes in brain tissue, especially in the hippocampus and other areas susceptible to epileptic activity. This explains the relation between inflammatory reactions and the occurrence of seizures. Therefore, in the treatment of refractory epilepsy, attention should be paid to the suppression of inflammatory response. In this study, the hippocampal CA3 area did not show any appreciable neuronal loss in the combination group. These results indicate

that PTZ-induced neuronal apoptosis in the hippocampal CA3 area may be the main cause of neuronal cell death. However, treatment with Wuling mycelia and VPA combination reversed the PTZ-induced hippocampal neuronal damage.

The HMGB1/TLR4/NF-κB signaling pathway is a significant route for the development of inflammation. Studies have showed that TLR4 activation is important in the activation of NF-κB. Activation of NF-κB upregulates the expressions of IL-6, IL-1β and other pro-inflammatory cytokines, thereby inducing an inflammatory response [6].

Inflammatory response is an important pathophysiological mechanism involved in epilepsy. In order to determine the inhibitory effect of VPA/Wuling mycelia combination on brain inflammation in PTZ-treated rats, the expressions of NF-κB/p65, TLR4 and IL-1β in rat hippocampus were assayed. The combination group showed significant reductions in the expressions of these inflammatory factors, when compared with the model group, demonstrating that Wuling mycelia combined with VPA effectively inhibited the expressions of pro-inflammatory cytokines. In summary, this study has demonstrated that the inhibitory effect of VPA combined with Wuling mycelia on neuronal damage is related to decreased expressions of NF-κB/p65, TLR4 and IL-1β in the rat hippocampus.

CONCLUSION

The findings of this study show that *Wuling mycelia* is an effective adjunct drug for the treatment of refractory epilepsy. In the PTZ-induced epilepsy model, the combination of *Wuling mycelia* and VPA is effective in controlling the occurrence of epilepsy, protecting against cognitive impairment and inhibiting hippocampal neuronal damage. *Wuling mycelium* exerts its anti-epileptic effect via a mechanism involving inhibition of the expressions of NF-κB/p65, TLR4 and IL-1β and other inflammatory factors, thereby affecting the HMGB1/TLR4/NF-kB signaling pathway. Therefore, the combination of *Wuling mycelia* and AEDs may be beneficial in clinical practice as a potential approach for treating refractory epilepsy.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Liming Wei and Haisheng Jiao conceived and designed the study. Liming Wei, Suqin Zhou, Chen Jia, Rui Zhang, Yuting Liu, Yujun Qiao, Yanan Li, and Chunxiang Liu collected and analyzed the data, while Boqun Cao wrote the manuscript. Liming Wei and Boqun Cao should be considered co-first authors.

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