

Original Article

Emodin plays an interventional role in epileptic rats via multidrug resistance gene 1 (MDR1)

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Received January 11, 2015; Accepted February 27, 2015; Epub March 1, 2015; Published March 15, 2015

Abstract: Objective: To observe the interventional effects of emodin in epileptic rats and elucidate its possible mechanism of action. Methods: Thirty-six female Wistar rats were randomly divided into normal control group, model group (intraperitoneal injection of kainic acid) and emodin group (intraperitoneal injection of kainic acid + emodin intervention). The rat epilepsy model was confirmed by behavioral tests and electroencephalography. The protein levels of P-glycoprotein and N-methyl-D-aspartate (NMDA) receptor in cerebral vascular tissue were analyzed by western blotting, and mRNA levels of multidrug resistance gene 1 (MDR1) and cyclooxygenase-2 (COX-2) were analyzed by real-time PCR. COX-2 and P-glycoprotein levels in the brains were detected by immunohistochemical assay. Results: The seizures were relieved in emodin group. Laser scanning confocal microscopy showed P-glycoprotein fluorescence increased significantly after seizures, indicating that epilepsy can induce overexpression of P-glycoprotein. Compared with control group, protein levels of P-glycoprotein and NMDA receptor in cerebral vascular tissue were significantly higher in model group, and mRNA levels of MDR1 and COX-2 were also significantly increased. Compared with model group, P-glycoprotein and NMDA receptor levels in cerebral vascular tissue were significantly decreased in emodin group ($P < 0.05$), and the levels of MDR1 and COX-2 were down-regulated ($P < 0.05$). In the rat brain, seizures could significantly increase COX-2 and P-glycoprotein levels, while emodin intervention was able to significantly reduce the levels of both. Discussion: These findings suggest that epileptic seizures are tightly associated with up-regulated MDR1 gene, and emodin shows good antagonistic effects on epileptic rats, possibly through inhibition of MDR1 gene and its associated genes.

Keywords: Emodin, MDR1 gene, epilepsy, animal model

Introduction

Understanding the mechanisms underlying drug-resistant epilepsy is one of the great challenges in epilepsy research. It has been confirmed that seizures can up-regulate multidrug resistance gene 1 (MDR1) mRNA expression, thereby increasing the synthesis of its product P-glycoprotein at the blood-brain barrier, and reducing the efficacy of antiepileptic drugs that are prevented by the blood-brain barrier from reaching neurons and glial cells at epileptic foci [1-3]. MDR1 gene expression is regulated by many factors, such as cyclooxygenase-2 (COX-2) [4]. Meanwhile, emodin has been proven in many studies to have an anti-inflammatory effect. Our previous studies have shown that

emodin significantly inhibits excitatory postsynaptic transmission and reduces cerebral hyperexcitability. To address the issue, in the present study, we aimed to produce a chemical kindling animal model for observing the effects of emodin in epileptic rats using real-time quantitative PCR and fluorescence quantitative positioning technology, and to investigate its mechanism of action, with the goal of identifying new and efficient antiepileptic drugs.

Materials and methods

Drug

Emodin was purchased from Xi'an Rongsheng Biotechnology Co., Ltd. (Xi'an, China), with a

purity of 98%. The molecular structure of emodin is $C_{15}H_{10}O_5$. Emodin solution was prepared by dissolving 8.3 mg of emodin in 1 ml of dimethyl sulfoxide + alcohol solution at a ratio of 2:8.

Reagents

Kainic acid was purchased from Sigma (St. Louis, MO). [N- ϵ (4-nitrobenzofurazan-7-yl)-D-Lys8]-cyclosporin A (NBD-CSA) was purchased from Novartis (Basel, Switzerland). P-glycoprotein, N-methyl-D-aspartate (NMDA) receptors, COX-2, and β -actin antibody were obtained from Abcam (Cambridge, England). The secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The streptavidin biotin complex (sABC) kit was purchased from Wuhan Boster Biotechnology Co., Ltd. (Wuhan, China). Trizol was purchased from Invitrogen (Carlsbad, CA). The reverse transcription kit, dNTP, Tag enzyme, and SYBR mix kit were purchased from Thermo Scientific (Waltham, MA). Primers were synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China).

Animals

Wistar rats were supplied by the Hubei Provincial Medical Experimental Animal Center (Wuhan, China), under license no. SCXK (e) 2008-0005.

Experimental grouping

A total of 36 female Wistar rats, specific pathogen-free grade, were randomly divided into three groups, normal control group, model group, and emodin intervention group. Rats in the normal control group received no treatment. Intraperitoneal injections of 12 mg/kg kainic acid were administered in the other two groups. No animal died during the modeling. After successful modeling, rats in the emodin group were intraperitoneally injected with 200 mg/kg emodin solution, and no animal died after 24 h.

Electroencephalogram (EEG) changes

EEG monitoring was performed. The recording electrode was positioned in the frontal cortex, while the reference electrode was placed in the cerebellum for EEG recording.

P-glycoprotein distribution under confocal microscopy

Rats were sacrificed at 3, 6, 9, 12 d after epileptic seizures to obtain brain tissues. The brain tissue was placed into PBS to remove the meninges, white matter and choroid plexus. The gray matter was homogenized and placed into Ficoll solution at a final concentration of 15% followed by refrigerated centrifugation at $5800 \times g$ at $4^\circ C$ for 20 min. Concentrated capillary microspheres were placed into 1% BSA-PBS and filtrated using 300-mesh nylon filter. The capillary supernatant was washed three times with PBS and then inoculated into 2 mM NBD-CSA for 1 h. After that, the capillaries were observed under the laser scanning confocal microscope for quantitative analysis of NBD-CSA fluorescence intensity.

P-glycoprotein and NMDA receptor levels in brain vessels detected using western blotting

Brain vascular tissue was isolated to extract protein. The bicinchoninic acid assay was used to determine protein levels by SDS-PAGE. After electrophoresis, the proteins in the gel were transferred to a PVDF membrane that was immersed in a TBS-balanced solution containing 5% skimmed milk powder for prehybridization at room temperature for 60 min. Following rinsing three times with TBS, the membrane was incubated with P-glycoprotein and NMDA receptor antibodies as primary antibodies at $4^\circ C$ for 16 h. The membrane was then washed a further three times with PBS and incubated with horseradish peroxidase-labeled secondary antibody at room temperature for 1 h. After sufficient washing and exposure, the signals on the membrane were detected using an enhanced chemiluminescence system. An image scanner (IMAGE 1000, Bio-Rad Company) was used for detection of hybridized signal intensity.

Real time-PCR detection of MDR1 and COX-2 mRNA expression in brain vessels

Rat brain tissue was isolated to extract total RNA, which was reverse transcribed into cDNA ($A_{260}/A_{280} = 1.994$). Primer sequences were as follows: MDR1, F: GACATGACCAGGTATGCCTA, R: CTTGGAGACATCATCTGTAAGTC; COX-2, F: CTGGAACATGGAATTACCCAGTTTG, R: TGGAACATTCTACCACCAGCA; Actin, F: TGGC-

Emodin in intervention of epileptic rats

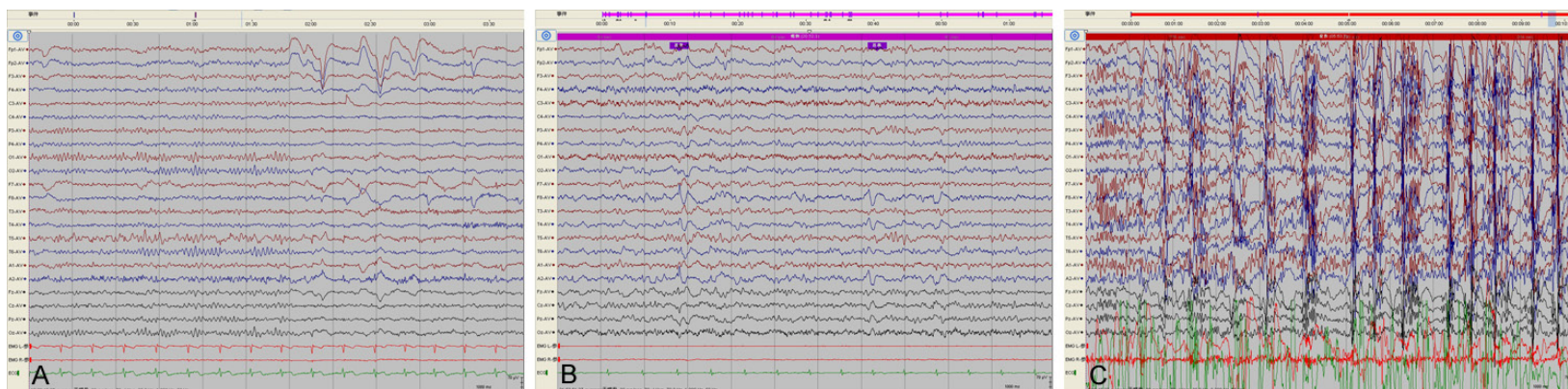


Figure 1. Rat EEG changes in different groups. A. EEG patterns in the normal control group. B. EEG patterns in the model group. C. EEG patterns in the emodin group.

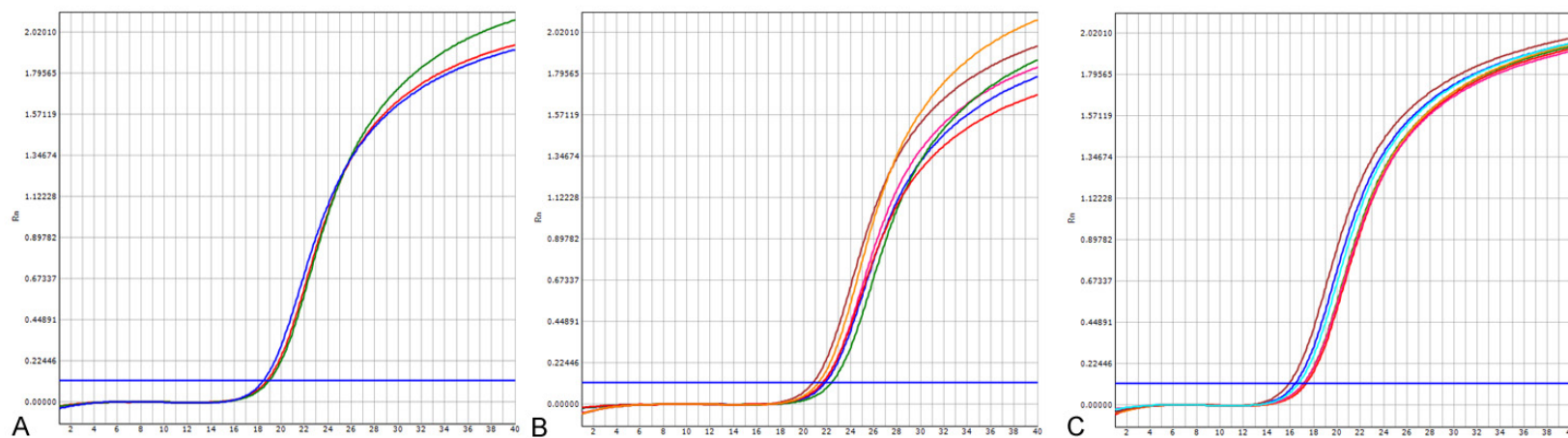


Figure 5. Real-time PCR amplification curves for actin (A), MDR1 (B), and COX-2 (C).

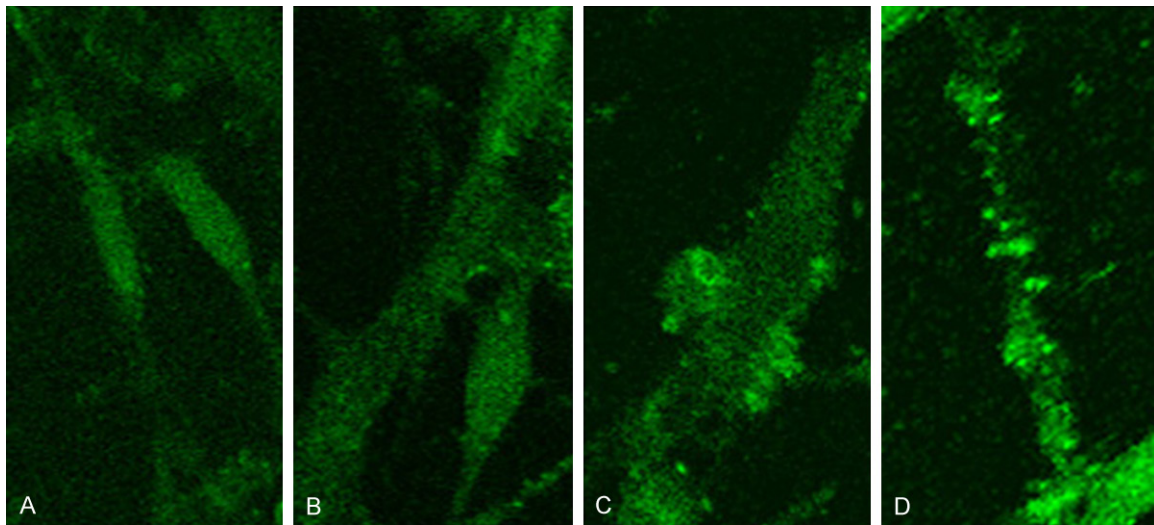


Figure 2. P-glycoprotein distribution in the microvessels of epileptic rats after 3 d (A), 5 d (B), 9 d (C), and 12 d (D) of seizures.

Table 1. Grayscale ratios of P-protein and NMDA receptor in each group

Group	Grayscale ratio of P-protein	$t_{P\text{-protein}}$	Grayscale ratio of NMDA receptor	$t_{NMDA\text{ receptor}}$
Control	0.1602 ± 0.04		0.1870 ± 0.02	
Model	$0.2519 \pm 0.07^{**}$	6.63	$0.4074 \pm 0.09^{**}$	8.38
Emodin	$0.2080 \pm 0.05^{\#}$	2.81	$0.2299 \pm 0.07^{\#\#}$	5.25

$^{**}P < 0.01$ versus the control group; $^{\#}P < 0.05$ versus model group.

ACCCAGCACAAATGAA, R: CTAAGTCAT AGTCCGC-CTAGAAGCA. A 25 μ l reaction system included SYBR Green mix 12.5 μ l, primers (each 2.5 pmol/ μ l) 2 μ l, ddH₂O 8 μ l, and cDNA (10-fold dilution) 2.5 μ l. Cycle conditions were predenaturation for 1 min at 95°C, followed by 40 cycles of 15 s denaturation at 95°C, 15 s annealing at 55°C, 45 s extension at 72°C, followed by a final extension for 10 min at 72°C. Fold differences in gene expression were calculated using analysis software (CFX-96, Bio-Rad Company).

Immunohistochemical detection of COX-2 and P-glycoprotein expression in brain tissue

Whole brain was removed and embedded in paraffin and sliced, and a methanol solution containing 0.3% H₂O₂ was used to remove endogenous catalase. The sections were washed and blocked with 10% goat serum for 20 min followed by incubation in COX-2 and P-glycoprotein antibodies at 4°C overnight. The

sections were rinsed three times with PBS and then incubated in secondary antibody (1:100 biotinylated goat anti-rabbit) at 37°C for 20 min. After a further three washes with PBS, bound antibody was detected using 3,3'-diaminobenzidine, and sections were counterstained, dehydrated, and mounted. Ten fields of vision from each section were selected randomly for immunohistochemical observation using an HPIAS-1000 high-resolution color pathological image analysis system, and the signal was expressed as positive units.

Statistical analysis

Data were expressed as the mean $\pm$ SD and analyzed using SPSS16.0 software (SPSS, Chicago, IL, USA). Comparisons of grayscale ratios of P-glycoprotein and NMDA receptor among groups, comparisons of MDR1 and COX-2 gene expression levels in brain vessels among groups, and comparisons of levels of COX-2 and P-glycoprotein in brain tissue among groups were performed using univariate analysis of variance and t-test. $P < 0.05$ was considered statistically significant.

Results

Behavior changes

Normal rats showed no abnormal changes in behavior. However, rats in the model group

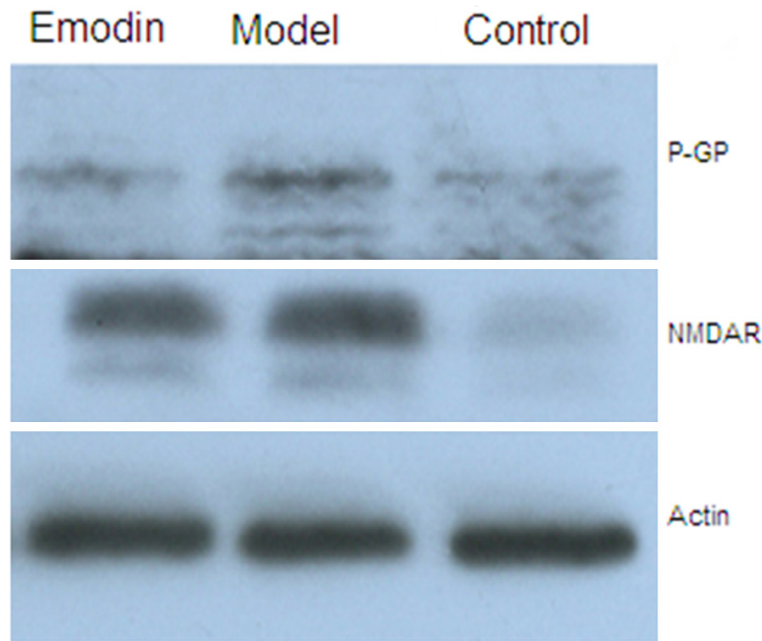


Figure 3. Expression of P-glycoprotein and NMDA receptor in brain vessels. (A) Normal control group, (B) model group, and (C) emodin group.

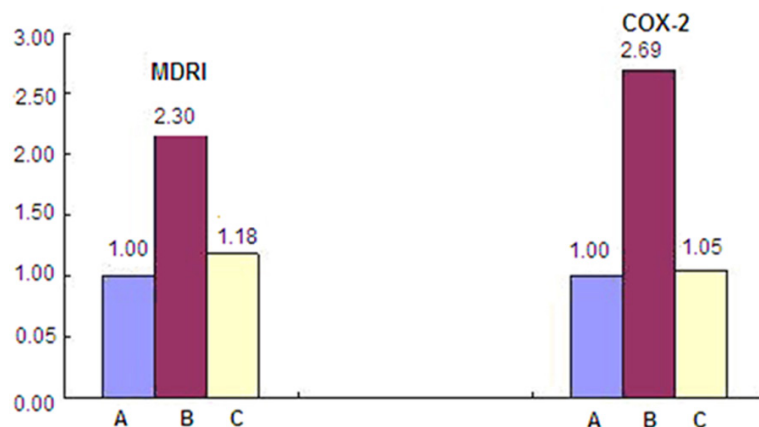


Figure 4. Expression of MDR1 and COX-2 genes in brain vessels. (A) Control group, (B) model group, and (C) emodin group.

showed significant symptoms of epilepsy: muscle tension in the tail, tail firming and obvious opisthotonus. In rats in the emodin group, the epileptic symptoms were alleviated significantly, and rats showed facial muscle twitching and nodding movement (*i.e.* neck muscle twitching).

EEG changes

EEG signals were distributed evenly in the normal control group, with no obvious signs of sei-

zures. In the model group, sleep stages III-IV were visible as 9-10 Hz spike waves, mainly in the forehead and involving the back of the head, with an onset time of 5-7 s. In the emodin group, sleep stages III-IV were visible as sharp waves in the left frontal pole and frontal region, possibly involving the left anterior-middle temporal region, as shown in **Figure 1**.

P-glycoprotein distribution changes in epileptic rats

As shown in **Figure 2**, P-glycoprotein expression was found to increase gradually with time, consistent with the fluorescence intensity, which was (98.8 ± 17.4) at 3 d, (118.8 ± 14.4) at 6 d, (144.7 ± 5.25) at 9 d, and (153.8 ± 6.67) at 12 d. These findings indicate that the seizures are closely related to P-glycoprotein over-expression.

P-glycoprotein and NMDA receptor expression in brain vessels

As shown in **Table 1**, the gray-scale ratios of P-glycoprotein and NMDA receptor were higher in the model group than in the control and emodin groups ($P < 0.05$ or $P < 0.01$). Western blot findings suggested that seizures significantly increase the levels of P-glycoprotein and NMDA receptor, but emodin

can significantly reduce the levels of both (**Figure 3**).

MDR1 and COX-2 gene expression in brain vessels

As shown in **Figure 4**, MDR1 and COX-2 gene expression levels in the model group were significantly higher than those in the control and emodin groups ($P < 0.05$ or $P < 0.01$). The results of these experiments suggested that seizures can significantly increase MDR1

Table 2. COX-2 and P-protein levels in brain tissue

Group	COX-2 positive unit (PU)	t _{COX-2}	P-protein positive unit (PU)	t _{P-protein}
Control	14.73 ± 1.84		30.62 ± 2.83	
Model	25.23 ± 3.45**	7.23	37.606 ± 4.49*	4.35
Emodin	19.38 ± 2.03#	4.81	32.27 ± 2.00#	2.87

P* < 0.05, *P* < 0.01 versus the control group; #*P* < 0.05 versus the model group.

and COX-2 gene expression levels, while emodin can significantly decrease these levels. Specific PCR amplification curves are shown in **Figure 5**.

COX-2 and P-glycoprotein expression in brain vessels

The levels of COX-2 and P-glycoprotein were higher in the model group than in the control and emodin groups (*P* < 0.05 or *P* < 0.01; **Table 2**), indicating that seizures can significantly increase COX-2 and P-glycoprotein levels in brain tissue but that these levels can be decreased by emodin intervention (**Figures 6 and 7**).

Discussion

Emodin is the main active monomer of rhubarb, with a variety of biological effects [5]. Our preliminary studies have shown that emodin has an inhibitory effect on central excitatory post-synaptic potentials that can be reversed by 8-(4-chlorophenylthio) adenosine 3',5'-cyclic monophosphate. This finding suggests that emodin functions to inhibit the release of central excitatory glutamate. Therefore, emodin could theoretically act as an epilepsy drug. In the present study, we also found that after the emodin intervention, epileptic rats appeared to show improvements in their behaviors and EEG findings, suggesting that emodin has a significant antiepileptic effect.

Epilepsy is a chronic brain dysfunction syndrome with many causes; it is manifested as recurrent, paroxysmal, transient cerebral dysfunction caused by repeated super-synchronous high-frequency discharges from groups of brain cells [6-8]. Numerous studies have confirmed an important role for MDR1 in the pathogenesis of epilepsy [9, 10]. MDR1 is located at human chromosome 7 q21.1, and plays a dominant role in the human multidrug resistance

gene family. MDR1 can be controlled by a variety of factors, among which is a regulatory gene [11]. This gene encodes a substrate for P-glycoprotein, which is a transmembrane glycoprotein with similar structure to channel proteins and functions as a membrane transport protein [12]. In our study, using real-time quantitative PCR, we observed 2.3-fold higher expression of MDR1 in the model group compared with the control and emodin groups. Experiments further confirmed that the MDR1 gene occupies an important position in the pathogenesis of epilepsy, while emodin can significantly reduce MDR1 gene activation and have an antiepileptic effect.

We also showed that P-glycoprotein levels in the capillaries at the blood-brain barrier increased with the duration of epilepsy. The level of P-glycoprotein in the model group was significantly higher than those in the control and emodin groups (*P* < 0.05 and *P* < 0.01, respectively). We also used immunohistochemistry to examine P-glycoprotein expression patterns in rat brain tissue, and observed significantly higher levels of P-glycoprotein in the model group than in the control and emodin groups (*P* < 0.05). P-glycoprotein is reported to be highly expressed in neurons, endothelial cells, brain parenchyma and perivascular astrocytes, and this high level of expression is positively correlated with multidrug resistance of intractable epilepsy. Therefore, the results of our experiments suggest that P-glycoprotein is critical for the pathogenesis of epilepsy, and that emodin can effectively reduce the expression of P-glycoprotein in brain vessels and the brain tissue of epileptic rat models to ensure the health of the blood-brain barrier and have an antiepileptic effect.

MDR1 gene activation and P-glycoprotein expression are regulated by NMDA receptor activation and regulation of COX-2, an inflammatory cytokine in the brain. In the present study, NMDA receptor expression in brain vessels and brain tissue was abnormally increased to enlarge NMDA biological effects, while expression of the COX-2 gene was also activated in brain tissue and brain vessels. Studies have shown that NMDA receptor activation is closely associated with COX-2 in the brain, and NMDA receptor-mediated cytotoxic responses can be blocked by COX-2 inhibitors, while the production of prostaglandin E2, the catalytic

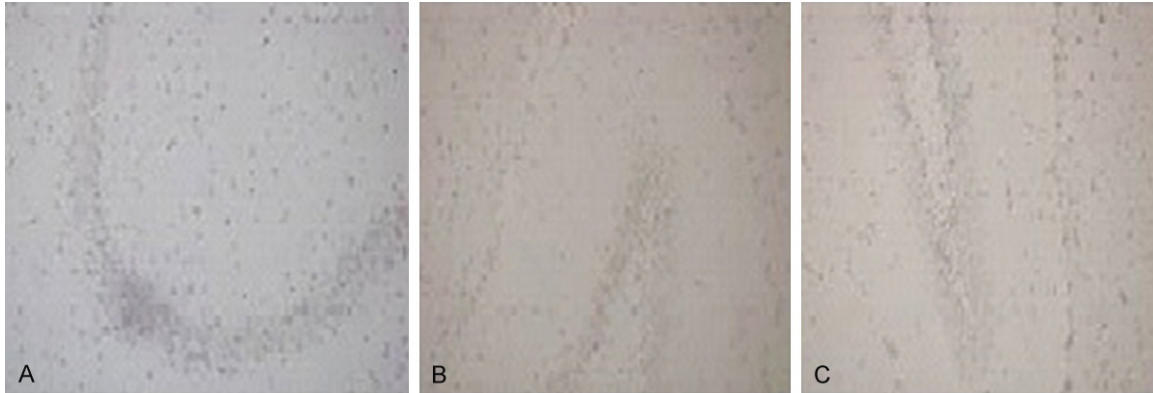


Figure 6. COX-2 expression in brain tissues (immunohistochemistry, $\times 200$). (A) Control group, (B) model group, and (C) emodin group.

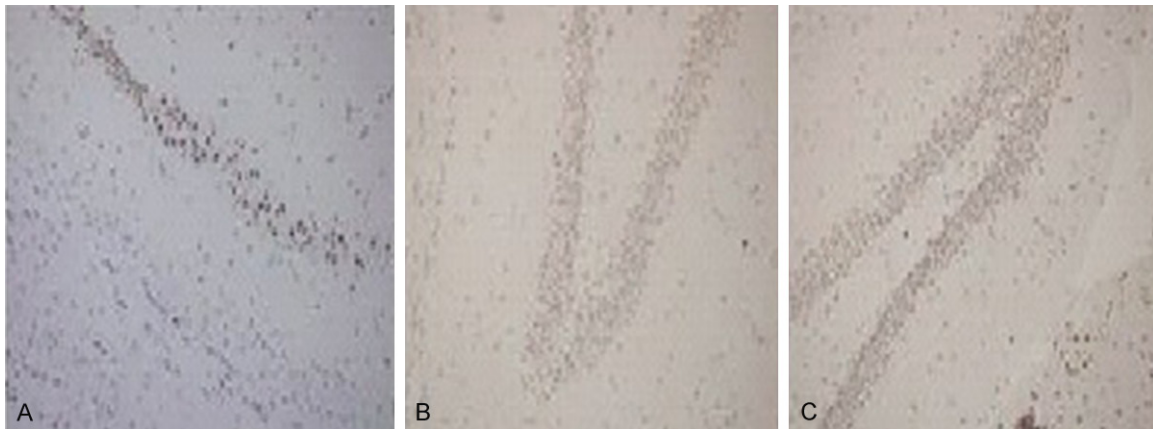


Figure 7. P-glycoprotein expression in brain tissues (immunohistochemistry, $\times 200$). (A) Control group, (B) model group, and (C) emodin group.

cyclooxygenase product in the central nervous system, can be blocked by NMDA receptor blockers [13, 14]. COX-2 inhibitors have been shown to have a similar effect to NMDA receptor blockers on endothelial cells at the blood-brain barrier; that is, glutamate-mediated overexpression of P-glycoprotein is inhibited [15]. Therefore, COX-2, NMDA receptor and P-glycoprotein have a synergic relationship in the pathogenesis of epilepsy. In our study, COX-2 expression in brain tissue and brain vessels was inhibited in the emodin group. Additionally, activity of NMDA receptor in the brain vessels was reduced in the emodin group, and significantly different from that in the model group ($P < 0.05$). Therefore, we hypothesized that the mechanism by which emodin inhibits overexpression of P-glycoprotein may involve its pharmacological effects against inflammatory reactions; that is, emodin can

influence the effects of COX-2 in brain tissue and thus block NMDA-mediated overexpression of MDR1, thereby achieving anti-epileptic effects.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (No. 81071037 and No. 81271395).

Disclosure of conflict of interest

None.

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