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Mechanism of *Erhuang* capsule for treatment of multiple sclerosis☆

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Abstract

Erhuang capsule, a typical formula based on traditional Chinese medicine theory, is widely used to ameliorate multiple sclerosis, inflammation and side effects of glucocorticoid treatment. Oligodendrocyte precursor cells are neural stem cells that are important for myelin repair and regeneration. In the present study, *Erhuang* capsule effectively improved clinical symptoms and neurological function scores, reduced mortality and promoted recovery of neurological functions of mice with experimental autoimmune encephalomyelitis. The mechanism of action involved significant increases in oligodendrocyte precursor cell proliferation in specific regions of the brain and spinal cord, increased oligodendrocyte lineage gene 2 expression and enhanced oligodendrocyte precursor cell differentiation.

Key Words

neural regeneration; traditional Chinese medicine; neurodegenerative diseases; oligodendrocyte precursor cells; *Erhuang* capsule; Chinese compound recipe; methods of tonifying the kidney; resolving phlegm and activating blood; oligodendrocyte lineage gene 2; experimental autoimmune encephalomyelitis; multiple sclerosis; central nervous system; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) *Erhuang* capsule reduced mortality, promoted recovery of neurological functions and improved clinical symptoms and neurological function scores of mice with experimental autoimmune encephalomyelitis.
(2) *Erhuang* capsule significantly promoted oligodendrocyte precursor cell proliferation in specific regions of the brain and spinal cord, increased oligodendrocyte lineage gene 2 expression and enhanced oligodendrocyte precursor cell differentiation to provide beneficial conditions for myelin sheath repair and regeneration.

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INTRODUCTION

Multiple sclerosis is a common inflammatory demyelinating disease in the central nervous system, which is pathologically characterized by multifocal inflammation[1-8], demyelination, axonal loss and gliosis in the white matter[9-10]. Experimental autoimmune encephalomyelitis is a commonly used mouse model of multiple sclerosis with similar pathological and clinical manifestations to the human disease[11-16].

In the central nervous system, oligodendrocytes are the resident cell type responsible for the production of myelin that consists of oligodendroglial plasma

membrane loops tightly wound concentrically around axons. Oligodendrocyte precursor cells are generated in the ventral neuroepithelium of the neural tube early in embryonic life. These proliferative cells migrate into the developing white matter[17-19], exit the cell cycle, undergo differentiation into mature oligodendrocytes and begin to express a subset of myelin-associated proteins[20-21]. Animal models of toxin-induced demyelination have demonstrated the remyelination process is mediated by newly infiltrating proliferative oligodendrocyte precursor cells that have differentiated into mature myelinating phenotypes, rather than previously myelinating post-mitotic mature oligodendrocytes within the lesion. Moreover, retroviral tracing indicated that proliferative cells eventually give rise to remyelinating oligodendrocytes[22]. Recent studies showed that the basic helix-loop-helix transcription factor oligodendrocyte lineage gene 2, which stimulates oligodendrocyte precursor cells to differentiate into oligodendrocytes, is strongly up-regulated in many pathological conditions including acute or chronic demyelinating lesions in the adult central nervous system[23-26].

Erhuang capsule is a typical formula based on traditional Chinese medicine theory of tonifying the kidney, resolving phlegm and activating blood. *Erhuang* capsule can ameliorate experimental autoimmune encephalomyelitis/multiple sclerosis symptoms, relieve inflammation, attenuate glucocorticoid side effects and lower the relapse rate[27-35]. The present study aimed to observe the effects of *Erhuang* capsule on the proliferation and differentiation of oligodendrocyte precursor cells and myelin repair and regeneration in the experimental autoimmune encephalomyelitis model.

RESULTS

Quantitative analysis of experimental autoimmune encephalomyelitis mice

A total of 125 C57BL/6 mice were equally and randomly assigned to normal, model, hormone (prednisone acetate tablets), *Erhuang* capsule high- and low-dose groups according to their body mass. Mice were subcutaneously injected with a water-in-oil emulsion composed of myelin oligodendrocyte glycoprotein 35–55 peptide and complete Freund's adjuvant in the model, hormone, *Erhuang* capsule high- and low-dose groups followed by intraperitoneal injection of pertussis toxin to establish experimental autoimmune encephalomyelitis. The normal group was injected with the same volume of physiological saline. From the first day of immunization, the normal and model groups were perfused with physiological saline, and the two *Erhuang* capsule groups were perfused with *Erhuang* capsule powdered extract suspension at varying concentrations. From day 9 post-immunization, model mice gradually exhibited depression, weight loss (Table 1), tail dragging, hind limb asthenia and even paralysis, accompanied by incontinence and quadriplegia. The neurological scores[36-37] remained at a high level from day 21 to 24 post-immunization. On day 22, the highest neurological score occurred in the *Erhuang* capsule high-dose group. From day 24 to day 42, the neurological score gradually decreased to a plateau. The incidence of experimental autoimmune encephalomyelitis was 96% (23/24, one mouse died from misoperation) in the model group, 88% (22/25) in the hormone group, 83% (20/24, one mouse died from misoperation) in the *Erhuang* capsule high-dose group, and 76% (19/25) in the *Erhuang* capsule low-dose group. The mortality rate was 8% in the model group, and 0% in the other four groups. The experimental autoimmune encephalomyelitis latency was 16.91 ± 4.07, 14.73 ± 3.91, 16.50 ± 3.20, and 17.68 ± 4.83 days in the model, hormone group, *Erhuang* capsule high- and low-dose groups, respectively.

Inflammation in mouse brain and spinal cord

The pathological changes in mouse brain and spinal cord were observed by hematoxylin-eosin staining. Results showed perivascular sleeve-like inflammatory infiltration and karyopyknosis of neurons in experimental autoimmune encephalomyelitis mice.

Table 1 Effect of *Erhuang* capsule (EH) on body mass (g) of experimental autoimmune encephalomyelitis mice

Group	*n*	Day 5	Day 12	Day 16	Day 22	Day 33	Day 40
Normal	25	18.80±1.52[b]	18.76±1.23	19.06±1.04[c]	19.95±1.17[c]	20.19±0.90[c]	21.17±0.98[c]
Model	24	17.79±1.28	18.48±1.78	19.05±1.79[c]	19.67±1.70[c]	22.02±0.78	22.64±1.10
Hormone	25	18.95±1.10[b]	19.64±2.40[a]	18.90±1.84[c]	19.88±1.28[c]	19.93±1.21[c]	20.18±1.25[c]
EH high-dose	24	18.50±1.24	19.69±1.20[a]	19.46±1.71	20.71±1.71	21.76±0.98	22.28±1.18
EH low-dose	25	19.37±1.52[b]	20.51±1.61[b]	20.40±1.97	21.38±1.64	21.83±1.15	22.65±1.21

[a]$P < 0.05$, [b]$P < 0.01$, *vs*. model group; [c]$P < 0.01$, *vs*. EH low-dose group (one-way analysis of variance). Data are expressed as mean ± SD.

Inflammatory foci were evident surrounding the lateral ventricle, CA2 and dentate gyrus region of the hippocampus, and the inflammatory reaction was alleviated in the two *Erhuang* capsule groups compared with the model group (Figure 1).

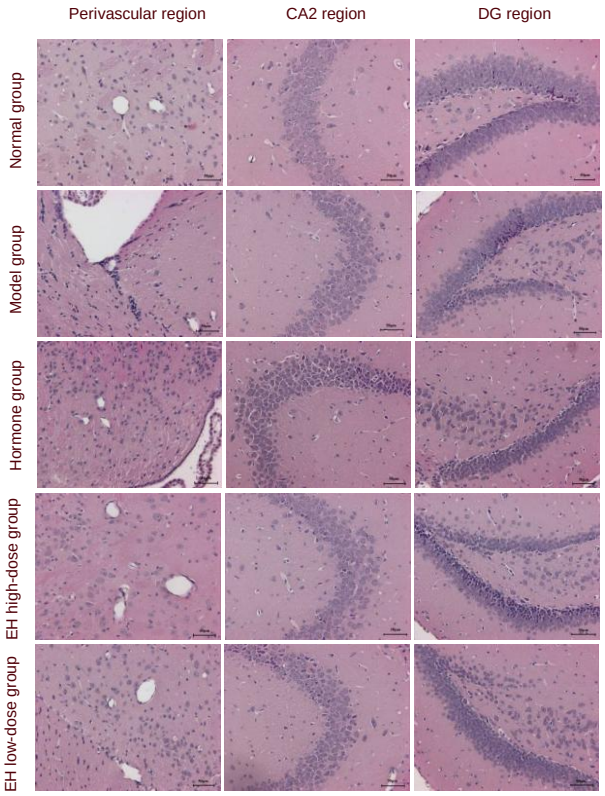

Figure 1 Influence of *Erhuang* capsule (EH) on inflammation in experimental autoimmune encephalomyelitis mice (hematoxylin-eosin staining, × 200). Scale bars: 50 μm.

Dilated capillaries, sleeve-like inflammatory infiltration and karyopyknosis of neurons were detected in the perivascular region, CA2 and dentate gyrus (DG) regions of the model group. These changes were also observed in the two EH groups, but the number of lesions was less than in the model group.

Expression of oligodendrocyte lineage gene 2 and 5-bromodeoxyuridine in different regions of mouse brain and spinal cord

The cellular localization of oligodendrocyte lineage gene 2, a marker for oligodendrocyte precursor cells, was present in the cytoplasm but was predominantly found in the nucleus. Positive expression of oligodendrocyte lineage gene 2 in the normal group showed a dispersed distribution, mainly in the white matter surrounding the lateral ventricle, as well as the ventral part of the spinal cord (Figure 2). In the region superior to the lateral ventricle, oligodendrocyte lineage gene 2 expression in the two *Erhuang* capsule groups was significantly greater than that in the other three groups ($P < 0.01$). In the region inferior to the lateral ventricle, oligodendrocyte lineage gene 2 expression in the two *Erhuang* capsule groups was significantly increased compared with the hormone group ($P < 0.01$). Oligodendrocyte lineage gene 2 expression in the *Erhuang* capsule high-dose group was greater than in the model group ($P < 0.05$).

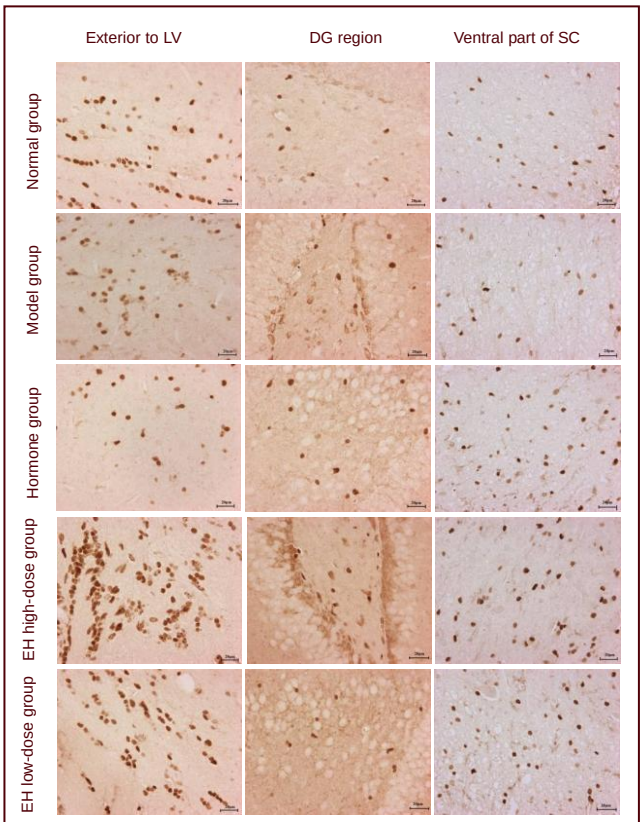

Figure 2 Influence of *Erhuang* capsule (EH) on oligodendrocyte lineage gene 2 (Olig2) expression in different regions of mouse brain and spinal cord (immunohistochemistry, × 400). Scale bars: 20 μm.

The Olig2-positive cells were stained brown-yellow. Olig2 positive cells in the two EH groups significantly increased in the surroundings of lateral ventricle (LV) and the ventral part of the spinal cord compared with the other three groups. DG: Dentate gyrus; SC: spinal cord.

In the region interior to the lateral ventricle, oligodendrocyte lineage gene 2 expression in the *Erhuang* capsule high-dose group was greater than in the hormone group ($P < 0.01$). In the region exterior to the lateral ventricle, oligodendrocyte lineage gene 2 expression in the *Erhuang* capsule high-dose group was greater than that in the model and hormone groups ($P < 0.01$; Table 2). In the ventral part of the spinal cord, oligodendrocyte lineage gene 2 expression in the two *Erhuang* capsule groups was greater than that in the model group ($P < 0.01$). In the dorsal part of the spinal cord, oligodendrocyte lineage gene 2 expression in the *Erhuang* capsule high-dose group was greater than that in the model and hormone groups ($P < 0.01$).

Table 2 Effect of *Erhuang* capsule (EH) on oligodendrocyte lineage gene 2 expression (integrated absorbance value) at different brain sites of experimental autoimmune encephalomyelitis mice

Group	Superior to LV	Inferior to LV	Interior to LV	Exterior to LV	DG
Normal	6.14±1.53	9.94±3.52[d]	11.40±3.67	4.53±1.51	1.66±0.55
Model	5.33±1.61	7.73±3.27	10.30±2.70	5.56±1.38	1.16±0.52
Hormone	6.25±2.56	5.82±2.93	9.24±2.70	4.44±2.09	1.94±0.72
EH high-dose	10.47±1.74[acd]	11.48±2.13[bd]	13.53±4.38[d]	12.05±5.81[acd]	2.03±1.10
EH low-dose	10.26±1.54[acd]	10.50±3.58[d]	10.91±2.46	9.67±4.09[abd]	1.74±0.02

[a]$P < 0.01$, *vs*. normal group; [b]$P < 0.05$, [c]$P < 0.01$, *vs*. model group; [d]$P < 0.01$, *vs*. hormone group (one-way analysis of variance). Data are expressed as mean ± SD ($n = 5$ sections per group). LV: Lateral ventricle; DG: dentate gyrus.

Table 3 Effect of *Erhuang* capsule (EH) on oligodendrocyte lineage gene 2 expression (integrated absorbance value) in different spinal cord sites of experimental autoimmune encephalomyelitis mice

Group	Ventral part of SC	Dorsal part of SC	Periphery of myelocele
Normal	5.78±2.16	4.67±0.92	2.83±0.00
Model	3.97±1.13[e]	3.20±0.81	2.64±0.44
Hormone	6.09±1.22[c]	4.29±0.87	4.67±1.48
EH high-dose	8.06±1.54[ad]	7.08±2.08[bdf]	ND
EH low-dose	7.09±0.75[d]	6.01±1.36[d]	4.06±1.95

[a]$P < 0.05$, [b]$P < 0.01$, *vs*. normal group; [c]$P < 0.05$, [d]$P < 0.01$, *vs*. model group; [e]$P < 0.05$, [f]$P < 0.01$, *vs*. hormone group (by one-way analysis of variance). The data were expressed as mean ± SD ($n = 5$ sections per group). The periphery of myelocele from the EH high-dose group was not analyzed because of technical issues. SC: Spinal cord; ND: not done.

Oligodendrocyte lineage gene 2 expression in the *Erhuang* capsule low-dose group was greater than that in the model group ($P < 0.01$; Table 3). The statistical data represent the results from day 23 (period I) and day 43 (period II) post-immunization. 5-bromodeoxyuridine was expressed on the exterior wall of the lateral ventricle and in the ventral white matter of the spinal cord in experimental autoimmune encephalomyelitis mice (Figure 3). In the normal group, there were few 5-bromodeoxyuridine-positive cells. On the exterior wall of the lateral ventricle, 5-bromodeoxyuridine expression in the two *Erhuang* capsule groups and model group was greater than that in the normal group ($P < 0.05$). In the ventral white matter of the spinal cord, 5-bromodeoxyuridine expression in the *Erhuang* capsule low-dose group was greater than that of the model group ($P < 0.05$). The expression in *Erhuang* capsule high- and low-dose groups was greater than that in the hormone group ($P < 0.05$, $P < 0.01$; Table 4). The statistical data represented the results of the two time points (period I and II).

In period I (day 23), oligodendrocyte lineage gene 2 gene expression in the *Erhuang* capsule low-dose and especially the *Erhuang* capsule high-dose group was greater than that in the hormone group ($P < 0.05$ and $P < 0.01$, respectively). In period II (day 43), oligodendrocyte lineage gene 2 gene expression in the *Erhuang* capsule low-dose group was greater than that in the hormone group ($P < 0.05$; Table 5).

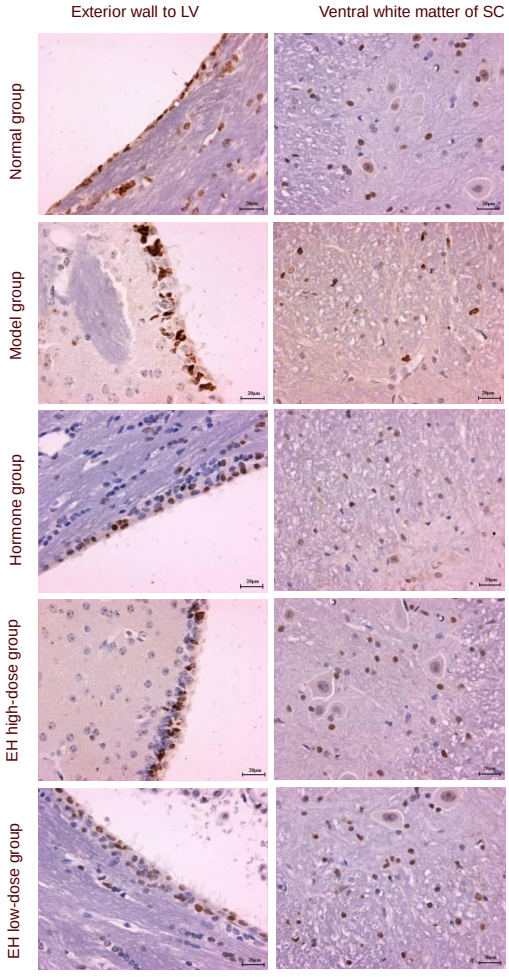

Figure 3 Influence of *Erhuang* capsule (EH) on 5-bromodeoxyuridine (BrdU) expression in different regions of mouse brain and spinal cord (immunohistochemistry, × 400). Scale bars: 20 μm.

The BrdU-positive cells were stained brown-yellow. On the exterior wall of lateral ventricle (LV), BrdU of two EH groups and model group was greater than that of the normal group. In the ventral white matter of spinal cord (SC), BrdU of the two EH groups was greater than that of the model group and hormone group.

Table 4 Effect of *Erhuang* capsule (EH) on 5-bromodeoxyuridine expression (integrated absorbance value) in different brain and spinal cord sites of experimental autoimmune encephalomyelitis mice

Group	Exterior wall to LV	Ventral white matter of SC
Normal	3.17±0.63	4.80±0.55[f]
Model	6.73±1.41[a]	3.86±0.69[f]
Hormone	5.92±1.31	1.98±1.20[bd]
EH high-dose	7.07±1.29[a]	3.62±0.17[e]
EH low-dose	6.51±1.94[a]	5.78±0.15[cf]

[a]$P < 0.05$, [b]$P < 0.01$, *vs*. normal group; [c]$P < 0.05$, [d]$P < 0.01$, *vs*. model group; [e]$P < 0.05$, [f]$P < 0.01$, *vs*. hormone group (one-way analysis of variance). Data are expressed as mean ± SD (n = 5 sections per group). LV: Lateral ventricle; SC: spinal cord.

Table 5 Effect of *Erhuang* capsule (EH) on oligodendrocyte lineage gene 2 mRNA expression ($2^{-\Delta\Delta Ct}$) in brain tissue of experimental autoimmune encephalomyelitis mice

Group	Day 23	Day 43
Normal	1.326±0.945	0.637±0.268
Model	2.167±1.276	1.309±1.399
Hormone	1.306±1.398	0.694±0.352
EH high-dose	3.008±1.440[ac]	0.734±0.186
EH low-dose	2.912±3.425[ab]	2.174±2.056[ab]

[a]$P < 0.05$, *vs*. normal group; [b]$P < 0.05$, [c]$P < 0.01$, *vs*. hormone group (one-way analysis of variance). Data are expressed as mean ± SD (n = 3 samples per group).

DISCUSSION

In the present study, the experimental autoimmune encephalomyelitis mouse model was successfully induced according to a previously described method[38]. The model was evaluated according to the incidence, clinical manifestations, neurological score, body mass and pathological observations. Experimental autoimmune encephalomyelitis was shown to be a useful model for human multiple sclerosis.

The transcription factor oligodendrocyte lineage gene 2 was first reported in 2000, and was shown to be expressed in oligodendrocyte precursor cells and mature oligodendrocytes[39]. The differentiation of oligodendrocyte precursor cells to mature oligodendrocytes was inhibited in the spinal cord of oligodendrocyte lineage gene 2 deficient mice[40]. In the central nervous system, the myelin sheath produced by oligodendrocytes winds tightly around the axon and is important for the protection of nerves and for correct conduction of neuronal signaling along the axon.

Demyelination is an important pathological change in the process of human multiple sclerosis and gradually leads to the dysfunction of the central nervous system. Thus, remyelination may represent a good strategy for the treatment of multiple sclerosis. The present study showed that oligodendrocyte lineage gene 2 was expressed mostly in the region interior to the lateral ventricle, and the ventral part of the spinal cord. Oligodendrocyte lineage gene 2 was increased in the two *Erhuang* capsule groups in the tissue surrounding the lateral ventricle and the ventral and dorsal parts of the spinal cord compared with the other three groups. BrdU staining showed the proliferating cells increased on the exterior wall to the lateral ventricle and in the ventral white matter of the spinal cord in the two *Erhuang* capsule groups. These results suggested that the number of oligodendrocyte precursor cells in the two *Erhuang* capsule groups markedly increased, which provided a premise for the maturation of oligodendrocytes.

Multiple sclerosis belongs to the category of "*Weizheng*" in Chinese medicine. Its pathogenesis is regarded as a deficiency of kidney, and phlegm-blood-stasis. *Erhuang* capsule is composed of Radix Rehmanniae, Radix Rehmanniae Preparata, Bulbus Fritillariae Thunbergii, Rhizoma Gastrodiae, Fructus Forsythiae Suspensae, Radix et Rhizoma Rhei (stir-fried with wine), Herba Leonuri Japonici, Hirudo, and Scorpio. The combination of these compounds yields synergy in tonifying the kidney, resolving phlegm and activating blood. The present study showed that *Erhuang* capsule could effectively improve the clinical symptoms of experimental autoimmune encephalomyelitis mice, prolong experimental autoimmune encephalomyelitis latency, lower mortality, alleviate inflammation of experimental autoimmune encephalomyelitis and promote the proliferation and differentiation of oligodendrocyte precursor cells.

We propose that *Erhuang* capsule may be involved in the proliferation and differentiation of oligodendrocyte precursor cells in specific regions of the brain and spinal cord.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

The experiment was performed at the School of

Traditional Chinese Medicine of Capital Medical University, China from December 2009 to January 2011.

Materials

Animals

A total of 125 healthy, female, specific pathogen-free, C57BL/6 mice, weighing 16–20 g, aged 7–8 weeks, were provided by Beijing Vital River Laboratories, Beijing, China (SCXK (Jing) 2007-0001). Experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[41].

Drugs

Erhuang capsule was composed of Radix Rehmanniae 15 g, prepared Radix Rehmanniae 15 g, Rhubarb (stir-fried with wine) 3 g, Radix Polygoni Multiflori Preparata 12 g, Chekiang Fritillary Bulb 9 g, Hirudo 3 g, Scorpion 3 g, Rhizoma Gastrodiae 5 g, Weeping Forsythia Fruit 9 g and Common Motherwort Herb 15 g, and was provided by Beijing Institute of Pharmaceutical Analysis, China (No. 071206). Prednisone acetate tablets (an effective drug generally accepted in multiple sclerosis clinical trials) were provided by Tianjin Pacific Pharmaceutical Co., Ltd., China (No. 061203). *Erhuang* capsule was dissolved in deionized water to prepare a suspension at a ratio of 41.6 g *Erhuang* capsule in 80 mL deionized water for *Erhuang* capsule high-dose group and 10.7 g *Erhuang* capsule in 80 mL deionized water for the *Erhuang* capsule low-dose group. A prednisone acetate tablet was dissolved in deionized water to prepare a suspension at a ratio of 41 mg Prednisone acetate tablets in 80 mL deionized water for the hormone group.

Methods

Induction of experimental autoimmune encephalomyelitis model and drug intervention

Experimental autoimmune encephalomyelitis was induced in mice by subcutaneous injection in the back with 0.2 mL emulsion, containing 50 μg myelin oligodendrocyte glycoprotein 35–55 peptide (No.C213406; Beijing Scilight Biotechnology Co., Ltd., Beijing, China) in 100 μL 0.9% physiological saline and 100 μL complete Freund's adjuvant (No. 049K8700; Sigma-Aldrich, St. Louis, MO, USA; to which 4 mg/mL *Mycobacterium tuberculosis* was added), followed by two intraperitoneal injections with 500 ng pertussis toxin (No.029K1314; Sigma-Aldrich) on days 0 (the first day of immunization was regarded as day 0) and 2 post-immunization. The mice from the normal group were given physiological saline. On day 1, the two *Erhuang* capsule groups were intragastrically administrated with *Erhuang* capsule 7.8 g/kg per day for the high-dose group and 2.0 g/kg per day for the low-dose group, once daily, for 42 consecutive days. The normal group and model group were intragastrically administrated with the same volume of saline. The dose of crude drug for humans was 23.47 g per day. The body weight of each person is regarded as 60 kg. Thus, the mean dose administered to humans was 23.47 g crude drug/60 kg body mass (that is, 0.39 g crude drug/kg body mass). The conversion ratio of body weight between mouse and human is 0.11. For the high-dose group, mice were given 7.8 g powdered extract/kg body mass, that is, 19.42 g crude drug/kg body mass. Taking the dose into consideration for humans, that would be equivalent to 19.42 g crude drug/kg body mass × 0.11 = 2.14 g crude drug/kg body mass, which is 5.5 times the mean dose given to humans. For the low-dose group, the mice were given 2.0 g powdered extract/kg body mass, that is, 4.98 g crude drug/kg body mass. Taking the dose into consideration for humans, that would be 4.98 g crude drug/kg body mass × 0.11 = 0.55 g crude drug/kg body mass, which is 1.4 times the mean dose for humans. On day 12, the hormone group was intragastrically administrated with prednisone acetate tablets at 7.714 mg/kg, once a day, for 31 consecutive days. Body mass, incidence, mortality rate and neurological scores were observed. Neurological scores were graded on a scale of 0 to 5 using previously established standard criteria[36-37]: 0: normal; 1: flaccid tail; 2: moderate hind or front leg weakness; 3: severe hind or front leg weakness; 4: complete paralysis of limb(s); 5: death.

5-bromodeoxyuridine administration

5-bromodeoxyuridine (No. B5002; Sigma-Aldrich) was dissolved in nonpyrogenic 0.9% physiological saline at a concentration of 5 mg/mL. Intraperitoneal injections of 5-bromodeoxyuridine (50 mg/kg) were given once daily for 4 consecutive days preceding tissue harvest. Twenty-four hours after the last injection, mice were sacrificed (on days 23 or 43 post-immunization, respectively).

Sample preparation

On day 23 (period I) and day 43 (period II) post-immunization, mice were anesthetized with an intraperitoneal injection of 10% chloral hydrate (5 mL/kg). The brain and spinal cord of mice were harvested, fixed, and paraffin embedded. Sections, 5 μm in thickness, were cut for hematoxylin-eosin staining and immunohistochemical analysis. Three mice from each group were prepared for harvest of brain tissues that were

stored at –80°C for real-time PCR analysis. The paraffin blocks for immunohistochemistry and the tissues for real-time PCR used the same region (the middle-third parts of mouse brain).

Hematoxylin-eosin staining

Sections were dewaxed using xylene I and II, dehydrated with gradient alcohol for 5 minutes each, and stained with Harris hematoxylin (No. HHS16; Sigma-Aldrich) for 1 minute, followed by eosin (No. E4009; Sigma-Aldrich) for 10 minutes. The sections were dehydrated in gradient alcohol, permeabilized with xylene, mounted on neutral gum, and observed by light microscopy (Nikon Eclipse 80i, Tokyo, Japan).

Immunohistochemistry detection for oligodendrocyte lineage gene 2

The sections were dewaxed using xylene I and II for 5 minutes each and dehydrated with gradient alcohol for 3 minutes each. The sections were treated with 3% hydrogen peroxide for 5 minutes at room temperature, and washed three times with PBS (pH 7.4) for 2 minutes each. The sections were pre-treated using heat mediated antigen retrieval with sodium citrate buffer (pH 6) for 20 minutes and then washed three times with PBS. The sections were then incubated with anti-oligodendrocyte lineage gene 2 antibody (1:150; rabbit polyclonal antibody to oligodendrocyte lineage gene 2, ab33427, No.735325; Abcam, Cambridge, UK) at 4°C overnight and washed three times with PBS. Secondary antibody (1 drop; two-step IHC Detection Reagent, PV-6001; Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China) was added, incubated at 37°C for 30 minutes, and then washed three times with PBS. DAB was used as the chromogen. The sections were then counterstained with hematoxylin. Five sections from each group were observed by light microscopy (Nikon Eclipse 80i, Tokyo, Japan). Six random high power fields (× 400) were selected for each section of the brain and spinal cord. The results were presented by integral optical density using NIS-Elements BR 3.0 software.

Immunohistochemistry detection for 5-bromodeoxyuridine

Sections were dewaxed using xylene I and II for 5 minutes each and dehydrated with gradient alcohol for 3 minutes each. The sections were treated with 3% hydrogen peroxide for 5 minutes at room temperature and washed three times with PBS. The sections were digested with pancreatin (diluted at a ratio of 3:1) at 37°C for 30 minutes. The sections were then treated with 2 N HCl at 37°C for 30 minutes and washed under running water for 15 minutes. Anti-BrdU antibody (1:100; sheep polyclonal to 5-bromodeoxyuridine, No.661267; Abcam) was added, incubated at 4°C overnight and then washed three times with PBS. Secondary antibody (one drop; two-step IHC Detection Reagent, PV-6001; Zhongshan Goldenbridge Biotechnology Co., Ltd.) was added, incubated at 37°C for 30 minutes and then washed three times with PBS. Diaminobenzidine was used as the chromogen. The sections were then counterstained with hematoxylin. Five sections from each group were observed by light microscopy (Nikon Eclipse 80i). Six random high power fields (× 400) were selected for each section in the brain and spinal cord. The results were presented by integral optical density using NIS-Elements BR 3.0 software.

Oligodendrocyte lineage gene 2 gene expression detected by real-time PCR

RNA from brain tissues was extracted using Trizol (CW0580, CWbio Co., Ltd., Beijing, China). Reverse transcription and real-time quantitative PCR were performed. The oligodendrocyte lineage gene 2 forward primer sequence was 5’-GGGGATTATGGGGAGTCGA-3’ and reverse primer 5’-GGAGTGTTCAGCCAAAGAGTCA-3’. β-actin served as the internal reference using the forward primer 5’-GCCTTCCTTCTTGGGTAT-3’ and reverse primer 5’-GGCATAGAGGTCTTTACGG-3’. The primers were synthesized by CWbio Co., Ltd.

Changes in gene expression were determined by the quantitative method ($2^{-\Delta\Delta CT}$). Following reverse transcription, quantitative PCR reaction was prepared as follows: 10 μL REAL SYBR Mixture (2 × ; CW0767, CWbio Co., Ltd), 0.5 μL upstream primer (10 μM), 0.5 μL downstream primer (10 μM), 2 μL cDNA template and dH_2O to a final volume of 20 μL. Sample loading was done on ice. The reaction solution was placed in 96-well plates, centrifuged at 5 000 × *g* for 15 seconds, followed by real-time PCR reaction in a quantitative PCR apparatus (ABI-7500, Carlsbad, CA, USA). PCR conditions were as follows: 95°C for 5 minutes; 40 cycles at 95°C for 10 seconds and 60°C for 31 seconds. β-actin and oligodendrocyte lineage gene 2 were quantified using PCR, and the relative expression of oligodendrocyte lineage gene 2 mRNA was calculated with the following formula: fold change = $2^{-\Delta\Delta Ct}$ = $2^{-(\Delta Ct(\text{treated samples}) - \Delta Ct(\text{control samples}))}$.

Statistical analysis

The data were analyzed using SPSS 13.0 (SPSS, Chicago, IL, USA), and were expressed as mean ± SD.

Intergroup differences were compared using one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

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