

Original Article

Icariin has synergistic effects with methylprednisolone to ameliorate EAE via modulating HPA function, promoting anti-inflammatory and anti-apoptotic effects

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Abstract: Background: High-dose methylprednisolone (MP) is a clinically recommended therapeutic regimen for Multiple Sclerosis (MS), whereas some dreadful complications induced by it remain inevitable. Studies implied that estrogens might play neuroprotective and anti-inflammatory roles in EAE and MS and promote glucocorticoid efficacy. Icariin (ICA), a primary active component of Epimedium extracts, also possesses neuroprotective and estrogen-like effects with less adverse complication than estrogen. However, rare study focuses ICA's effects on MS or EAE. Objective: Our purpose is to determine whether ICA has synergistic effects with MP in treating EAE and explore the possible mechanisms. Methods: C57BL/6 EAE mice were received different dose of ICA combined with MP and single MP treatment. Then, the clinical scores and serum Interleukin-17 (IL-17), Corticosterone (CORT), Adrenocorticotropic Hormone (ACTH) concentrations were analyzed. Western blot and Flow Cytometry were used to investigate the expression of glucocorticoid receptor (GR) and cell apoptosis. Results: ICA has cooperative effects with MP in decreasing serum IL-17 and CORT concentrations, up-regulating the expression of GR in cerebral white matter and attenuating the cell apoptosis in spinal cord, especially high-dose ICA combined with MP. Conclusion: ICA has synergistic effects with MP to ameliorate EAE via modulating hypothalamic-pituitary-adrenal (HPA) function, promoting anti-inflammatory and anti-apoptotic effects. ICA could be considered as a promising therapeutic option for MS.

Keywords: Icariin, methylprednisolone, experimental autoimmune encephalomyelitis, multiple sclerosis, hypothalamic-pituitary-adrenal

Introduction

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). At present, corticosteroid has been considered as a successful agent for the therapy of relapses, exacerbations or attacks of MS [1]. Administration of high dose methylprednisolone (MP) is a clinically recommended therapeutic regimen. Previously, our study demonstrated that increase the dosage of MP may not improve efficacy in experimental autoimmune encephalomyelitis (EAE) rats [2]. In the light of high dose or long term corticosteroids treatment may lead to severe complications or

glucocorticoid (GC) resistance [3], researchers are fascinated to explore another approach which could decrease the dosage of corticosteroid, or as an alternative for corticosteroid.

Abundant studies have confirmed that estrogens exert many effects on immunomodulation which could down-regulate the level of cytokines TNF- α , IFN- γ , IL-2 and up-regulate IL-4, IL-10 in EAE or MS patients [4, 5]. EAE animals treated with estrogens experienced significantly delayed onset and decreased disease severity [6]. Thus, estrogens may play neuroprotective and anti-inflammatory roles in EAE and MS. Additionally, evidence also indicated that estro-

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gen could positively regulate GC. Treatment with estrogen in ovariectomized female mice increased corticotropin-releasing hormone (CRH) gene expression and resulted in elevated GC level [7]. Therefore, we speculate that estrogen may promote GC efficacy by modulating hypothalamic-pituitary-adrenal (HPA) and ameliorate EAE or MS by the synergistic activities with GC.

However, high dose or long term estrogens treatment may result in hypercoagulability, hypertension or edema and increase the risk of tumor such as breast and endometrial cancer, which hindered the application of estrogen. Meanwhile, researchers alternate their interests in an analogue of estrogen-Phytoestrogen, a non-steroidal plant-derived xenoestrogen, which structurally or functionally mimics circulating estrogen. So far, there is emerging evidence that phytoestrogens have beneficial effects in diverse health disorders, including prostate, breast, bowel, and other cancers [8, 9], cardiovascular disease [10], Alzheimer's disease [11] and osteoporosis [12], with some irreplaceable advantages in less adverse complication than endogenous estrogen.

Epimedium Herba, a genus of flowering plants in the family Berberidaceae, is a kind of phytoestrogen which has been confirmed possessing strong estrogenic activity [13]. Icariin (ICA), a primary active component of Epimedium extracts, also possesses neuroprotective and estrogen-like effects which has been linked to a possible therapeutic role in the treatment of major age-related diseases, like neurodegeneration [14], memory and depressive disorders [15], chronic inflammation [16], diabetes [17], and osteoporosis [18]. However, rare study focuses ICA's effects on MS or EAE. Consequently, our purpose is to determine whether ICA has synergistic effects with MP in treating EAE and explore the possible mechanisms including anti-inflammatory, anti-apoptotic and HPA axis modulation. Our results demonstrated that ICA has cooperative effects with MP in decreasing serum Interleukin-17 (IL-17) and Corticosterone (CORT) concentrations, modulating HPA function, up-regulating the expression of glucocorticoid receptor (GR) in cerebral white matter and attenuating the cell apoptosis in spinal cord.

Materials and methods

Experimental animals

C57BL/6 mice (female, 6-9 weeks old, weight 18 to 22 g) were purchased from Guangdong Medical Laboratory Animal Center (License No. 44007200003436). They had free access to food and water. All animals were treated in accordance with institutional animal ethics guidelines and approval.

EAE induction

The method to induce EAE was applied according to the published protocol [19]. C57BL/6 female mice were induced by immunization with an emulsion of MOG35-55 (Tocris Bioscience) in complete Freund's adjuvant (CFA) (Sigma-Aldrich), followed by administration of pertussis toxin (PTX) (Enzo Life Sciences) in Phosphate Buffered Saline (PBS), first on the day of immunization and then again the following day. Weight and neurological signs were evaluated daily. Neurological signs were scored as following criteria [20]: 0, no detectable signs of EAE; 0.5, limp distal tail; 1, complete limp tail; 1.5, limp tail and hind limb weakness; 2, unilateral partial hind limb paralysis; 2.5, bilateral partial hind limb paralysis; 3, complete bilateral hind limb paralysis; 3.5, complete hind limb paralysis and unilateral forelimb paralysis; 4, total paralysis of both forelimbs and hind limbs; 5, death.

Histological identification of EAE

The peak onset time of EAE was 12 days after immunization when three mice were chosen randomly to be identified by pathological staining. Anesthetized mice were fixed by cardiac perfusion with 4% paraformaldehyde in PBS over 15 min. Spinal cords were postfixed overnight at 4°C and embedded in paraffin. Paraffin sections were cut at 5 µm and stained with hematoxylin and eosin (HE) to assess inflammation, with solochrome cyanine staining for demyelination.

Experimental groups and treatment protocols

EAE mice were divided randomly into five groups named group A, B, C, D, M, six normal mice were fed at the same condition as group N, as described in **Table 1**. ICA (Sigma-Aldrich) or carboxymethylcellulose (CMC) (Sigma-Aldrich) was given by gavage, MP (Pfizer Manufacturing Be-

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Table 1. Experimental groups and treatment protocols

Group	n	Treatment	Protocol	
			Intragastric administration	Tail vein injection
A	6	high-dose ICA combined with MP	ICA 300 mg/kgd	MP 150 mg/kgd
B	7	mid-dose ICA combined with MP	ICA 150 mg/kgd	MP 150 mg/kgd
C	6	low-dose ICA combined with MP	ICA 75 mg/kgd	MP 150 mg/kgd
D	6	placebo combined with MP	CMC 0.3 ml	MP 150 mg/kgd
M	7	placebo	CMC 0.3 ml	Saline 0.1 ml
N	6	placebo	CMC 0.3 ml	Saline 0.1 ml

ICA: Icariin, MP: methylprednisolone, CMC: carboxymethylcellulose. M: EAE model control group; N: normal control group.

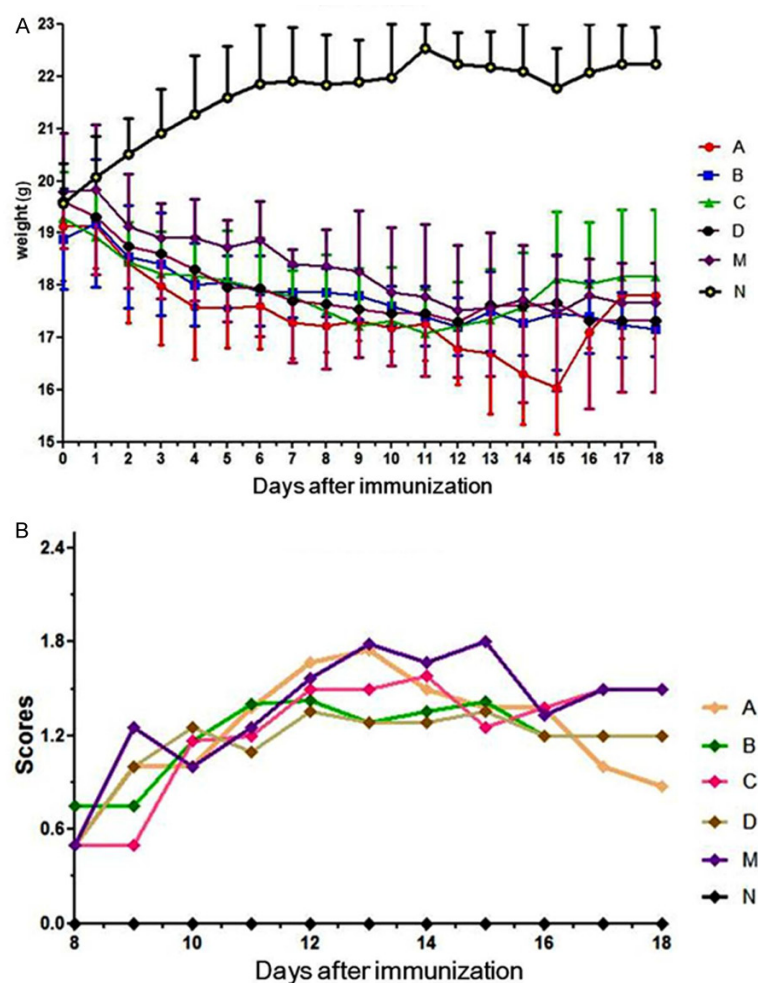


Figure 1. Daily weights and clinical scores of mice. A. Weights of EAE mice were declined after immunization, while normal control mice increased gradually. The weight loss of EAE mice was recovery in different degree followed by ICA and MP treatment, particularly by the treatment of high-dose ICA combined with MP (group A). B. The onset of EAE in mice was 9 days after immunization, neurological signs developed at 11-12 days and ameliorated in different degree after ICA or MP treatment. Treatment with high-dose ICA combined with MP (group A) showed a significantly reduced clinical score.

Igium NV) or saline was injected into tail vein. Treatment was started at 13 days after inoculation when EAE mice presented severe clinical

symptoms. Animals were treated once daily for five days.

Serology test for IL-17, CORT, ACTH

The mice were sacrificed 6 days after treatment and the sera were abstracted at the same time point. Serum concentrations of interleukin-17 (IL-17) was assayed by specific Enzyme-linked immunosorbent assay (ELISA) Kit (Wuhan Boster Biological Technology Co., Ltd, China); Serum CORT and Adrenocorticotrophic Hormone (ACTH) were measured using specific radioimmunoassay kits (Sino-UK Institute of Biological Technology, Beijing, China) and GC-911 γ -ray radioactive immunity analysis instrument (USTC Holdings Co., Ltd, Hefei, China).

Western blot analysis GR in cerebral white matter

Cerebral white matter in lateral ventricle was dissected, the corpus callosum and internal capsule tissues were collected and lysed in a RIPA buffer (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) containing protease inhibitors. Protein concentration was determined by BCA protein assay kit (Thermo Scientific, Pittsburgh, PA, USA). Protein samples were separated by 10% sodium dodecyl sulfatepolyamide gel electrophoresis (SDS-

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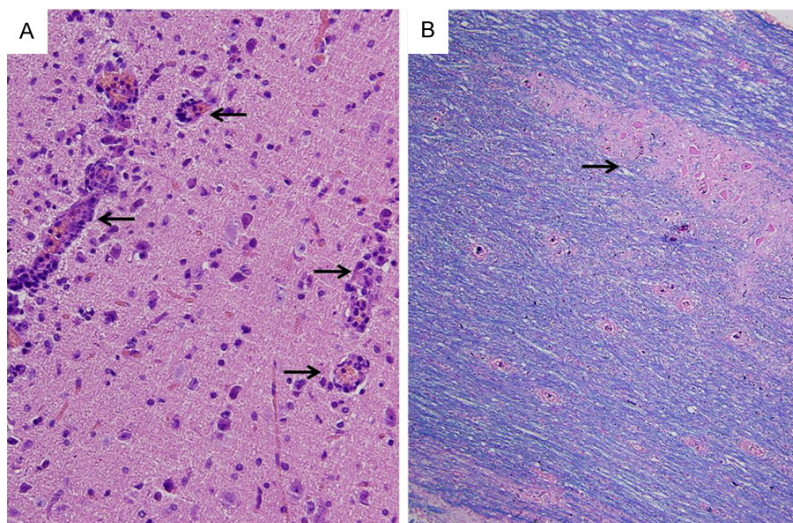


Figure 2. Pathological staining. A. HE×400 (arrows indicate perivascular and parenchymal mononuclear cell infiltrates or cuffs). B. Solochrome cyanine ×100 (arrows indicate demyelinations).

Table 2. Comparison of clinical scores in EAE (X ± SD)

Group	n	Before treatment	After treatment
A	6	1.667±0.516	1.000±0.408*
B	7	1.429±0.535	1.200±0.274
C	6	1.500±0.633	1.500±0.707
D	6	1.357±0.378	1.200±0.274
M	7	1.571±0.535	1.500±0.500

EAE: experimental autoimmune encephalomyelitis.

*P=0.026, vs. before treatment (Wilcoxon signed rank test); There were no significant difference of clinical scores between before and after treatment in group B, C, D, M.

PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 and then incubated with mouse monoclonal anti-GR antibody (1:1000, Millipore). Mouse monoclonal anti-β-actin antibody (1:6000, Millipore) was used as an internal control. Protein bands were detected using enhanced chemiluminescence with horseradish peroxidase-conjugated secondary antibodies (1:10000, Abcam). Scanned images were analyzed by Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc, USA).

Flow cytometry for cell apoptosis

Mice were sacrificed and the lumbar spinal cords were isolated. A single-cell suspension was prepared by passing the tissue through a

400-mesh screen (Cellec-tor, Bellco Glass, Inc., Vineland, NJ). The cells were washed with cold PBS and centrifuged for 6 min at 1,000 rpm, the supernatant was discarded, and the cells were stained with 1 µg/mL of Annexin V and PI. The cells were incubated for 30 min at 4°C and finally, the fluorescence was measured by flow cytometry (BD Biosciences, San Diego, CA, USA).

Statistical analysis

Data are presented as means ± SD. The differences were analyzed by one-way ANOVA followed by the

least-significant difference-multiple comparison test, Wilcoxon test or t test. P<0.05 was considered statistically significant.

Results

Clinical and pathological manifestations of EAE

The onset of EAE in mice was 9 days after immunization. The first sign of illness was decreased appetite and weight loss. Afterward, the mice presented with distal tail weakness, which gradually developed into complete tail paralysis and various degrees of limb paralysis. Neurological signs developed at 11-12 days. Some EAE mice deteriorated quickly and died of severe illness. The weight loss of EAE mice was recovery in different degree followed by ICA and MP treatment, particularly by the treatment of high-dose ICA combined with MP (group A) (**Figure 1A**).

In the HE-stained EAE spinal cord, perivascular and parenchymal mononuclear cell infiltrates were observed in the gray and white matter, and a number of perivascular cuffs were seen (**Figure 2A**). Solochrome cyanine staining showed various degrees of demyelination in EAE mice (**Figure 2B**).

Comparison of clinical scores

The neurological signs were ameliorated in different degree after ICA or MP treatment. But

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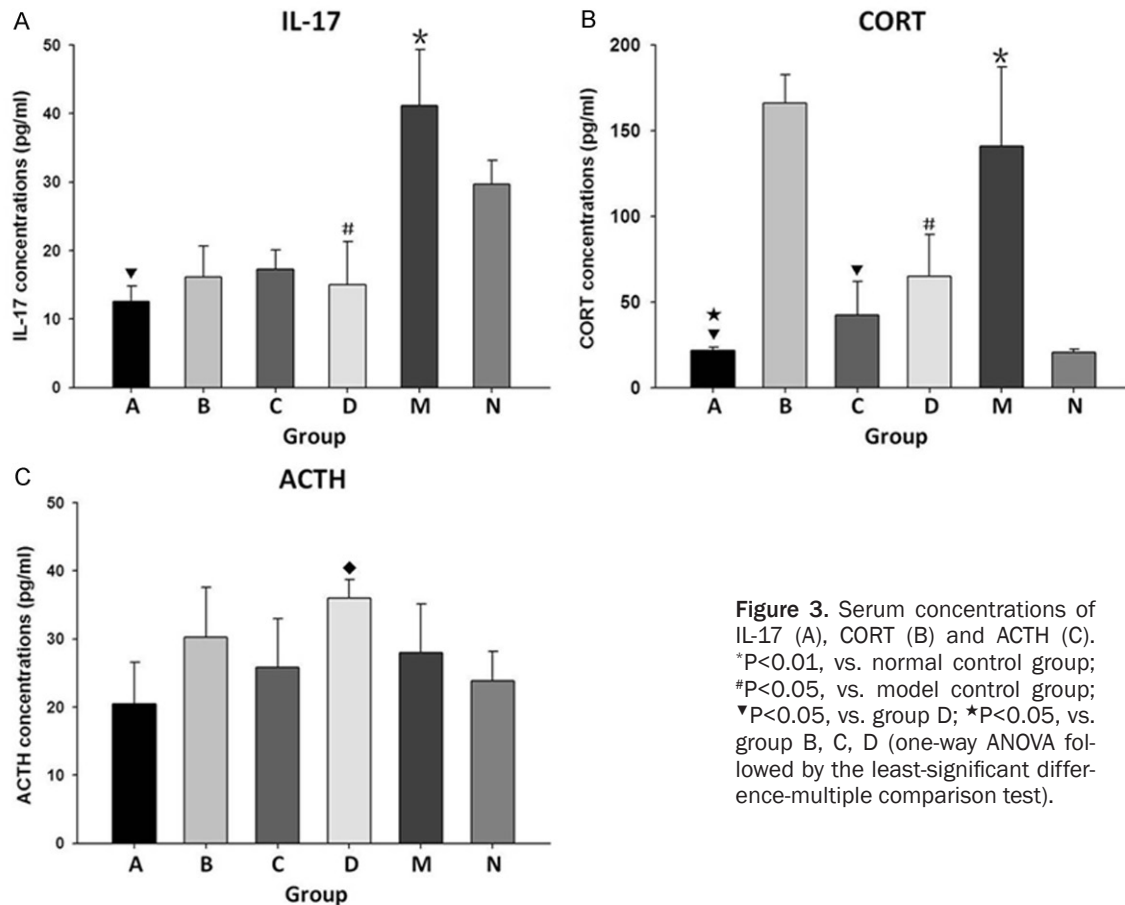


Figure 3. Serum concentrations of IL-17 (A), CORT (B) and ACTH (C). * $P < 0.01$, vs. normal control group; # $P < 0.05$, vs. model control group; $\nabla P < 0.05$, vs. group D; * $P < 0.05$, vs. group B, C, D (one-way ANOVA followed by the least-significant difference-multiple comparison test).

only treatment with high-dose ICA combined with MP (group A) showed a significantly reduced mean clinical score (Table 2; Figure 1B). There were no significant differences of clinical scores between before and after treatment in other groups. These data indicated that high-dose ICA combined with MP may be superior to mid-dose or low-dose of ICA combined with MP in clinical efficacy.

Comparison of IL-17, CORT, ACTH

In the model control group, concentrations of IL-17 and CORT were apparently higher than that in the normal control group ($P < 0.01$). Mice treated with MP exhibited significantly reduced IL-17 and CORT concentrations in comparison to the model control group ($P < 0.01$). In contrast to single MP treatment, administration of high-dose ICA combined with MP further decreased the levels of IL-17, while mid-dose and low-dose of ICA group showed similar levels to single MP group (Figure 3A). High-dose and low-dose ICA group also showed significantly lower levels of CORT compared with single MP group, whereas

high-dose ICA reduced CORT levels markedly as compared to low-dose of ICA group. There was no significant difference in CORT levels between mid-dose of ICA group and model control group (Figure 3B). In view of ACTH concentrations, the levels in single MP group elevated remarkably in comparison with that in other groups. Treatment with different dose of ICA combined with MP produced unchanged ACTH concentration compared to the model control group and normal control group (Figure 3C).

Expression of GR

Densitometric analysis of Western blot revealed that the GR levels decreased in model control group in comparison to normal control group. Treatment with single MP triggered a significant increase in GR levels compared with model control group. It was surged further by the administration of different dose of ICA combined with MP. Notably, treatment with high-dose ICA elicited the elevation more remarkably (Figure 4).

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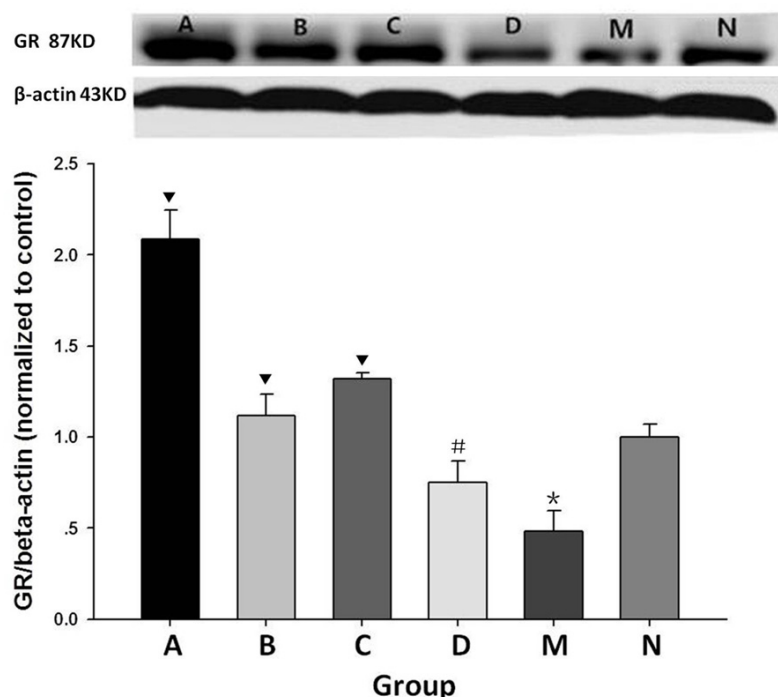


Figure 4. Densitometric analysis of GR expressions. * $P < 0.01$, vs. normal control group; # $P < 0.05$, vs. model control group; ▼ $P < 0.01$, vs. group D (one-way ANOVA followed by the least-significant difference-multiple comparison test).

Cell apoptosis

Flow cytometry assay with Annexin V/PI double staining showed that the apoptotic rate increased to $37.33 \pm 3.308\%$ in model control group, while, the rates of normal control group was only $4.15 \pm 0.636\%$. Statistic analyses revealed that treatment with different dose of ICA combined with MP or single MP declined the apoptotic rate markedly in comparison with that in model control group, particularly in high-dose ICA group, the rate decreased more significant than the other treatment groups (**Figure 5**).

Discussion

The effects of IL-17 on the progression of MS have been concerned greatly at present. IL-17 produced mainly by Th-17 cells which is involved in the development of many autoimmune diseases. It was found that IL-17 mRNA was augmented in the blood and CSF of MS patients [21], and the increased production of IL-17 was detected in the brain during early EAE [22]. The development of EAE was markedly suppressed in IL-17 gene knock-out mice [23]. Thereby,

these observations suggest that IL-17 is crucial for the pathogenesis of MS and EAE. Currently, ICA has been reported to have potential anti-inflammatory activities and associated with immunomodulation, including IL-17. Studies illustrated that ICA could decrease Th17 cells and suppress the production of IL-17 in mice of collagen-induced arthritis [24]. ICA also triggered a significant reduction in IL-6, IL-17 and TGF- β level in bronchoalveolar lavage fluids cell [25]. Moreover, recent study demonstrated that ICA ameliorated EAE and inhibited Th1 and Th17 cell differentiation by the modulation of dendritic cells [26]. Hence, it implies that ICA has therapeutic potential for neuroinflammatory diseases

via the regulation of IL-17. Our results also found that IL-17 was enhanced in the serum of EAE mice, which could be down-regulated by the treatment of MP and decreased deeply by combining with ICA in a dose-dependent manner. Thus, it suggested that ICA has synergistic effect with MP in down-regulating IL-17, especially high-dose ICA.

Nowadays, increasing studies in MS and its animal models have shown disruptions in the HPA axis. Decreased HPA function may play an important role in the increased susceptibility and severity of MS [27, 28]. Hyper- and hypoactivity of the HPA axis have been described to be associated with more severe courses [29]. ACTH is an important hormone of HPA axis plays a key role in regulating adrenal cortex to release CORT. In animals, CORT is the primary GC whose level responses to the activity of HPA axis. There is clear evidence that disruption of HPA axis results in up-regulated CORT with the purpose of constraining the progression of inflammatory state in the occurrence of EAE. However, over-expressed CORT may lead to structural and functional changes in brain regions including hippocampus and induce

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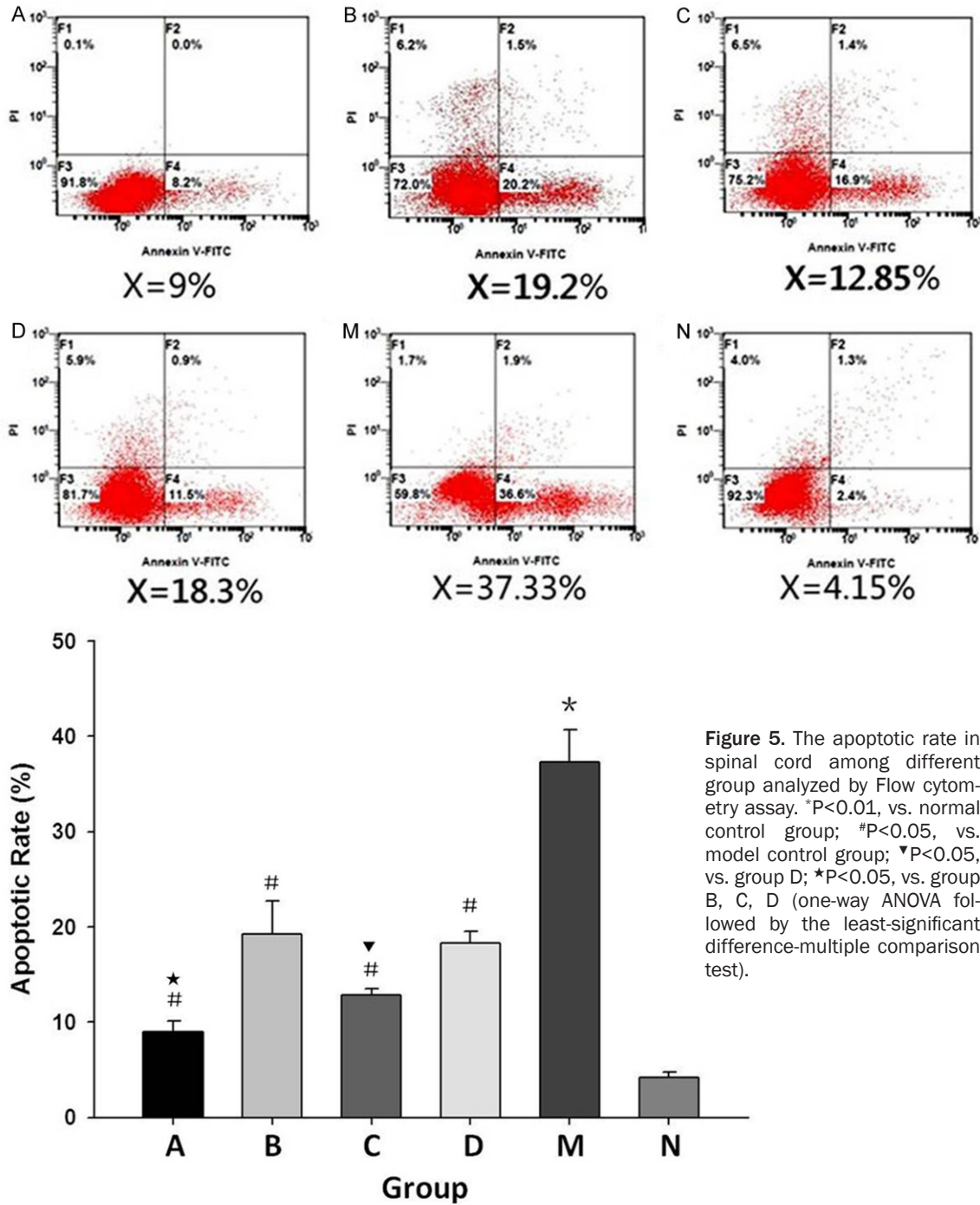


Figure 5. The apoptotic rate in spinal cord among different group analyzed by Flow cytometry assay. *P<0.01, vs. normal control group; #P<0.05, vs. model control group; ▼P<0.05, vs. group D; *P<0.05, vs. group B, C, D (one-way ANOVA followed by the least-significant difference-multiple comparison test).

emotional or cognitive deficits, even exacerbate the disease [30, 31]. In the present study, the serum CORT level was also elevated in EAE mice, while ACTH was unchanged. After given exogenous GC, the elevated CORT was attenuated by negative feedback regulation and further decreased by the treatment of ICA combined with MP, whereas ACTH remained

unaltered. As a result, it suggests that ICA has cooperative effect with MP in inhibiting CORT to regulate HPA axis function. Nevertheless, we didn't found ICA could dose-dependently down-regulate CORT and affect serum ACTH level. Likewise, other studies also demonstrated that administration of ICA could reverse the abnormal increases of serum CORT levels and had no

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appreciable effect on ACTH [32-34]. Thus, we inferred that it probably caused by other factors which may contribute to the modulation of CORT and ACTH since the underlying mechanisms in ICA regulating HPA axis remain elusive.

Recently, much work has been reported that ICA induced GR modulation. Studies revealed that ICA significantly increased GR mRNA and protein expression in the lungs of mice exposed to smoke, stress and allergen [32, 35]. ICA also attenuated social defeat-induced down-regulation of GR in the liver and hippocampus of mice model for depression [36, 37]. Similarly, our data also manifested that administration of ICA combined with MP further promoted the expression of GR in cerebral white matter of EAE mice in contrast to single MP treatment, especially high-dose ICA. Accordingly, we speculated that ICA has synergistic effect with MP to restore EAE-induced down-regulation of GR and normalize GR function, so as to improve the anti-inflammatory activities of GC.

Additionally, previous studies indicated that ICA possesses cardioprotective and anti-osteoporotic efficacy, which is associated with its anti-oxidative and anti-apoptotic effect [38, 39]. In our work, we found that ICA combined with MP attenuated the apoptosis of spinal cord neuron more significant than single MP treatment. It suggests that the cooperative activity of ICA with MP in anti-apoptosis greatly contribute to the recovery of EAE.

In conclusion, ICA has synergistic effects with MP in decreasing serum IL-17 and CORT concentrations, modulating HPA function and up-regulating the expression of GR in cerebral white matter, enhancing the anti-inflammatory and anti-apoptotic effects of MP in ameliorating EAE. As a major constituent of flavonoids from the Chinese medicinal herb *Epimedium brevicornum*, ICA exerts neuroregulatory and neuroprotective activities as well as estrogen, but less adverse complication than estrogen, thus it is considered as a potential therapy against neuroinflammatory diseases. Admittedly, MP is a pervasively applied first-line drug for MS, whereas some dreadful complications induced by it remain inevitable. The synergistic effects of ICA combined with MP will make it feasible to shrink MP dosage and reduce the side effects of corticosteroid such as GC-

induced osteoporosis [40]. Hence, ICA may be considered as a promising therapeutic option for MS.

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Disclosure of conflict of interest

None.

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