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Full Length Research Paper

Evaluation of 11-hydroxy-Δ8-THC-dimethylheptyl effects on cytokines profile and locomotor tests in experimental autoimmune encephalomyelitis

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One of the components that activate cannabinoids (CB) receptors is 11-hydroxy-Δ8-THC-dimethylheptyl (HU-210). The present study was conducted to evaluate the effectiveness of HU-210 treatment to cytokines profile and locomotor tests in the experimental autoimmune encephalomyelitis (EAE) model in the mice. A total of 48 male C57BL/6 mice were placed in 8 therapeutic groups. Mice were immunized with myelin oligodendrocyte glycoprotein (MOG) 35–55. When EAE was observed¸ HU-210 was subcutaneously administered in 3 groups with different doses: 3,10,30 mg/kg. Control groups were treated to vehicle or HU-210. Drug or vehicle was given once on the following days: 1, 3, 5, 7, 9, 11, 13, 15, 17. During the study clinical scores of disease and balance beam test were analyzed. After 17 days of treatment, the animals sacrificed and, TNFα, IL-12, IL-4 level in serum was measured using enzyme-linked immunosorbent assay (ELISA). Our results showed that HU-210-treated mice had significantly less clinical score of EAE than non-treated EAE induced mice ($p<0.05$). Data revealed that dose 30 mg/kg HU-210 in EAE induced mice significantly decreased serum TNFα, IL-12 ($p<0.05$). Also, Serum IL-4 levels increased significantly in mice receiving 3, 10, 30 mg/kg dose of HU-210 ($p<0.05$). Present study revealed the beneficial effects of HU-210 in the model of EAE, and suggests its potential use as a drug for the treatment of multiple sclerosis (MS).

Key words: Experimental autoimmune encephalomyelitis, 11-hydroxy-Δ8-THC-dimethylheptyl, cytokine, cannabinoids.

INTRODUCTION

Multiple sclerosis (MS) is a disorder of the central nervous system, manifesting as acute focal inflammatory demyelination and axonal loss with limited remyelination, culminating in the chronic MS plaques from which the disease gets its name. More recently, there are indications that cannabinoids (CB) may slow progressive neurodegeneration (Zajicek et al., 2005). (–)3–(1,1-dimethylheptyl)-(–)11-hydroxy-delta 8 tetrahydro cannabinol (HU-210) belongs to the group of classic CBs, which present the tricyclic benzopyran structure as their skeletons (Hill and Gorzalka, 2004; Bologov et al., 2010). The marked lipophilic properties of HU-210 allow it to pass across the blood-brain barrier. To date, two different CB receptors have been characterized and cloned from mammalian tissues: the CB1 receptor, which is found primarily in the central nervous system and testis (Devane et al., 1988), and the CB2 receptor, which is located in the periphery, predominantly in the immune system (Rieder et al., 2010). Study of CB1 (Devane et al., 1988) and CB2 (Rieder et al., 2010) receptors clearly established the molecular determinants of ligand binding

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Table 1. Experimental design of study.

Intervention / Group	MOG33-55 peptide injection	Days to EAE induction	Drug/ vehicle was given once on the days 1, 3, 5, 7, 9, 11, 13, 15, 17 (After EAE induction)	Animal were sacrificed	IL-12,TNFα,IFNγ, IL-4 were measured Using ELISA
EAE+3 mg/kg	Yes	22±2	3 mg/kg HU-210	Yes	Yes
EAE+10 mg/kg	Yes	22±2	10 mg/kg HU-210	Yes	Yes
EAE+30 mg/kg	Yes	22±2	30 mg/kg HU-210	Yes	Yes
EAE+PBS	Yes	22±2	Phosphate buffer saline	Yes	Yes
Healthy mice	No	-	NO	Yes	Yes
noEAE+3 mg/kg	No	-	3 mg/kg HU-210	Yes	Yes
noEAE+10 mg/kg	No	-	10 mg/kg HU-210	Yes	Yes
noEAE+30 mg/kg	No	-	30 mg/kg HU-210	Yes	Yes

and selectivity, as well as of their activation. CB receptors, activate multiple intracellular signal transduction systems, such as inhibition of adenylate cyclase (Marriott and Huffman, 2008; Howlett et al., 2010) and stimulation of the microtubule associated protein kinase (MAPK) cascade (Powles et al., 2005; Lipina et al., 2010). HU-210 has been found to be much more potent than tetrahydrocannabinol (THC) at binding to the neuronal CB1 receptor and inhibiting adenylate cyclase (Devane et al., 1988; Howlett et al., 1990). CB has been associated with suppression of the immune function (Croxford and Yamamura, 2005; Klein and Cabral, 2006).

HU-210 suppresses the productive phase of the primary humeral immune response by impairing B cells macromolecular synthesis, which is chirally dependent (Pertwee, 2005; Basavarajappa et al., 2009). HU-210 has been found to inhibit the activity of macrophages (Smith et al., 2000). The above described mechanisms combined with the safe profile of the drug encouraged us to hypothesize that the use of HU-210 could be beneficial as a novel treatment approach for neurological damage of MS. The present study was conducted to evaluate the effectiveness of HU-210 treatment in the experimental autoimmune encephalomyelitis (EAE) model in the mice.

MATERIALS AND METHODS

Animals

All experiments were conducted on male mice aged 4–6 weeks belonging to the C57BL/6 strain (Central Animal Breeding House, Pasture Institute of IRAN) weighing 17–20 g and housed in groups of six. Standard laboratory rodent chow and tap water were supplied ad libitum. Room temperature of 25±0.5°C and humidity between 50 and 70% were maintained during a 12/12 h (7 a.m. /7 p.m.) light–dark cycle. Ethics approval was obtained from the animal ethics committee of Kashan University of medical sciences.

EAE induction

Mice were immunized with myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (MEV GWY RSP FSR VVH LYR NGK; ALEXIS) for development of EAE. For sensitization protocol, each animal was immunized by subcutaneous injection in the flank with 300 µl of a 1:1 emulsion of 250 µg of MOG prepared in phosphate buffer saline (PBS) and complete freund's adjuvant (CFA)(Sigma–Aldrich) containing *Mycobacterium tuberculosis* H37Ra. Mice were also intraperitoneally (i.p) injected with 400 ng of pertussis toxin (Sigma–Aldrich) in 100 µL of PBS. A second, identical injection of pertussis toxin was given after 48 h (Costa et al., 2003). The disease course of animals was evaluated with the clinical EAE-scoring scale (Meyer et al., 2001; Kerschensteiner et al., 2004). Clinical signs of EAE appeared 22±2 days after MOG35–55 peptide injection.

Experimental design

Altogether 48 mice were used in this study, among which 24 were immunized with MOG 35–55 peptide for development of EAE. The animals were divided into two big groups (experimental and control).

The experimental group consisted of 3 immunized subgroups (EAE; n=6), And control group consisted of 5 subgroups. Experimental design is shown in Table 1.

It was possible that the side effects of the drug on animals with EAE not clear due to EAE complications, therefore to study the side effects of drug three control groups were considered (noEAE+3 mg/kg HU-210, noEAE+10 mg/kg HU-210, noEAE+30 mg/kg HU-210, respectively).

Locomotor tests

EAE clinical score

For the evaluation of the disease course, all animals were examined daily from the onset of the clinical signs of EAE for 17 days. Researchers were blinded when doing this test. An adapted EAE-scoring scale was used in this study (Meyer et al., 2001; Kerschensteiner et al., 2004).

Score 0, no clinical disease; Score 0.5, partial tail weakness or slight loss of muscle tone; Score 1.0, tail weakness; Score 1.5, slightly clumsy gait; Score 2.0, hind limb paresis; Score 2.5, marked hind limb paresis and partial dragging of the hind limbs; Score 3, hind limb paralysis; Score 3.5, hind limb paralysis and forelimb paresis; Score 4.0, complete paralysis (tetraplegy); Score 5.0, moribund or dead.

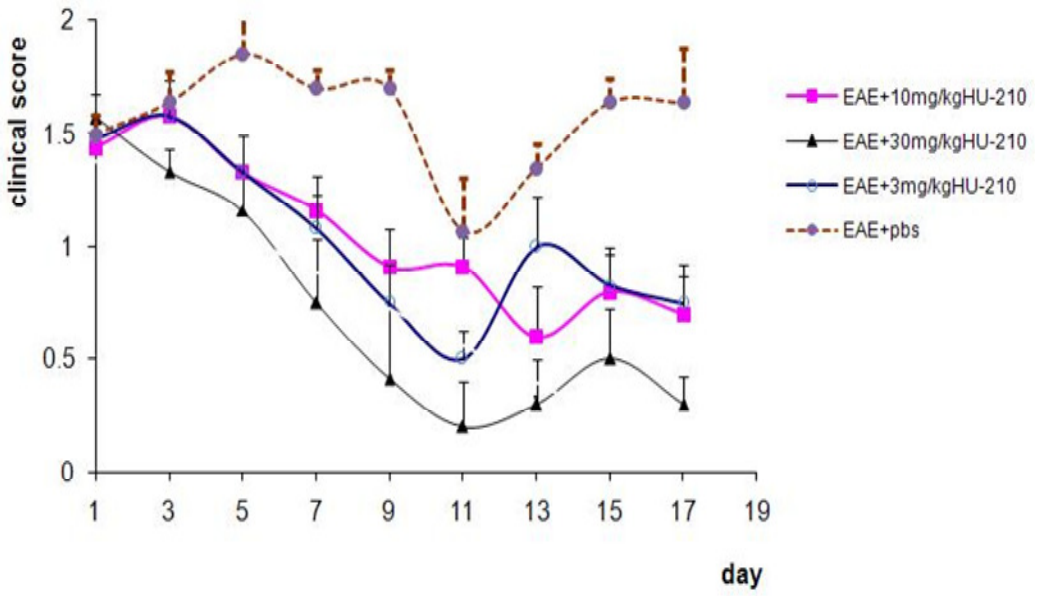

Figure 1. Effects of three doses of HU-210 given after disease onset on EAE severity and duration. EAE+3 mg/kg HU-210: Mice with EAE received HU-210 in dose of 3 mg/kg body weight. Clinical scores in this group has significant difference with control (EAE + PBS) on the ninth day only ($p < .05$). EAE+10 mg/kg HU-210: Mice with EAE received HU-210 in dose of 10 mg/kg body weight. Clinical scores in this group in comparison with control showed no significant difference in any of the days. EAE+30 mg/kg HU-210: Mice with EAE received HU-210 in dose of 30 mg/kg body weight. Clinical scores in this group in comparison with control had a significant difference on day 5, 7,9,11,13,15,17 ($p < .05$).one-way ANOVA with repeated measures followed by Duncan's post hoc test. Filled Circles represent vehicle control animals with EAE and other symbols represent treatment with 3, 10, 30 mg/kg of HU-210.

Balance beam

This paradigm was performed to assess the ability of the animals to balance across a 1-m long beam. The balance beams were built to custom specifications by medium-density fiberboard (MDF). Four beams were constructed, at widths of 9, 12, 15, and 18.0 mm. Thus, the difficulty to balance across increased from beam to beam. All beams were 100 cm in length and mounted on supports that provided a vertical distance of 40 cm from the top of the beam to the table below. The beam was textured to provide slight traction. Pads were placed under the beam to provide cushioning if an animal fell. Animals were trained for 2 days before baseline evaluation. For baseline and postoperative testing, animals had to cross each beam three times and were then evaluated by a scoring system previously described (Metz et al., 2000). For traversing each beam with full weight support and normal plantar paw placing, a score of 2 was applied. If plantar placing of the paw was only partly possible, the animal scored 1.5. A score of 1 was given if the animal could traverse the entire beam but without plantar placing of the hind paws. When only half of the beam could be crossed, score of 0.5 was assigned, and for complete inability to cross the beam, a score of 0 was applied. For each beam, the average score was calculated. Average scores of all four beams were then added up so that a maximum of 8 points could be reached (Buddeberg et al., 2004).

Analysis of cytokines by ELISA

After 17 days of treatment, the animals were sacrificed and, serum IFN-γ, TNFα, IL-12, levels as inflammatory factors, and serum IL-4 level as a anti-inflammatory factor were measured by Sandwich ELISA kits (axxora co.).

Statistical analyses

The results are presented as the mean±standard error (S.E.M.). Fisher least significant difference (LSD)-test was applied for multiple testing in this study. ANOVAs and Tukey post hoc with repeated measurements were carried out using statistical package for the Social Sciences (SPSS) and $P<0.05$ was considered statistically significant**.**

RESULTS

The Results of intraperitoneal administration of HU-210 to mice with experimental autoimmune EAE on clinical symptoms are shown in Figure 1. Treatment with HU-210 (30 mg/kg) ameliorates severity of clinical signs established experimental autoimmune (EAE) when administered at the onset of clinical signs of disease. Treatment with 3, 10, 30 mg/kg doses of HU-210 in healthy mice showed no significant effect on clinical symptoms. There were no specific side effects in these groups (data are not shown).

The results of administration of HU-210 to mice with experimental autoimmune EAE on balance beam scores

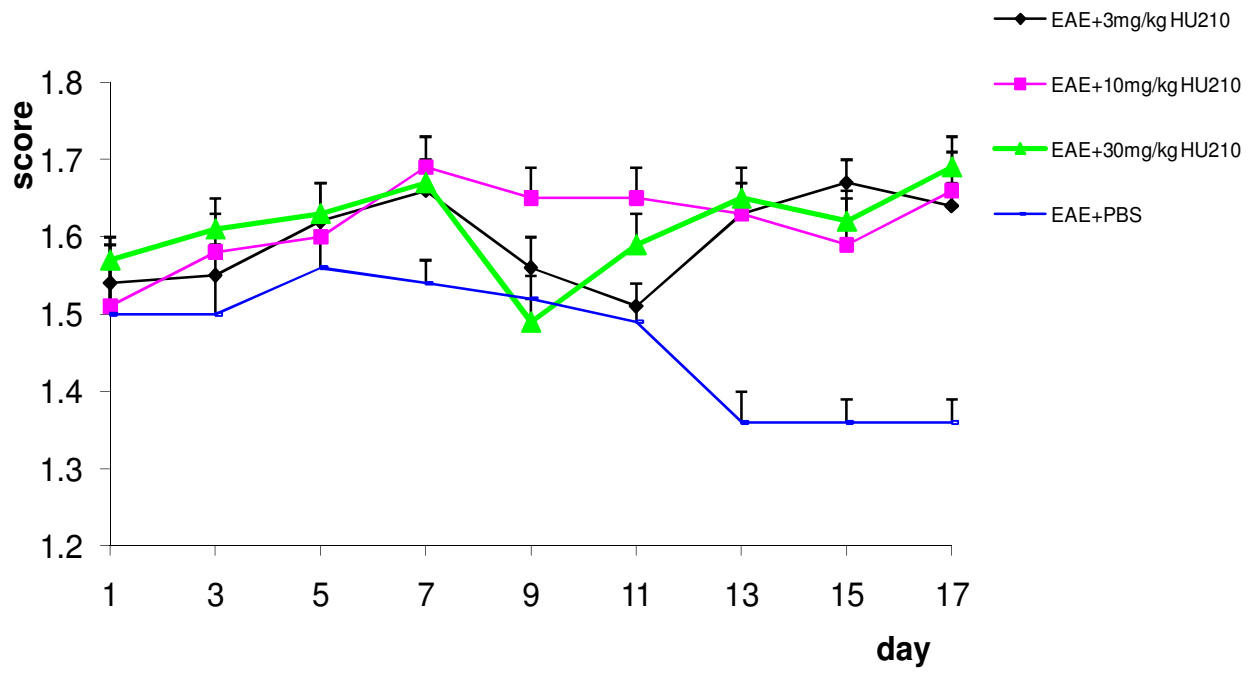

Figure 2. Effect of three doses of HU-210 given after disease onset on balance beam scores. EAE+3 mg/kg HU-210: Mice with EAE received HU-210 in dose of 3 mg/kg body weight. Balance beam scores in this group has significant difference with control (EAE + PBS) on days 7, 13, 17($p < .05$). EAE+10 mg/kg HU-210: Mice with EAE received HU-210 in dose of 10 mg/kg body weight. Balance beam scores in this group has significant difference with control (EAE + PBS) on days 3, 7, 9, 13, 15, 17($p < .05$). EAE+30 mg/kg HU-210: Mice with EAE received HU-210 in dose of 30 mg/kg body weight. Balance beam scores in this group has significant difference with control (EAE + PBS) on days 3, 7, 13, 15, 17($p < .05$).

are shown in Figure 2. In the first group, administration of HU-210 with dose of 3 mg/kg resulted in the improvement of balance beam score in comparison with control (EAE+PBS) on days 7, 13, 17 ($p < .05$). In the EAE+10 mg/kg group balance beam scores were significantly higher than those in EAE+PBS group on day 3, 7, 9, 13, 15, 17 ($p<.05$). The EAE+10 mg/kg group balance beam scores were significantly higher than those in EAE+PBS group on days 3, 7, 13, 15, 17 ($p < .05$). The effects of HU-210 on IL-12, TNFα and IFN-γ levels are shown in Table 2. Treatment with 30 mg/kg dose of HU-210 in EAE mice significantly reduced the IL-12, TNFα and IFN-γ levels compared with control group (EAE+PBS) ($P<0.5$), While other doses couldn't significantly reduced these cytokines levels. Treatment with 30 mg/kg dose resulted in a considerable reduction in levels of inflammatory cytokines (IL-12, TNFα, IFN-γ) so that cytokines levels differences in these groups are not significant in comparison with healthy mice group. The effects of HU-210 on IL-4concentration in control and experimental groups are shown in Table 2. Treatment with dose of 3, 10, 30 mg/kg significantly increased IL-4 concentrations in EAE mice and healthy mice compared with control (EAE+PBS) mice. It is noteworthy that IL-4 concentrations in EAE mice (treatment with dose of 3, 10, 30 mg/kg) and healthy treated mice are significantly higher than those in healthy mice. Drug injection in healthy mice did not show negative side effects on clinical score test and inflammatory cytokines and All three doses caused a significant increase in anti-inflammatory factor IL-4 compared with untreated healthy mice ($P<0.5$) (Table 2).

DISCUSSION

Results indicated HU-210 positive effects on Locomotor tests and cytokines profile thus it can affect inflammation caused by EAE. This study showed that the clinical signs in the high dose treated group (EAE +30 mg/kg HU-210) had a significant difference with control group (EAE+PBS) on days 5, 7, 9, 11, 13, 15, 17 ($p < .05$). However the first group (EAE +3 mg/kg HU-210) on the ninth day showed only significant difference with control group (EAE+ PBS) ($p<.05$).The data analysis showed that injection of this compound in dose 10 mg/kg in EAE mice reduced clinical symptoms but this reduction wasn't significant in any of the days. The results showed that the effects of HU-210 are dose-dependent. When HU-210 was administered at 30mg/kg dose, the severity of clinical scores reduced between 37 and 81% in days 11 and 17.

Buccellato et al. in 2007 studied the effect of CB

Table 2. Effect of three doses of HU-210 given after disease onset on IL-12, TNFα, IFN-γ, IL-4 concentrations in control and experimental groups.

Factor / Group	IL-12	TNFα	IFN-γ	IL-4
EAE+3 mg/kg	163.6±2.18	208±9.5	144.8±2.15	**385.6±3.29
EAE+10 mg/kg	178±23.70	209.2±8.77	167.8±19.77	**396.2±7.33
EAE+30 mg/kg	*144.8±6.34	*170.2±5.05	*124.2±8.23	**426.6±4.87
EAE+PBS	194±4.87	234.4±4.65	178.8±5.34	351.6±4.08
Healthy mice	134.5±2.71	162.3±2.87	119.8±3.97	350.6±6.34
noEAE+3 mg/kg	133.3±3.81	155.8±6.99	117±2.81	392.5±5.76
noEAE+10 mg/kg	128.8±6.83	146±4.65	114.6±6.28	431.8±4.08
noEAE+30 mg/kg	114.66±4.49	137.6±6.21	112.3±6.50	448.4±6.55

IL-12, TNFα, IFN-γ, IL-4 concentrations in experimental groups are compared to IL-12, TNFα, IFN-γ, IL-4 concentrations in control group (EAE+PBS). *Treatment with HU-210 (30 mg/kg) showed that concentrations of IL-12, TNFα, IFN-γ significantly reduced compared with PBS treated EAE mice (P<0.5). **Treatment with three doses of HU-210(3, 10, 30 mg/kg) significantly increased IL-4 concentration compared with PBS treated EAE mice (P<0.5).

extracts administration on motor function in a EAE model of multiple sclerosis. According to their results during the relapse phase a significant decrease of neurological scores was observed (Buccellato et al., 2011). In another study Achiron et al. (2000) investigated the effects of Dexanabinol (HU-211) on experimental autoimmune EAE. Histological studies supported the clinical findings demonstrating reduction in the inflammatory response in the brain and spinal cord in animals treated with dexanabinol. The results suggest that dexanabinol may provide an alternative mode of treatment for acute exacerbations of MS.

Narrow beam provides valuable option for the evaluation of balance and descending motor control in animals with targeted EAE. The results of our study on the balance beam score showed that treatment with 3 mg/kg dose of the drug led to improvement of balance at the end of the study period while treatment with 10, 30 mg/kg doses led to significant increase in balance beam scores in at the beginning of the study and continued until the end. Many cytokines have been shown to be involved in the pathogenesis or the resolution of MS and EAE. Results on the cytokine profile showed that the dose 30 mg/kg HU-210 significantly reduced levels of inflammatory cytokines IL-12, TNFα and IFN-γ. During the acute phase of EAE, first cytokines that appear in the central nervous system are lymphtoxin and IL-12. Subsequently, IFN-□, IL6 and TNFα appeared and their expression levels are in parallel with clinical symptoms and infiltration of inflammatory cells (Tanasescu and Constantinescu, 2010).

IL-12 is the major enhancer of the Th1 response and its presence propagates the production of cytokines such as IFN-γ and TNFα. Immediately prior to the onset of EAE symptoms, production of IL-12 is elevated. Addition of IL-12 enhances disease severity while depletion of IL-12 suppresses EAE (Hart et al., 2005; Hart et al., 2008). Regulation Th1 differentiation through blocking IL-12 or IL-12 receptor may be a critical mechanism for regulating EAE. 30 mg/kg dose treatment was effective in reducing the level of IL-12. A significant difference is not observed between the third group (EAE mice that received treatment with dose of 30 mg/kg) and the fourth group (healthy mice that did not have any treatment), (Table 2).

One important Th1 cytokine, which has been demonstrated to be responsible for significant pathology in the central nervous system (CNS), is IFN-γ. Expression of IFN-γ in the CNS is observed at the onset of disease, which increases as disease progresses and declines as disease resolves (Beggolka et al., 1998). In addition, over expression of IFN-γ in the CNS leads to progressive demyelination and increased disease severity. Similarly, Newton et al. reported that the injection of THC to mice, can cause suppression of interferon gamma production (Newton et al., 1994). The pro-inflammatory cytokine TNFα acts like IFN-γ in that it tends to be correlated with EAE and MS severity (Maimone et al., 1991; Sharief and Hentges, 1991).

Thus, as shown in our study, it can be assumed that HU-210 reduced severity of EAE through TNF- α inhibition. IL-4 is an anti-inflammatory immune factor. Injection of three doses of HU-210 (3, 10, 30 mg/kg) significantly increased levels of interleukin-4. Both IL-10 and IL-4 have been correlated with protection in EAE. Increased levels of both cytokines have been found in the CNS and in lymphoid cells during remission. Also, IL-4 treatment is correlated with disease amelioration and remission (Begolka et al., 1998).

Many evidences show that the injection of THC, that is similar to HU-210 can affect CB1 and CB2 receptor function, and decreases production of IL-12 and its receptor activities thus TH1 activity are suppressed (Klein et al., 2000). HU-210 through a similar mechanism improved EAE symptoms in mice. Likely CB1via adrenal - pituitary - hypothalamic axis affect the TH1 activity (Visser et al., 1998). On the other hand it is possible that

the expressed CB2 receptors in immune cells regulate the environmental cytokines and TH1 cell maturation (Guindon and Hohmann, 2008). Most studies conducted in animal models indicate that CB induced immunosuppression is dose-dependent and this suppression mainly is attributed to the CB2 receptor activation. CB2 receptors specifically are expressed on peripheral cells such as lymphocytes, macrophages and mast cells (Nagarkatti et al., 2009; Reider et al., 2010). Therefore HU-210 with 30 mg/kg dose inhibits activity of macrophages and due to the capacity of macrophages as antigen presentation cells and positive and negative regulatory proteins producer, this combination can suppress the immune system. It seems that HU-210 injection especially with dose of 30 mg/kg to EAE mice reduces inflammation, improves clinical symptoms and balances status through regulation of inflammatory and anti-inflammatory factors.

Conclusion

The present study revealed the beneficial effects of HU-210 in the model of EAE, and suggests its potential use as a drug for the treatment of MS.

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