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Alleviation of Experimental Allergic Encephalomyelitis in C57BL/6 Mice by Soy Daidzein

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ABSTRACT

Experimental allergic encephalomyelitis (EAE) is considered as the murine model of multiple sclerosis. Daidzein a phytostrogenic compound of soy is known to impose immunomodulatory and antioxidative effects. We conducted this study to assess the potential protective and therapeutic effects of daidzein on allergic encephalomyelitis.

C57BL/6 mice were induced with allergic encephalomyelitis using myelin oligodendrocyte glycoprotein (35-55) and received daidzein or dimethyl sulfoxide as the vehicle control. To assess the protective effect of daidzein, the mice were administered with 20 mg/kg of daidzein from 21 days prior to 21 days post EAE induction on a daily basis. To evaluate the therapeutic effect of daidzein, mice were fed with 300 mg/kg daidzein after the appearance of the first clinical signs for 10 days. One day after the last gavage, the mice were sacrificed. Spleen and brain were removed for further histological and immunological analysis.

Feeding mice with low dose of daidzein prior to disease induction did not affect disease severity. However, treating with high dose of daidzein after the onset of the disease reduced interferon-γ and interleukin-12 secretion, enhanced interleukin-10 production, suppressed lymphocyte proliferation, and decreased cytotoxicity as judged by lactate dehydrogenase release.

In conclusion, daidzein reduced the extent of demyelination and disease severity. Chronic oral therapy with low dose of daidzein did not prevent experimental autoimmune encephalomyelitis. However, high doses of daidzein could prohibit disease exacerbation.

Keywords: Daidzein; Experimental allergic encephalomyelitis (EAE); Immunomodulation; Interferon-gamma; Isoflavones; Multiple sclerosis; Soy

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INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated inflammatory disease of the central nervous system (CNS) that can lead to irreversible clinical disability in most severe cases.[1] Most of the current approved therapies possess some disadvantages that can cause non-adherence to treatment including administration via injection, costs, partial effect, and potential toxicity.[2] Moreover, although current therapies are effective at decreasing the frequency and severity of relapses, they have little impact on progressive forms of the disease. Experimental allergic encephalomyelitis (EAE) is a T-cell mediated autoimmune disease, validated as the animal model of MS.[3] Both CD4+ and CD8+ T-cells were reported to be important in the development of EAE.[4] In EAE many T helper (Th)1-type cytokines (proinflammatory) contribute to the process of demyelination, while several Th2-type cytokines have been implicated in remyelination and survival of neurons.[5]

Daidzein is one of the most potent isoflavones of soy bean. Soy bean is known as the main dietary source of isoflavones and has been traditionally consumed in Far East countries.[6] Isoflavones, as a group of phytoestrogens, appeared to possess weak estrogenic and/or antiestrogenic actions.[7] Considering the immunomodulatory actions of estrogen, it may be probable that daidzein also influence the immune system. A study, treating ovariectomized balb/c mice with daidzein resulted in a reduction of splenic CD4+ T cells and tumor necrosis factor-alpha (TNF-α) secretion.[8] Daidzein was also reported to suppress Interferon-gamma (IFN-γ) production in splenocytes isolated from C57BL/6 mice.[9] Treating mice with 20 mg/kg of daidzein was also reported to reduce plasma levels of Interleukine (IL)-6 and TNF-α in an animal model of rheumatoid arthritis.[10] In addition daidzein is known to possess antioxidative properties.[11] Moreover, intraperitoneal injection of 200 mg/kg of genistein, another soy isoflavone, to EAE-induced C57BL/6 mice has recently been reported to modify Th1/Th2 balance and reduce rolling and adhering of leukocytes.[12,13] Regarding the structural similarity of genistein and daidzein and the immunomodulatory and antioxidative roles of daidzein, we conducted this study to investigate potential protective and therapeutic effects of oral daidzein on the progressive model of EAE.

MATERIALS AND METHODS

Mice

The study was performed on 10-12 week old female C57BL/6 mice (Pasture institute, Iran). The animals were housed with free access to food and water on a 12 hour light-dark cycle. All protocols used in this experiment were approved by the Tehran University of Medical Sciences committee on laboratory animals. The mice were assigned into four groups, two groups in each set of experiments. In the first set of the experiment, the protective effect of oral daidzein was studied. In second set, the therapeutic effect of daidzein was the subject of the study.

Daidzein Administration

To assess the protective effect of daidzein, the mice (n=6/group) were: 1) administered with 20 mg/kg daidzein dissolved in 0.1 ml of Dimethyl sulfoxide (DMSO, Sigma Aldrich, USA) (11%) (daidzein-protection), 2) gavaged with 0.1 ml DMSO 11% as the vehicle control (n= 6/group) (control-protection). Oral gavage began 21 days before immunization and continued up to 21 days after immunization on daily bases (43 days including day 0). Twenty mg/kg of daidzein has been reported to have immunomodulatory effects in collagen induced arthritis (CIA) and consequently protect rats against this autoimmune disease.[10] As CIA and EAE have similar pathology, we adopted this dose for assessing the protective potency of daidzein.

To study the therapeutic effect of daidzein, the mice were induced with EAE. After the appearance of the first clinical sign (score=1), the mice were administered with 1) 300 mg/kg daidzein dissolved in 0.1 ml DMSO 11% (daidzein-therapy), or 2) 0.1 ml DMSO as vehicle control (control-therapy). As the bioavailability of oral daidzein is about 50%, for assessing the therapeutic effect of daidzein, we administered mice with 300 mg/kg of daidzein.[14]

EAE Induction

The mice were immunized with a 1:1 ratio of myelin oligodendrocyte glycoprotein (MOG) 35-55 (Alexis, Switzerland) dissolved in Phosphate buffered saline (PBS) (300 µg MOG in 100 µL PBS) and complete Freund's adjuvant (CFA) (100 µL) containing 0.4 mg of mycobacterium tuberculosis (Sigma-Aldrich, USA). In the first set of the experiments, after 21 days of gavage,

200 µL/ mouse MOG-CFA emulsion was subcutaneously administered on the four sites of the upper flanks (day 0). The additional immune adjuvant, pertussis toxin (PTX) (Sigma-Aldrich, USA) in 10% PBS was intraperitoneally injected (500 ng/mouse) on the initial day of immunization and again 48 hours later. In the second set of the experiments, after a week of adaptation, the EAE was induced in mice by protocol.

Clinical Evaluation of EAE

The following grading scheme was used to clinically score the animals: 0, for no clinical signs, 0.5, hook tail, 1, flaccid/floppy tail, 2, walking deficits, 2.5, unilateral hind limb paralysis 3, bilateral hind limb paralysis, 3.5, paraplegia with forelimb weakness, 4, quadriplegia and 5, for moribund.

In the first set of the experiments, the clinical scores were assessed daily for 21 days post immunization. In the second set of the experiments, clinical scores were assessed daily up to 10 days after the appearance of the first clinical sign.

Histological Assessments

Twenty-four hours after the last treatment, the mice were sacrificed. Brain of the animals were removed and post-fixed in 10% paraformaldehyde and stored at 4^{O} C for 48 hours. Prior to sectioning, the tissues were put in PBS 10% for 24 h and imbedded in paraffin. The brain sections (8 microns) were cut on the cryostat and stained. For assessing the percentage of demyelination luxol fast blue- LFB (Merck, Germany)/ Cresyl fast violet (BDH, England) staining was used. Demyelination was assessed in at least 5 sections for each mouse.

Spleen Cytokine Assessment

The mice were sacrificed 24 hours after the last gavage and their splenocytes were isolated. Mononuclear cells obtained from spleens of the immunized mice were prepared and incubated with 1.5 ml in RPMI-1640 medium at a concentration of 2×10^{6} cells/well in 24-well plate (Nunc, Denmark) for two days. The RPMI was supplemented with 10% FCS, 1% L-glutamine, 1% HEPES, 0.1% 2ME, and 0.1% penicillin/streptomycin, and pulsed with 10µg/ml daidzein. The cell supernatants were collected. The amount of cytokines (INF-γ, IL-10 and IL-12) in cell supernatants was assessed using sandwich-based ELISA kits (ELISA Ready-SET-Go, eBioscience, USA). The tests were performed according to the manufacturer's protocol. All tests were performed in triplicate for each mouse.

Brain Cytokine Assessment

The concentration of brain cytokines (INF-γ, IL-10 and IL-12) was assessed 24 hours after the last gavage. For this purpose, 100 mg of the brain tissue of each mouse was dissolved in 10 ml of extracting solution consisting of 50 µM tris (Sigma-Aldrich, USA), 2 mM EDTA (Merck, Germany), 0.1 M NaCl (Sigma-Aldrich, USA), 1 mM dithiotheritol (Merck, Germany), and 200 µM phenylmethylsulfonyl fluid (Merck, Germany), 1 µg/ml chymostatin (Sigma-Aldrich, USA), and 1 µg/ml trypsin inhibitor (Sigma-Aldrich, USA) (15) . Brain homogenate was spun at 4^{o}C for 10 minutes at 10000 g. Level of INF-γ, IL-12 and IL-10 were determined in brain supernatant using sandwich-base ELISA kit (eBioscience, USA) following the manufacturer's instruction. All tests were performed in triplicate for each mouse.

Lymphocyte Proliferation Assay (LPA)

Twenty four hours after the last oral administration, single cell suspension of mononuclear cells was obtained from the immunized mice and used for lymphocyte proliferation assay. Briefly, the suspension of isolated spleen cells was treated with lysis buffer [0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA, (pH 7.2)]. In 96-well flat-bottom culture plates (Nunc, Denmark), $2x10^{5}$ cells/ well were cultured. The preparations were cultured with RPMI-1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine, 1% HEPES, 0.1% 2ME, 0.1% penicillin/streptomycin and incubated in the presence of 10 µg/ml daidzein. T cell mitogen phytohemagglutinin-PHA (Sigma chemicals, Australia) at a concentration of 5 µg/ml was used as the positive control. After 3 days, MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma chemicals, Australia) was added per well in concentration of 5 µg/ml and was incubated for 5 hours at 37 ºC in 5% CO_2. DMSO (100 µl) was added to dissolve the produced formazan crystals.

The plates were read at 540 nm, and the results were expressed as stimulation index (SI). The SI was determined as follows: OD values of stimulated cells (Cs) minus relative cell numbers of unstimulated cells

(Cu) by relative OD values of unstimulated cells.

SI = (Cs _ Cu)/Cu

All tests were performed in triplicate for each mouse.-[16]

Cell Cytotoxicity Assay

Twenty-four hours after the final administration, the mice were sacrificed and their splenocytes were isolated. For each sample obtained from an individual mouse, single cell suspension of mononuclear cells (used as the effecter cells) was co-cultured in RPMI 1640 medium with washed target cells, EL4 at various effector-to-target cell for 4 h in phenol red-free RPMI 1640 containing 3% FCS. For preparation of the target cells, EL-4 cells were stimulated with daidzein antigen.

After centrifugation, the supernatants (50 µl/ well) were transferred to the 96-well flat-bottom plates, and lyses of the target cells were determined by measuring Lactate dehydrogenase (LDH) release using Cytotoxicity Detection Kit (LDH) according to the procedures stated by the manufacturer (Takara Company, Japan). Several controls were used for the cytotoxicity assay.

"High control" was the total LDH released from the target cells, and all EL4 cells were lysed by a medium containing 1% Triton X-100. "Low control" was the natural release of LDH from the target cells, which was obtained by adding EL4 cells only in the assay medium. "T-cell control" was used to measure the natural release of LDH from T cells obtained by adding different ratios of T cells only in the assay medium. The assay was performed in triplicates for all samples, including the controls.

The LDH-mediated conversion of the tetrazolium salt into red formazan product was measured at 490 nm after incubation at room temperature for 30 min. The percentage of specific cytolysis was determined by the following formula:

$$\%\ \text{Cytotoxicity} = [(\text{experimental value} - \text{effector cell control}) - \text{low control}/\text{high control} - \text{low control}] \times 100$$

Statistical Analysis

The data were analyzed with SPSS 17. One-way analysis of variances (ANOVA) was used to compare clinical and histological signs of the disease, as well as, cytokine concentrations between the studied groups. Sheff's honesty significant intergroup comparison at the 0.05 level of significance was adopted for all comparisons.

RESULTS

Clinical Assay

In the first set of the experiments, daidzein administration (20 mg/kg) did not prevent mice from EAE. The low dose of daidzein did not significantly affect the day of onset, mean daily, commutative, or maximum clinical score (Figure 1).

In the second set of the experiments, the mice were gavaged with dadizein or DMSO up to 10 days after the appearance of the first clinical sign. 9 to 14 days following immunization, mice developed clinical signs of EAE. No significant differences in the mean onset time were observed between the two groups. The mean daily clinical score of the daidzein-treated group was always lower than the controls; the differences became significant on days 17, 18, and 19. Daidzein also significantly reduced cumulative and maximum disease score (Figure 1 and Table 2).

In the first set of the experiments, demyelinated lesions were scattered widely throughout the brain in DMSO-treated mice. The daidzein-treated mice did not show milder CNS pathology compared to controls. In the second set of the experiments, demyelination was notably absent in the daidzein treated mice (Figure 2).

Table 1: Effect of oral administration of daidzein or DMSO on clinical signs of experimental allergic encephalomyelitis (EAE)

		Incidence‡	Onset (day)	Commutative disease score	Maximum clinical score
Protection	Control	6/6	11.33±1.5	25.25±7.99	3±0.45
	Daidzein	5/6	11.25±0.51	22.25±3.88	2.87±0.62
Therapy	Control	5/6	11±1.67	23.25±5.33	2.91±0.37
	Daidzein	6/6	11.2±1.6	12.5±5.67*	1.6±0.82*

The mega-dose of daidzein (300 mg/kg) decreased commutative and maximum disease score significantly. All data is represented as mean± SD. ‡ratio, *P<0.05 compared to control-therapy

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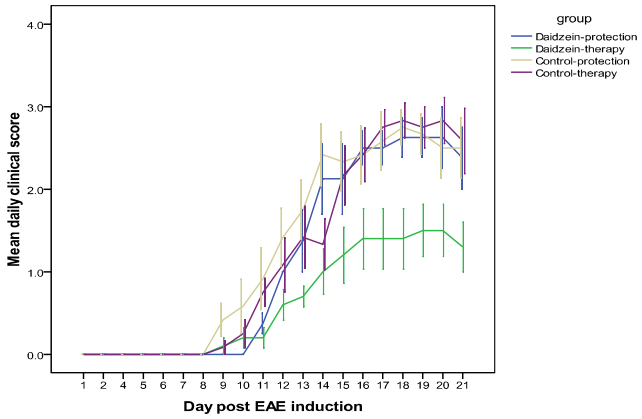

Figure 1. Effects of oral administration of daidzein on the mean daily clinical scores of experimental allergic encephalomyelitis (EAE). Administering with 20 mg/kg (n=6) from 21 days prior to 21 days post immunization did not reduce the severity of clinical signs compared to the vehicle controls. Gavaging with daidzein after the onset of clinical signs for 10 days reduced the mean daily clinical score. The difference became significant (p<0.05) from day 17 to 19 compared to control group.. All data is represented as mean± SD.

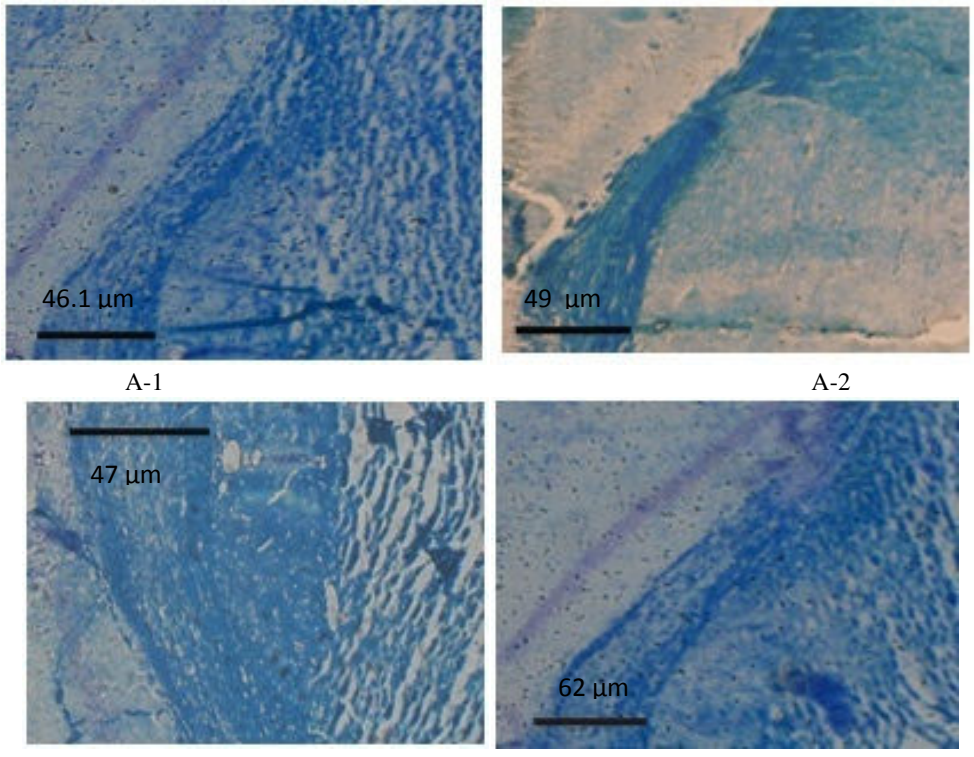

Figure 2. Demyelination of the brain. A, brain sections of control-protection (A-1), daidzein-protection (A-2), control-therapy (B-1), and daidzein-therapy (B-2) groups are stained using luxol fast blue & Cresyl fast violet. 20 mg/kg of daidzein was used as a protective dose from 21 days prior to 21 days post induction. 300 mg/kg of daidzein was used as a therapeutic dose for 10 days after disease onset. Therapeutic dose of daidzein reduced the extent of demyelination.

Immunological Assay

Cytokine Profile

The animals were sacrificed 24 hours after the last treatment. In the first set of the experiments, brains and spleens of the animals were dissected. In splenocytes isolated from daidzein-treated animals after daidzein exposure, no significant differences were detected in levels of IFN-γ, IL-12, and IL-10. The comparable effect was noted on brain cytokine profile (Figure 3).

As presented in figure 3, in the second set of the experiments, in the splenocytes removed from daidzein-treated group, after daidzein exposure, IFN-γ and IL-12 levels were significantly down regulated compared to vehicle-control (p=0.02 and 0.002, respectively). Daidzein also reduced IFN-γ (p=0.024) secretion in brain. It enhanced IL-10 secretion in brain and splenocytes (p= 0.014 and 0.001, respectively).

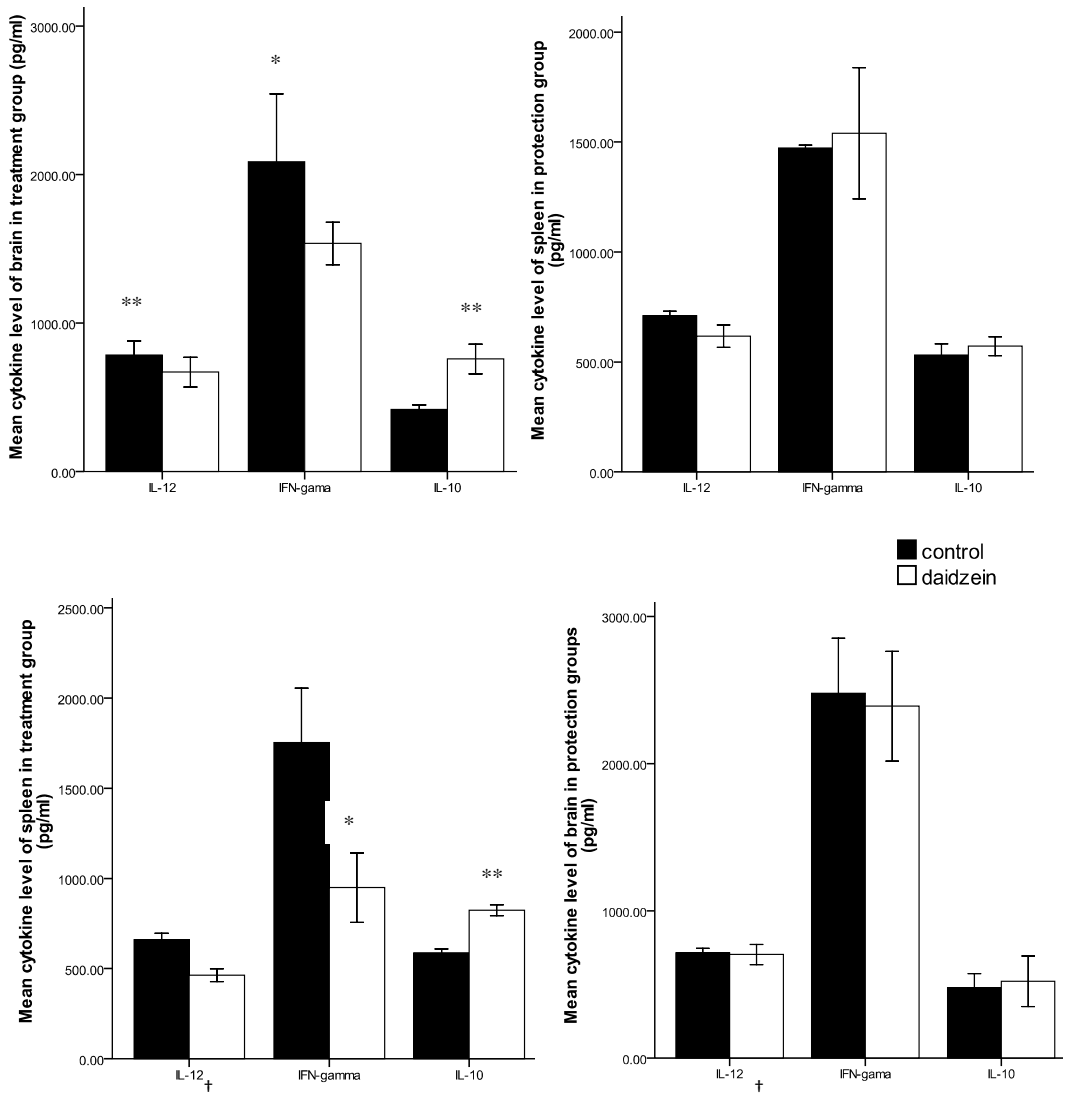

Figure 3. The effect of daidzein on cytokine profile (IFN-γ, IL-10 and IL-12) of spleen and brain. To assess the protective role of daidzein, the mice either received daidzein (20 mg/kg) or DMSO as the vehicle control from 21 days before to 21 days after immunization. To evaluate the therapeutic effect of daidzein, mice were either gavaged with daidzein (300 mg/kg) or DMSO after the onset of clinical signs for 10 days. Treating mice with daidzein after the onset of the disease notably reduced IFN-γ level in brain and splenocytes. It also significantly decreased IL-12 level in splenocyte. Moreover, therapeutic dose of daidzein enhanced IL-10 secretion in brain and splenocytes. All data are represented as mean± SD. *P<0.05, **P<0.005. †ng/dl

Lymphocyte Proliferation

Proliferative response of splenocytes' lymphocytes was assessed using MTT test. In the first set of the experiments, in the immunized mice with daidzein, the proliferative response of lymphocytes was suppressed, but the difference was not significant in comparison to the control group. In the second set of the experiments, the stimulation index of lymphocytes was reduced significantly (Figure 4).

LDH Cell Cytotoxicity

Twenty-four hours after the final treatment, single cell suspension of mononuclear cells (effecter cells) was co-cultured with pulsed EL-4 cells (target cell).

As shown in Figure 5, in lymphocytes of mice that received the therapeutic dose of daidzein, specific cytolytic activity was suppressed at an E/T ratio of 50:1 significantly as compared to that of DMSO-treated animals (25.2±2.8% v.s. 33.7±1.09%) group. The most significant differences between all studied groups were at 50:1 E/T ratios (Figure 5).

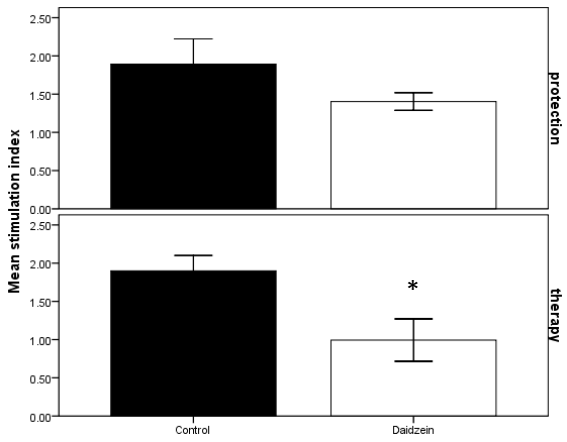

Figure 4. Lymphocyte proliferation evaluation, using MTT assay. Lymphocyte proliferation response was measured in daidzein-treated (20 mg/kg or 300 mg/kg) mice and compared with DMSO-treated animals. In mice treated with the therapeutic dose of daidzein, lymphocyte proliferation response was significantly lower, in comparison to the DMSO group. All data are expressed as mean± SD. *P<0.05.

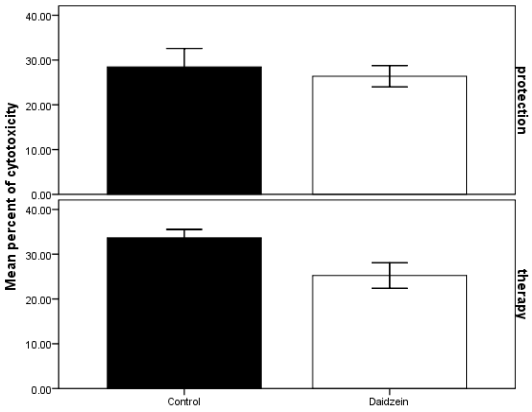

Figure 5. Cell cytotoxicity assays in mice treated with daidzein using LDH release assay. After oral treatment with DMSO or daidzein (20 mg/kg from 21 days before EAE induction or 300 mg/kg after disease onset), the spleens were harvested as described in materials and methods. The data were collected from LDH results at various E/T ratios (E/T=25:1, 50:1, 100:1) and expressed as cytotoxicity percentage ± SD. The data shown here are from three independent experiments with triplicates. The percent of cytotoxicity was significantly lower in the daidzein-treated group compared to vehicle-control. *P<0.05 compared to control.

DISCUSSION

Few evidences exist on the immunomodulatory effect of daidzein. Up to the best of our knowledge, no study has previously been performed on the effect of daidzein on EAE. We found that administrating mice with high doses of daidzein after the onset of the disease can attenuate EAE severity. However, gavaging with a low dose of daidzein prior to EAE-induction does not provide protection against disease induction.

The therapeutic dose of daidzein reduced the stimulation index of lymphocytes which mainly represents memory CD4+ T-cell proliferation. High dose of daidzein also decreased the percent of cytotoxicity which mainly manifested CD8+ activity. EAE is mainly characterized by infiltration of myelin-auto reactive CD4+ and CD8+ T cells into the CNS.[16] We also showed that gavaging with high dose of oral daidzein resulted in suppression of IFN-γ and IL-12 production, as well as the enhancement of IL-10 secretion. IFN-γ also controls the differentiation of CD4+ cells into Th1 effectors as well as, initiating the production of pro-inflammatory cytokines.[17] IFN-γ was found to be

increased in the CSF and CNS of patients with MS and IFN-γ administration to MS patients exacerbates the disease.[18] IL-10 is an anti-inflammatory cytokine that is produced by macrophages, monocytes, B cells and Th2 cells. It inhibits the production of pro-inflammatory Cytokines (IL-1 and TNF-α) by macrophages.[19] In EAE models, mice with IL-10 deficiency developed more severe EAE compared to the controls and even to IL-4 deficient mice, suggesting IL-10 has a unique role that cannot be substituted by other Th2 cytokines.[20] Treatment with IL-10 has also been shown to protect against EAE progression in mice.[20]

In agreement with our results, a previous in vitro study reported that high doses of daidzein (50 μm) reduced IFN-γ production and lymphocyte proliferation of concanavalin A-stimulated splenocytes.[21] Also, Tyagi AM. et al. reported that gavaging ovariectomized Balb/c mice with daidzein reversed the ovariectomy induced CD4+ T cell expression in spleen.[8] As mentioned previously, De paula et al. reported that intraperitoneal administration of 200 mg/kg genistein, after the onset of disease, reduced EAE severity by reducing IFN-γ and IL-12 production, decreasing the rolling and adhering of leukocytes on brain microvasculature, as well as enhancing IL-10 secretion.[12] Our results showed that similar to genistein, therapeutic dose of daidzein can attenuate EAE severity.

Few in vivo studies were performed on the immunomodulatory effects of daidzein. One of the best examples was a study on CIA. Mohammad-Shahi et al. reported that treating CIA-induced Spraugue-Dawley rats with 20 mg/kg daidzein postponed disease onset and reduced its severity.[10] Also in an in vitro study, 10^{-8} M daidzein reduced IFN-γ production in splenocytes isolated from C57BL-6 mice.[9] In contrast to Mohammad-shahi results and the previous in vitro study, in our study low-dose daidzein did not show the predicted protective effect on EAE. The observed difference with the previous in vivo study may be due to the difference in the pathological feature of EAE and CIA. They reported that the low dose of daidzein protect rats against CIA by reducing the serum concentration of TNF-α, IL-6, and leptin. Ideas on the role of IL-6 in EAE are controversial. IL-6 signaling has been identified as a stimulant that directs cells toward Th17. In a number of recent studies, Th17 (IL-17A-secreting-Th cells) was considered essential in developing EAE. On the other hand, in another study greatly increased levels of T cell-derived IL-17A expression was reported to have no effect on the development of EAE and the quality and quantity of CNS inflammation.[22] TNF-α was also playing a dual role during EAE. The complete elimination of TNF-α may induce disease severity. However, incomplete modulation of TNF-α expression may provide protection against EAE.[23] So reducing IL-6 and TNF-α level might not necessarily induce tolerance to EAE. A major difference between our in vivo animal study and the previous in vitro study was the bioavailability of daidzein that could have been significantly influenced the results. Furthermore, physiological and pathological factors in autoimmune disease including other immune cells may play a role in predicting lymphocyte potentiation and cytokine action.

To mention our limitations, we did not evaluate the activity of CD4+ and CD8+ T cells with more elaborate methods. However, we cannot rule out daidzein effect on CD4+ and CD8+ T cells measured by MTT and LDH tests. Further studies on protective and therapeutic effects of different doses of daidzein are warranted.

In conclusion, Oral administration of low dose of daidzein did not prevent EAE progression. However, treating with high dose of daidzein at the onset of clinical signs modulated T-cell response and consequently alleviated disease symptoms.

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