Prevention of Animal Model of Multiple Sclerosis by oral genistein, extracted from soy bean.

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Iranian Journal of Neurology, Vol.7, No.26 & 27 , Summer & Autumn 2009, 505-517

Abstract

Introduction: Multiple sclerosis (MS) is a chronic and often debilitating demyelinating disease of the central nervous system (CNS). Many of the current treatments are costly, limited in efficacy, and possess unpleasant side effects. These drawbacks bring the protective strategies into the center of attention. Genistein is one of the components of soy bean which happen to have anti-inflammatory, antioxidant and neuroprotective properties**.** We conducted this study to assess the potential role of genistein in protection against MS.

Methods and Materials: We used Experimental Autoimmune Encephalomyelitis (EAE) -an inducible $CD4^+$ T cell-mediated autoimmune disease with pathological features similar to MS. Oral administration of 20 mg/ kg genistein to MOG-immunized mice was started 21 days before the induction of disease and continued for 42 days.

Results: It significantly prevented mice from prolonged neurological sequel by postponing the disease onset, attenuating EAE course and alleviating the severity of clinical and histological signs of EAE. Genistein impose its beneficial role by modulating T-helper1/T-helper2 balance by decreasing $CD4^+$ T-helper 1 cells. Furthermore it inhibited encephalogenic and systemic release of IFN-γ and IL-12 and encephalogenic secretion of TNF-α. On the contrarily it increase the production of IL-10 in central nervous system and spleen.

Conclusion: Taking together, these results suggest that genistein may represent a new protective approach for MS by anti-inflammatory and immune-modulating properties.

Keywords: **Genistein, Experimental Autoimmune Encephalomylitis(EAE), Anti-inflamatory**

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Introduction

Multiple sclerosis (MS) is a complex demyelinating disease of the central nervous system that afflicts approximately two-and-a-half million individuals worldwide3.[1] It identified as the most debilitating neurological disease in young adults that can lead to irreversible clinical disability.[2] The exact etiology and pathology of MS remains unknown. Different genetic and environmental factors have been implicated in the etiology of MS. Regardless of etiology, most experts agree that MS is an immune-mediated disease.[3,4] This immune attack is mediated by unregulated autoreactive T cells that enter CNS through a disrupted blood-brain-barrier[5] and attack oligodendrocytes as well as the myelin surrounding axons.[6] $CD4^+$ T cells are considered essential in the initial stages of CNS inflammation; however, both adaptive ($CD8^+$ T cells and antibodies) and innate (microglia/macrophages) immunity also play crucial roles to inflammation and tissue injury.[7,6] Cytokines are involved in many of the key pathological feature of MS. Many Th1 cytokines are increased while Th2 cytokines are decreased.

Most of the currently approved medications are only partially effective, given parenterally, and often associated with adverse effects and potential toxicities. They also tend to be relatively expensive. Furthermore no approved medication is available for progressive form of MS.[8] These drawbacks bring the protective strategies into the center of attention.

There are a number of experimental and clinical evidences to support a protective role for estrogen in MS. The protective effects of estrogen may be mediated by its immunomodulatory mechanism.[9] Phytostrogens comprise a family of biologically active plant agents, possessing estrogenic effect.[10] Phytosterogens have the potential to protect against other disease of immune origin such as diabetes and cancer.[11,12] The most widely distributed phytosterogens are isoflavons, which found in high-concentration in soy bean. Gensitein is the most potent isoflavone of soy bean which happen to have anti-inflammatory, antioxidant and neuroprotective properties that may potentiate it in protection against MS.[12-14] Experimental autoimmune encephalomyelitis (EAE) is an inducible T cell-mediated autoimmune disease. The pathological features of EAE are reminiscent of MS.[15] We conducted this study to assess the ability of genistein to protect against EAE.

Methods and Materials

Induction of EAE

EAE was performed on 10-12 weeks female C57Bl/6 mice (22.4 gr; Pasteur Institute of Iran). Animals were housed in Shefa Neuroscience research center, with access to food and water ad libitum on 12 h light-dark cycle. All protocols used in this experiment were conducted in accordance with approval by the Tabriz University of medical

sciences committee on laboratory animals. Mice were immunized with a 1:1 ratio of MOG 35-55 (Alexis, Switzerland) dissolved in Complete Freund's adjuvant (CFA) containing 0.4 mg of mycobactererium tuberculosis (Sigma-Aldrich, USA). For this purpose 300 µg of MOG dissolved in 100 µl PBS and mixed with equal volume of CFA. On day 0, the MOG-CFA emulsion was injected subcutaneously into two sites of the upper flunks (200 µg/ mouse). The additional immune adjuvant, pertussis toxin (PTX), (Sigma-Aldrich, USA) was injected i.p (400 ng/mouse) on day 0 and two.

Genistein administration

Genistein (20 mg/kg) (LC laboratories, USA) dissolved in DMSO 4% (Sigma Aldrich, USA) or DMSO 4% as the vehicle control, was administered daily by oral gavage beginning on 21 days before immunization (n= 6 mice/group) and continued for 42 days. A second control group was not induced with MOG (n= 6 mice/group).

Clinical evaluation of EAE

The clinical score of each mouse were assessed daily over the duration of 21 days. The following grading scheme was used to clinically score the animals: 0, 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, moribund.

Histological evaluation

To examine for the presence of cellular infiltrate and demyelination, hematoxylin (MERK, Germany) and eosin (MERK, Germany) and Luxol Fast Blue (Merk, Germany)/Crystal violet (BDH, England) stain was performed respectively. Mice were sacrificed 22 days after immunization. The brains and spinal cords of the animals were removed and post-fixed in 10% paraformaldehid and stored at 4^{O} C for 48 hours. Prior to sectioning, tissues were put in phosphate buffer saline 10% for 24 h and imbedded in paraffin. Sagittal lumbar spinal cord sections (8 microns) and cross sectional forebrain sections (8 microns) were cut on the cryostat and stained with hematoxylin and eosin and Luxol Fast Blue/Crystal violet. The number of leukocytes and demyelinated foci per mm^2 were determined by assessing at least 10 sections for each mouse.

Determination CNS concentration of cytokines

For determining the cytokine concentration of CNS, 100 mg of brain tissue of each mouse were homogenized in 10 ml of extraction solution contained: 50 µM tris (Sigma-Aldrich, USA), 2 mM EDTA (MERK, Germany), 0.1 M NaCl (Sigma-Aldrich, USA), 1 mM dithiotheritol (MERK, Germany), and 200 µM phenylmethylsulfonyl fluride (MERK, Germany), and sonicated for 60 seconds at 40000 hertz on ice. Brain homogenate was centrifuged (10000*10 min at 4^{o} C) and supernatant was analyzed for TNF-γ, TNF-α, IL-10 and IL-12 concentration

using sandwich-based ELISA kits (ELISA Ready-SET-Go! (eBIOSCIENCE, USA) following manufacturer's instruction. All tests were performed in triplicate for each mouse.

Determination systemic concentration of cytokines

24 hours after the last oral administration, mice were sacrificed and their splenocytes were isolated.

Mononuclear cells from spleens of immunized mice at a concentration of 2 $\times 10^6$ cells/well in 24-well plates (Nunc, Denmark) were incubated for 2 days in a total volume of 1.5 ml of RPMI-1640 supplemented with 10% FCS, 1% L-glutamine, 1% HEPES, 0.1% 2ME, 0.1% penicillin/streptomycin and pulsed with 10 µg genistein. The cell supernatants were collected and assayed for the presence of cytokines using commercially available sandwich-based ELISA kits (ELISA Ready-SET-Go! (eBIOSCIENCE, USA) following manufacturer's instruction. All tests were performed in triplicate for each mouse.

Lymphocyte proliferation assay (LPA)

24 hours after the last oral administration, single cell suspension of mononuclear cells obtained from immunized mice was used for lymphocyte proliferation assay. Briefly, the suspension of isolated spleen cells was treated with lyses buffer [0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA, (pH 7.2)] in order to clear the red blood cells. In 96-well flat-bottom culture plates (Nunc, Denmark) $2x10^5$ cells/well were cultured in 96-well flat-bottom culture plates (Nunc, Denmark). The preparations were cultured with RPMI-1640 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 genistein. T cell mitogen PHA (phytohemaglotinin, Sigma chemicals) at a concentration of 5 µg/ml, was used in as positive control. After 3 days, MTT (3-(4,5-dimethyl tetrazolyl-2) 2,5 diphenyl) tetrazolyumbromide (Sigma chemicals) in concentration of 5 µg/ml was added per well and incubated for 5 h at 37 0 C in 5% CO2. DMSO (dimethyl sulfoxide) (100 µl) was added to dissolve produced formazan crystals.

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.

Data Analysis

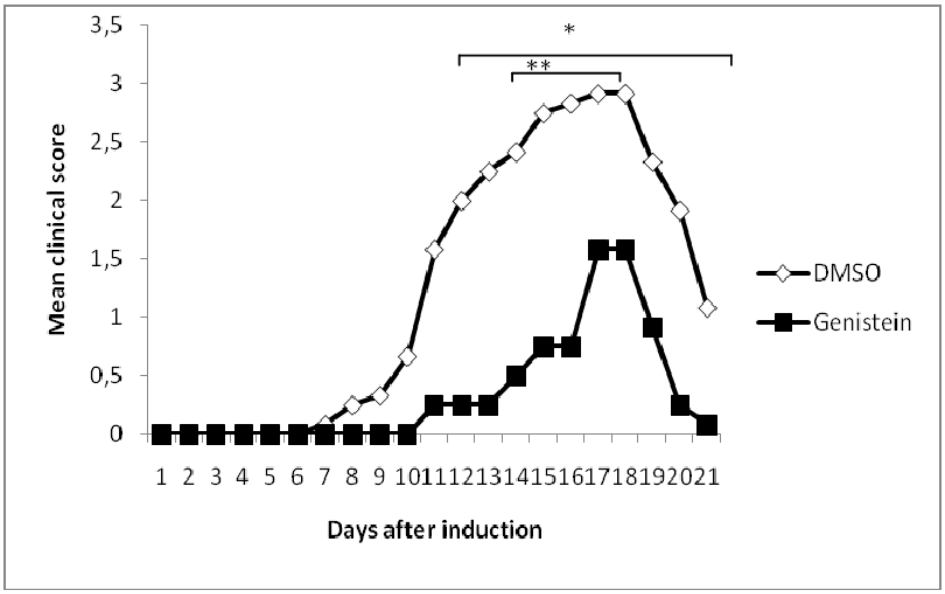
The effect of genistein on clinical scores and histological characteristics (inflammation and demyelination) was analyzed with independent-student-t-test between genistein and vehicle treated groups. One way analysis of variance (ANOVA) with scheff post hok comparison was performed to compare cytokine concentration

between three groups. Data were analyzed with SPSS 15.

Results

Clinical score assay

To evaluate the protective effect of genistein in EAE, C57Bl/6 mice were put on oral therapy with genistein form 21 days before immunization with MOG to day 21 following induction. Genistein prevented mice from prolonged neurological sequel as shown by a delay in disease onset (on day 14±2.7 vs 9.17±1.72 post induction) and EAE course attenuation (5.2±3.49 vs 11.17±1.47 days) (fig.1). It also alleviates the severity of the disease. The mean daily score of genistein group was always lower than vehicle group and they have significantly lower maximum (1.9±0.81 vs 3.17±1.03) and mean disease score (fig.1).



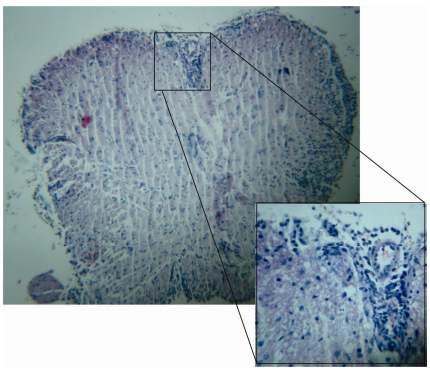
	Incidence	Onset	Peak	Course of disease (days)- mean± Std. Error	Mean clinical score
DMSO	6/6	9.17±1.72	3.17±1.03	11.17±1.47	1.81±0.26
Genistein	5/6	14±2.74*	1.9±0.81*	5.2±3.49**	0.42±0.2**

Figure 1: Effects of orally-administered genistein (20mg/kg) on clinical scores of EAE-induced mice after the onset of clinical signs. All mice received genistein or vehicle (6 mice/ group) from 21 days before induction, were induced with EAE on day 0 and continued treatment for 21 days post immunization on daily bases. Significant differences in clinical scores between DMSO and genistein treated group were observed from day 11 to 21. Genistein treated mice had significant difference in mean clinical score, peak score, and mean duration of EAE compared to vehicle. All date, except for disease course, were expressed as mean±SD. P. values are in comparison with vehicle-treated group. *p<0.05, **p<0.005

Histological assay

Histological examination of brain and spinal cord of mice demonstrated significant lower level of inflammation and demyelination in genistein-treated mice (fig.2). Demyelinated lesions scattered widely throughout spinal cord and brain in DMSO-treated mice which covers 39.84±8.09 % and 45.98±5.6% of spinal cord and brain white matter respectively compared to 11.62±5.16% and 7.19±2.96% in genistein-treated animals (fig.2).

A-1



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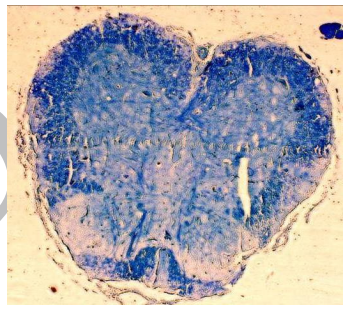

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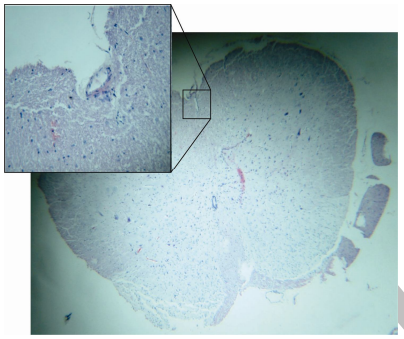

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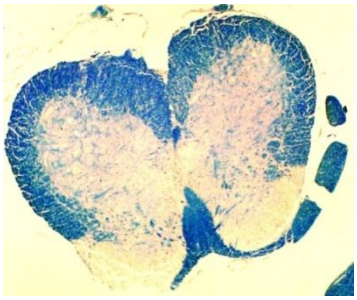

C

		Number of infiltrates/mm^2 (Mean± SD)	Percent of demyelination (mean± SE)
DMSO group	Spinal cord	66.7±33.02**	39.84±8.09 **
	Brain	42.16±12.14**	45.98±5.67**
Genistein group	Spinal cord	4.93±2.4	11.62±5.16
	Brain	2.77±1.44	7.19±2.96

Figure 2: Cellular infiltration and demylination in the spinal cords of treated (with DMSO or genistein) mice. Spinal cord sections were stained from vehicle-treated and genistein(20mg/kg)-treated EAE mice and stained using H&E (A-1 and B-1) and LFB& crystal violet(A-2 and B-2). Massive demyelination and Infiltration were noticed in vehicle treated mice(A), while cellular infiltrates and demyelination were noticeably absent in genistein treated mice (B). Number of infiltrates in each mm2 and percent of demyelination were significantly lower in genistein-treated group. . P. values are in comparison with vehicle-treated group. *p<0.05, **p<0.005

Encaphalogenic and systemic cytokine assay

To determine the underling mechanism of genistein protective effect, we evaluated the cytokine pattern of splenocyte. After genistein exposure, lower levels of INF-γ and IL-12 and higher level of IL-10 were detected in spelenocytes that isolated from genistein-treated mice compared to DMSO-treated ones. No significant difference in TNF-α level was noted between genistein and DMSO treated group (fig.3). To evaluate whether genistein has a comparable effect on the CNS cytokine profile, we measured IL-10, IL-12, TNF-α and IFN-γ in brain supernatant. As presented in figure 4, genistein reduced the production of IL-12, TNF-α and IFN-γ. It further increased IL-10 level. These data revealed that genistein imposes its beneficial role by decreasing Th1 and

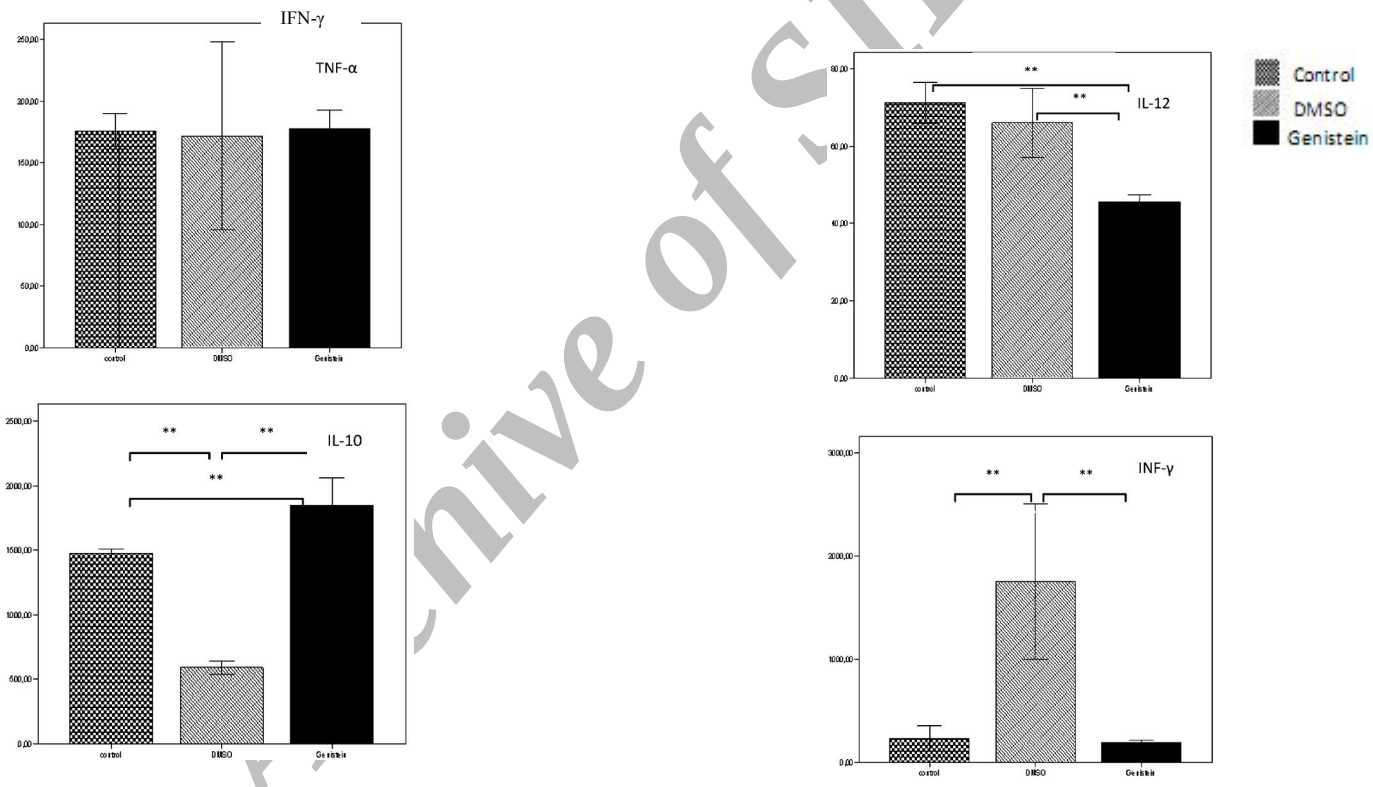

Figure3: Spleen concentration of cytokines (IFN-γ, TNF-α, IL-10 and IL-12). The concentration of cytokines was measured in three groups of mice after restimulating with genistein. These groups were included of control group (not immunized with MOG) (6 mice/ group), immunized with MOG and treated either with DMSO (6 mice/ group) or with genistein (6 mice/ group) from 21 days before to 21 days after immunization. All data expressed as the mean±SD. *p<0.05, **p<0.005

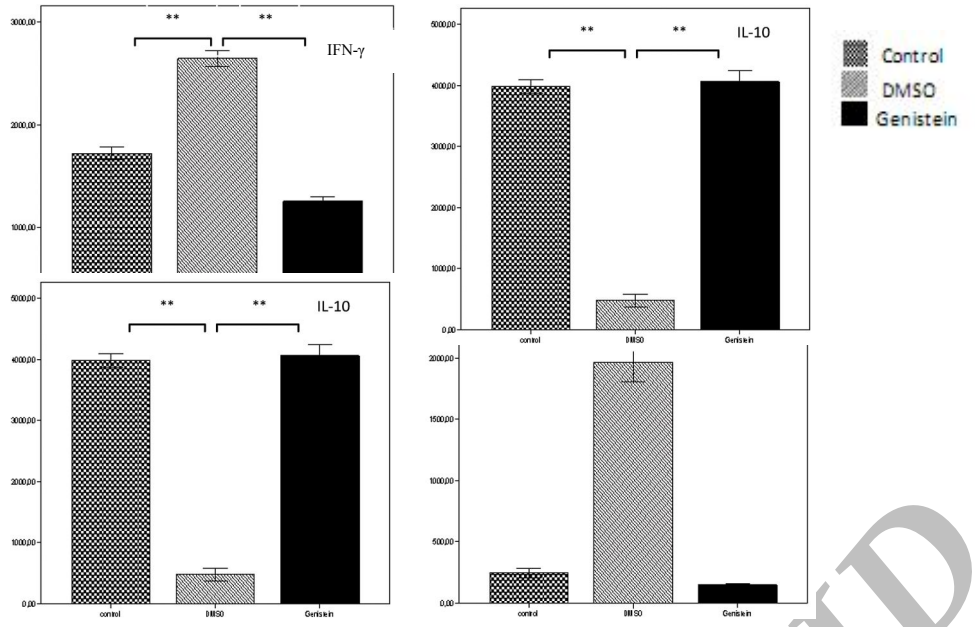

Figure 4: Brain concentration of cytokines (IFN-γ, TNF-α, IL-10 and IL-12). The concentration of cytokines was measured in three groups of mice including control group (not immunized with MOG) (6 mice/ group), immunized with MOG and treated either with DMSO (6 mice/ group) or with genistein (6 mice/ group) from 21 days before to 21 days after immunization. All data expressed as the mean±SD. *p<0.05, **p<0.005

Lymphocyte proliferation assay

Since lymphocyte proliferative responses are generally considered as measure of cell-mediated immunity, genistein specific lymphocyte proliferation was evaluated using MTT assay. As presented in Fig.5, immunized mice with genistein, induced significantly less specific proliferation response, in comparison with control groups which received DMSO. This data indicate the immunomodulatorory capability of genistein

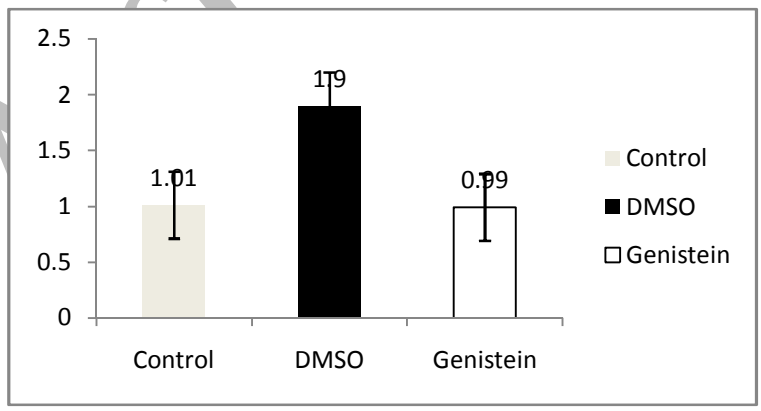

Figure 5: Lymphocyte proliferation evaluation, using MTT assay. Lymphocyte proliferation response was measured in non-immunize, DMSO-treated and genistein-treated group. Genistein-treated mice, induced less specific proliferation response, in comparison with control groups which received DMSO (p<0.05). All data expressed as the mean±SD.

Discussion

We have demonstrated that although oral administration of genistein, the major isoflavone of soy bean, didn't prevent from EAE, it significantly delayed the onset, decreased the severity and shorten the course of disease (Fig. 1). More ever it ameliorates inflammation and subsequent demylination in CNS (Fig. 2). Cellular infiltrate, the hallmark characteristics of EAE, and subsequent demyelination is noticeably absent in genistein-treated group (Fig. 4). According to our results, genistein protective properties can be ascribed to its immmunomodulatory effects. As mentioned in results genistein suppressed the systemic and CNS production of inflammatory cytokines (IL-12 and IFN-γ) production (Fig. 3 and 4). Furthermore it lowered TNF-α secretion in CNS (Fig. 3). In contrast it evidently raised the production of IL-10, an immune-suppressing cytokine, in spleen and CNS (fig.3and4). As shown in MTT test, it also decreased CD4+ T. helper 1 cells proliferation which means genistein can modulate the balance of Th1/Th2.

In the majority of cases, many Th1 cytokines are increased in MS and EAE, while Th2 cytokines are decreased. Several Th2 cytokines have been implicated in survival of neurons. In contrast one of the key factors which involved in the process of demyelination is Th1 cytokines.[15] Th1 proinflammatory cytokines, such as TNF-α, IL-1, IL-6, and cellular immunity enhancers such as IFN-γ have been shown to enhance demyelination, oligodendrocyte apoptosis, and axonal loss.[16-20] In MS and EAE IL-12 is believed to promote Th1 polarization by stimulating IFN- γ secretion,[21] anti-IL-12 can augment EAE signs.[22] On the other hand anti-inflammatory (Th2) cytokines such as IL-10 has been identified to contribute in EAE remission.[23] It may therefore explain why therapies that promote a Th1 to Th2 cytokine-shift are beneficial in MS. In Ms, all approved therapies, in addition to many of those under investigation appear to possess immunomodulatory and anti-inflammatory roles as the main mechanism of action. Beta-interferons and Glatiramer acetate are on top of this list.[24, 26] The results of our study showed that genistein targets this key mechanism of disease and works like approved treatments. IFN-γ enhances cellular immunity. In our study genistein decrease the systemic and encephalogenic secretion of IFN-γ. This result was confirmed by MTT test. As MTT test showed, genistein decreased the number of Th-1 lymphocyte in the periphery. Genistein also decreased the production of TNF-α in the brain, without influencing systemic mononuclear cell activity which differentiates it from other protective and therapeutic agents. The ability of genistein to raise IL-10 level and reduce

the IL-12 production, strengthen its effect.

Previously parenteral admission of 200 mg/kg genistein, two weeks after immunization, was reported to decrease the severity of clinical signs of EAE.[27] In their study genistein injection decreased IFN-γ, TNF-α, and IL-12 level in brain and elevated IL-10 secretion in brain and spleen. Splenocyte from genistein-treated group has also produced lesser amount of IFN-γ. Our results clarified that oral genistein in much smaller dose (20 mg/kg) can have protective effect against the prolonged sequel of the disease by having a similar effect on cytokine pattern.

Genistein anti-inflammatory properties were also observed in collagen-induced rheumatoid arthritis (CIA). Genistein modulate Th1/Th2 balance in CIA by inhibiting IFN-γ secretion and heightening IL-4 production.[28] More ever genistein ability to induce apoptosis, suppress cell proliferation, attenuate osteoclast and lymphocyte activities, and impose antioxidant effects, was previously documented.[14,29]

The results presented in this article demonstrate that genistein can be effective in protecting against prolonged and severe neurological sequel of MS by imposing anti-inflammatory and immune modulating effects. Considering that soy bean is commonly consumed in Japan, one can hypothesize that the beneficial characteristics of soy bean presented here contribute to the lower prevalence of MS in Japanese population.

Acknowledgment

This study was supported by research vice-chancellor, Tabriz University of Medical Ssciences. The authors thank the staff of Shefa Neuroscience Research Center especially Mrs. Alizadeh and Mrs.Eshaghabadi for their kind cooperation. We also thank Dr.Ghaemi for his enthusiastic technical supports and Prof.Gorji for providing the materials. None of the authors had any financial or personal conflicts of the interest.

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اثرات جنیستئین خوراکی استخراج شده از سویا در پیشگیری از مدل حیوانی MS

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فصلنامه علوم مغزواعصاب ایران، سال هفتم، شماره۲۶ و ۲۷، تابستان و پاییز ۱۳۸۸، ۵۱۷-۵۰۵

چکیده

سابقه و هدف: بیماری اسکلروز منتشر(MS) شایعترین بیماری مزمن دمیلینه کننده دستگاه عصبی مرکزی است که با کاهش برگشت ناپذیر توان عملکردی همراه است. تا کنون دارویی کاملاً اثربخش برای این بیماری عرضه نگردیده است و روشهای درمانی حاضر پر هزینه، دارای عوارض جانبی و نیازمند تزریق های متعدد هستند. مشکلات موجود در درمان بیماری MS، اهمیت پیشگیری از بیماری را بیشتر کرده است. جنیستئین یکی از ترکیبات سویا است دارای خواص ضد التهابی، آنتی اکسیدانی و نروپروتکتیو می باشد. این مطالعه با هدف بررسی اثر احتمالی جنیستئین در پیشگیری از بیماری MS طرح ریزی گردید.

روش بررسی: در این بررسی از انسفالومیلیت آلرژیک آزمایشی (EAE) در موشهای C57BL/6 استفاده شد. عوارض پاتولوژیک این بیماری اتوایمیون مشابه MS است و به عنوان مدل حیوانی MS استفاده می شود. برای انجام این بررسی موشها در گروههای ۶ تایی قرار گرفتند. به یک گروه از موشهای تحت بررسی از ۲۱ روز قبل از ایجاد بیماری تا ۲۱ روز پس از آن ۲۰ میلی گرم به ازاء هر کیلوگرم وزن بدن جنیستئین به صورت خوراکی داده شد. از DMSO به عنوان حلال جنیستئین استفاده شد و به یک گروه از موشها به عنوان گروه کنترل بیمار از ۲۱ روز قبل تا ۲۱ روز بعد از ایجاد EAE، DMSO داده شد. در گروه سوم بیماری ایجاد نگردید. در طول دوره بررسی شدت علائم بالینی در دو گروه ثبت گردید. در پایان بررسی های هیستولوژی (التهاب و دمیلیناسیون) و ایمونولوژی (میزان IFN-γ، TNF-α، IL-10 و IL-12 در مغز و طحال) انجام شد

یافته ها: دریافت خوراکی جنیستئین با کاهش معنی دار شدت و مدت بیماری همراه بود. همچنین به طور معنی‌دار بروز بیماری را به تعویق انداخت. اثر جنیستئین در کاهش شدت بیماری با بررسی های هیستولوژی (التهاب و دملیناسیون در برشهای مغز و نخاع) نیز تأیید شد. بررسی وضعیت سایتوکین ها نشان داد جنیستئین اثر خود را با کاهش سیستمیک (بررسی طحال) و انسفالوژنیک سیتوکین های پیش التهابی (IFN-γ، TNF-α و IL-12) و افزایش سیتوکین های ضد التهابی (IL-10) اعمال می کند.

نتیجه گیری: نتایج این بررسی نشان داد، جنیستئین با دارا بودن خواص ضد التهابی می تواند به پیشگیری از بیماری MS کمک کند.

واژگان کلیدی: جنیستئین، انسفالومیلیت آلرژیک آزمایشی، ضد التهابی