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Effects of Intermittent Fasting on Experimental Autoimmune Encephalomyelitis in C57BL/6 Mice

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ABSTRACT

Several religions recommend periods of fasting. One of the most frequently asked questions of MS patients before the holy month of Ramadan is whether fasting might have an unfavorable effect on their disease course. This debate became more challenging after the publication of experimental studies suggesting that calorie restriction prior to disease induction attenuates disease severity. We conducted this study to assess early and late effects of fasting on the animal model of MS, known as autoimmune encephalomyelitis.

EAE was induced in the C57BL/6 mice, using Myelin Oligodendrocyte Glycopeptide (MOG) 35-55 and they fasted every other day either after the appearance of the first clinical sign or 30 days after disease induction for ten days. Thereafter, the mice were sacrificed for further histological and immunological evaluations.

Intermittent fasting after the establishment of EAE did not have any unfavorable effect on the course of disease. Moreover, fasting at the early phase of disease alleviated EAE severity by ameliorating spinal cord demyelination. Fasting suppressed the secretion of IFN- γ , TNF- α and raised IL-10 production in splenocytes. Fasting was also associated with a lower percent of cytotoxicity.

Intermittent fasting not only had no unfavorable effect on EAE but also reduced EAE severity if started at early phase of disease.

Keywords: Calorie restriction; Experimental autoimmune encephalomyelitis; Fasting; Immunity; Inflammation; Multiple sclerosis

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INTRODUCTION

Several religions including Islam suggest times of fasting. Although sick Muslims are exempted from fasting, many individuals with mild, moderate, and severe medical conditions choose to fast. Furthermore, recent evidences suggest that there is no contraindication to Islamic fasting for patients with many of the type-2 diabetes, asymptomatic peptic ulcer, intestinal motility disorders, stable asthma, valvular problems and mild coronary artery disease.¹

Multiple sclerosis (MS) is one of these medical conditions. MS is a complex demyelinating syndrome with an unclear etiology that affects approximately two-and-a-half million individuals worldwide and is identified as the most common debilitating neurological disease in young adults.² MS is an immune-mediated inflammatory disease of the central nervous system (CNS) that, in the most severe cases, can lead to irreversible clinical disability.³ The concern for Muslim MS patients is whether Ramadan fasting might have an unfavorable effect on their disease course. This issue became more conflicting after the work of Esquifino et al. on the animal model of MS, known as Experimental Autoimmune Encephalomyelitis (EAE). They reported that calorie restriction⁴ improves signs of EAE.⁵ Hence, we aimed to assess the possible impact of intermittent fasting (IF) on mice induced with EAE, either after the appearance of the clinical signs or after the establishment of the disease.

MATERIALS AND METHODS

Mice

The study was performed on 8-12 week old female C57BL/6 mice, 6 mice per each group (Pasture Institute, Iran). The animals were maintained on a 12 hour light-dark cycle. All study protocols were in accordance with Shefa neuroscience center guidelines for laboratory animals.

EAE Induction

The mice were immunized with a 1:1 ratio of MOG 35-55 (Alexis, Switzerland) dissolved in complete Freund's adjuvant (CFA) prepared from non-metabolizable oil (paraffin oil and mannide monooleate) containing 0.4 mg of mycobacterium tuberculosis (Sigma-Aldrich, USA). For this purpose, 300 µg of MOG was dissolved in 100 µl PBS (phosphate buffered saline 10%, Sigma-Aldrich, USA) and mixed with an equal volume of CFA. On day 0, the MOG-CFA emulsion was subcutaneously injected into two sites of the upper flanks (200 µg/mouse). The additional immune adjuvant, pertussis toxin (PTX), (Sigma-Aldrich, USA) was injected intraperitoneally (IP) (400 ng/mouse) on days 0 and 2.⁶

Administering with IF

The effect of intermittent fasting on EAE severity was assessed at two stages of the disease. In the first experiments, the mice were either fed every other day

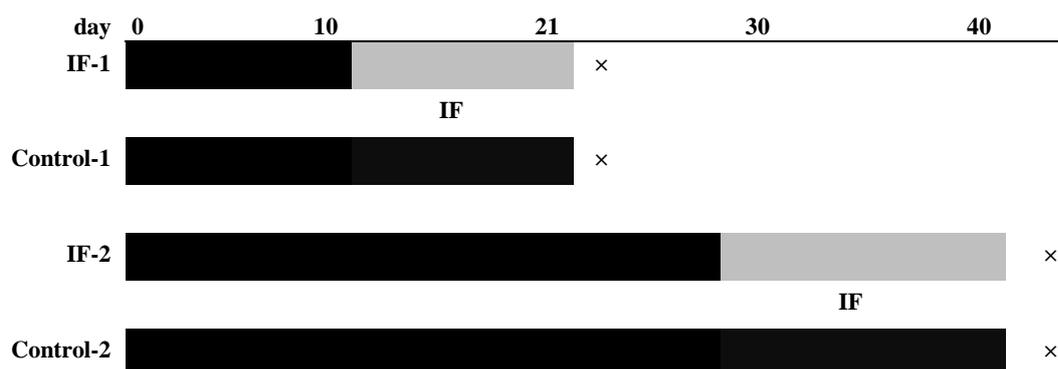


Figure 1. The schematic chart for EAE induction and treatment of female C57BL/6 mice with intermittent fasting (IF). The first clinical sign of EAE appeared between days 9 and 11 in the IF-1 group (10 ± 0.89). Mice in this group were fed every other day after the appearance of the first clinical sign for 10 days. Mice in the control-1 group continued daily feeding for 21 days. The IF-2 group was put on intermittent fasting from 30 days post-immunization for 10 days. The control-2 group continued daily feeding for 40 days. All mice were sacrificed for further assessments, one day after the last treatment.

after the appearance of the first clinical sign for 10 days (IF-1) or daily with normal rodent chew for 21 days (control-1). In the second experiment, the mice were either put on IF from 30 days post-immunization for 10 days (IF-2) or fed daily for 40 days (control-2). Rodent chew contained: 17.5% protein, 2% fat, 12% fiber, 12% water, and 56.5% carbohydrate. Mice in the IF groups were subjected to fasting every other day (from 10 AM, for 24 hours). Figure 1 shows the schematic chart for EAE induction.

Clinical Evaluation of EAE

The clinical scores were recorded daily in all studied groups. In the IF-1 group, daily recording of the clinical scores of each mouse started on day 0 and continued for 10 days after the onset of the clinical signs. In the IF-2 group, daily scoring continued for 40 days. The following grading scheme was applied to score the clinical signs of EAE: 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. The Clinical examiners were not blind to the treatment.

Histological Evaluations

At the end of the experiment, cervical dislocation (CD) method was used for animal scarification. Immediately after CD with a longitudinal section on midline of animal body, we had access to the vertebral column. The vertebral column was removed from the body and the spinal cord was extracted using a bone crusher. The tissues were fixed with paraformaldehyde 4% for 24 hours; then, they were transferred to the tissue processor device (Payroll process), and after dehydration and clearing, they were embedded in paraffin. Axial lumbar spinal cord sections (8 microns) were cut using rotary cryostat. A series of 4-5 cuts were mounted on one slide using albumin glue. The samples were stained with Luxol Fast Blue (Merck, Germany)/Cresyl violet (BDH, England) for assessing the demyelination.

Spleen Cytokine Concentration

At the end of the experiment, the spleen of the mice were dissected and homogenized to obtain single cell suspensions. Mononuclear cells were suspended in 1.5 ml of RPMI-1640 (Gibco-BRL, UK) supplemented

with 10% FCS (Gibco), 1% L-glutamine, 1% HEPES, 0.1% 2ME, and 0.1% penicillin/streptomycin, and were incubated at the concentration of 2×10^6 cells/well in 24-well plates (Nunc, Denmark) for 2 days at 37 °C in 5% CO₂ and 90% humidity incubator. The cells were then pulsed with MOG 35-55. Cell supernatants were then collected and analyzed for the presence of cytokines using commercially available sandwich-based ELISA kits according to the manufacturer's protocol (eBioscience, USA). All tests were performed in triplicate for each mouse.

Lymphocyte Proliferation Assay

At the end of the experiment, mononuclear cells were obtained from the treated mice and were used for the 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)]. 2×10^5 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 were incubated in the presence of 1 µg/ml MOG. T-cell mitogen PHA (phytohemagglutinin, Sigma chemicals) at the concentration of 5 µg/ml was used as positive control. After 3 days, MTT (3-(4,5-dimethyl tetrazolyl-2) 5 diphenyltetrazolium bromide) (Sigma chemicals, Australia) at the concentration of 5 µg/ml was added per well and incubated for 5 hours at 37 °C in 5% CO₂ and 90% humidity incubator. DMSO (dimethyl sulfoxide) (100 µl) was added to dissolve the produced formazan crystals.

The plates were read at 540 nm using ELISA method and intensity of the color reaction product was quantitated on a microplate reader (ELx800, Biotek Instruments, Inc., Winooski, VT, USA). 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) divided by relative OD values of unstimulated cells: $SI = (Cs - Cu) / Cu$

All tests were performed in triplicate for each mouse.

Data Analysis

Parametric variables were assessed using independent sample T-test with Levene's Test for checking the equality of variances. Mann-Whitney U

Test was used to compare non-parametric variables. Differences were considered significant at $p < 0.05$. Data were analyzed with SPSS version 17.

RESULTS

Clinical Assessments

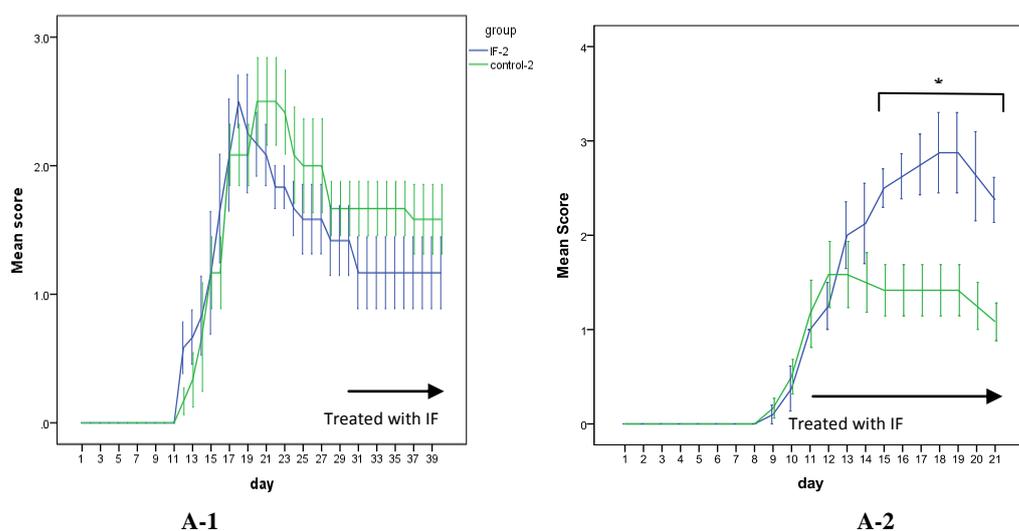
After the induction of EAE, female C57BL/6 mice were fasted every other day in early (IF-1), and late phases (IF-2) of the disease. Fasting every other day in the late phase of disease had no significant effect on the mean and maximum clinical score of the disease (Figure 2). Moreover, mice that fasted every other day in the early phase of the disease showed significantly lower scores between days 15 and 21 post induction ($p < 0.05$).

Histological Evaluations

On the day after receiving the last treatment, the mice were sacrificed, and their spinal cords were removed. As presented in Figure 3, demyelinated lesions were apparent in the white matter of the spinal cord of the controls and the IF-2 group. A lack of demyelinated foci in spinal cord was observed in IF-1 animals.

Cytokine Assay

One day after receiving the last treatment, the spleens of the mice were removed. The level of IFN- γ , TNF- α , IL-10, and IL-12 were assessed in splenocytes. Intermittent fasting in the early phase of the disease decreased IFN- γ and TNF- α levels and enhanced IL-10 production in comparison to the control group. No significant differences were observed between IF-2 and control groups (Figure 4).



B

	Control-1	IF-1	<i>p</i> value	Control-2	IF-2	<i>p</i> .value
Incidence	5/6	6/6	0.3‡	6/6	6/6	
Day of onset	10.25±0.96	10±0.89	0.76¥	14±1.54	14±3.35	0.81¥
Maximum clinical score†	2.87 (2-4) †	1.58 (0.5-2.5) †	0.067¥	2.58 (1-3) †	2.42 (1-3) †	0.48¥

Figure 2. Clinical signs of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice administered with IF for 10 days either after the appearance of the first clinical sign or 30 days post immunization. **A)** The mean daily clinical scores of IF-1 animals were significantly lower than the control group between days 15 and 21 post immunization ($p < 0.05$). **B)** also, IF-1 mice had a lower maximum disease scores ($p = 0.048$) compared to the control group. The data represents as mean±SD. †) mean score (minimum-maximum), ‡ level of significance using Pearson Chi-square Test, ¥ level of significance using Mann-Whitney U Test. IF-1: Intermittent Fasting after the appearance of first clinical sign, IF-2: Intermittent Fasting from 30 days post immunization, Control-1: normal chew for 21 days, Control-2: normal chew for 40 days.

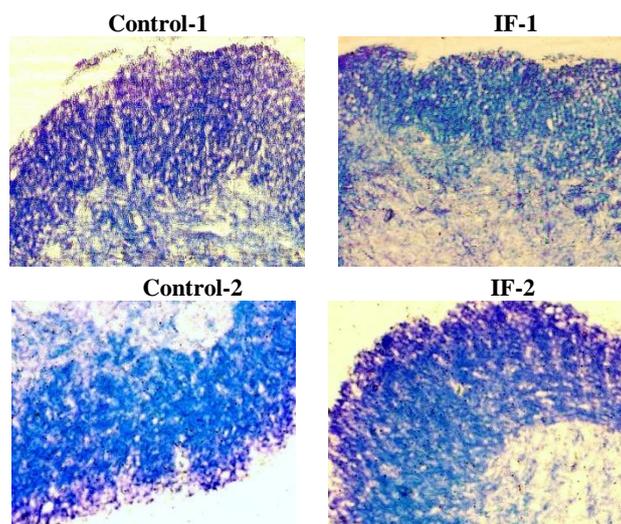


Figure 3. Demyelination of the spinal cords in mice induced with experimental autoimmune encephalomyelitis and treated with IF or continued daily feeding. Luxol Fast Blue/ Cresyl violet staining was applied to assess the ratio of demyelination of spinal cord. Myelin became blue and demyelinated areas became violet. As shown in pictures above, demyelinated areas are apparent in the outer layer of the white matter of IF-2 and Control groups. IF-1: Intermittent Fasting after the appearance of the first clinical sign for 10 days, IF-2: Intermittent Fasting from 30 days post immunization for 10 days, Control-1: normal chew for 21 days, Control-2: normal chew for 40 days

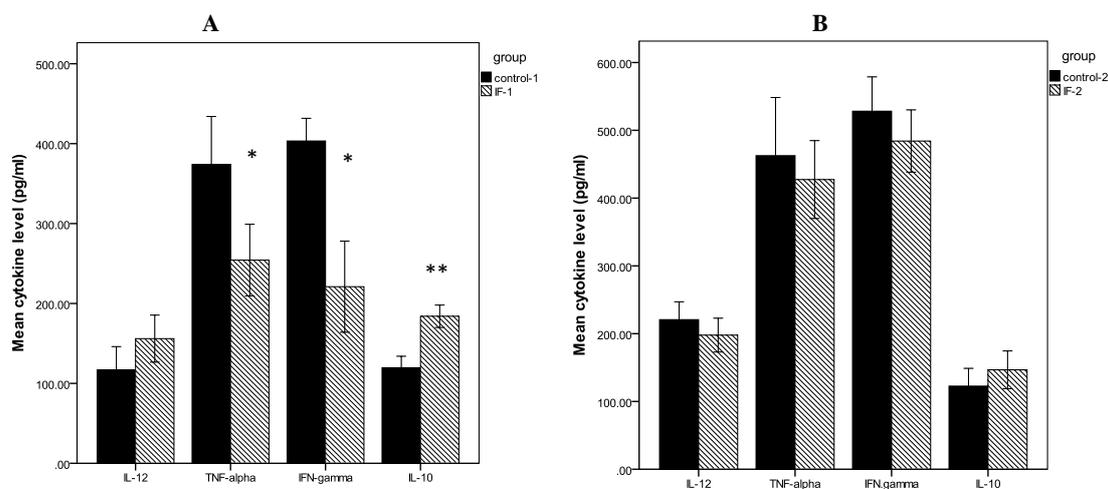


Figure 4. Cytokines level (pg/ml) in splenocytes of experimental allergic encephalomyelitis (EAE) induced C57BL/6 mice. IFN- γ , TNF- α , IL-12, and IL-10 were measured in the supernatant of cultured splenocyte as described in methods. A) Intermittent fasting after the onset of clinical signs (IF-1) modified spleen level of IL-10, TNF- α and IFN- γ compared to the control group. All data were expressed as mean \pm SD *) $p < 0.05$ in comparison to control-1 and **) $p < 0.005$ in comparison to control-1. IF-1: Intermittent fasting after the appearance of the first clinical sign, IF-2: Intermittent Fasting from 30 days post immunization, Control-1: normal chew for 21 days, Control-2: normal chew for 40 days.

MTT Assay

The mice were sacrificed 24-hours after the last treatment and the spleen cells were harvested. The lymphocytes were stimulated in-vitro by 1 μ g/ml MOG and evaluated by MTT methods. As presented in Figure

5, the proliferative response of T-cells was suppressed in IF-1 mice compared to the control group ($p = 0.003$). No significant difference was observed between IF-2 mice and the control group.

Periodic Fasting and EAE

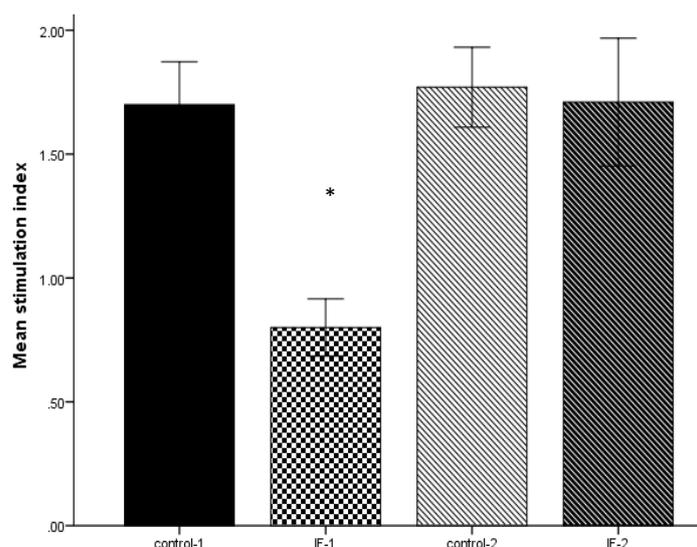


Figure 5. Lymphocyte proliferation response in control and intermittent fasting (IF) groups. The Intermittent fasting mice induced less specific proliferation response, in comparison to the control group. All data were expressed as mean±SD. *) $p < 0.05$ in comparison to control-1. IF-1: Intermittent fasting after the appearance of first clinical sign, IF-2: Intermittent fasting from 30 days post immunization, Control-1: normal chew for 21 days, Control-2: normal chew for 40 days.

DISCUSSION

In this study, we showed that fasting every other day did not aggravate the EAE course. Furthermore, fasting in the early phase of the disease, notably alleviated the severity of the clinical signs of the disease, confirmed by less severe CNS pathology. Esquifino et al. have previously reported that daily calorie restriction⁴ attenuates EAE course in Lewis rats.^{5,7} They reported that restricting energy intake by 33% or 66%, 15 days prior to immunization decreased the severity of EAE.⁷ This was in accordance with the results of the current study.

In the current study, we observed that IF, after the appearance of the first clinical signs, modified the immune response by lowering the level of IFN- γ , and elevating the level of IL-10 in cultured splenocytes. Moreover, IF diminished T-cell proliferation. Esquifino et al. reported impairment in lymphocyte proliferation, a decrease in CD4⁺ cells of lymphoid organs, and suppressed IFN- γ production, which was in agreement with our results.⁵ IFN- γ , which is predominantly secreted by T-cells and natural killer cells, differentiates T-cells from Th1 cells; activates macrophages, and microglia cells.⁸ The accumulated data pointed to the principal role of IFN- γ in progressive demyelinating diseases, including MS and

EAE;⁹ IFN- γ becomes detectable in the CNS at the onset of EAE/MS. This cytokine is involved in many of the pathological features of MS/EAE^{10,11} and partial elimination of IFN- γ enhances tolerance to EAE.¹² The systemic administration of IFN- γ to MS patients worsens the disease.¹³ One of the MS therapeutic effects attributed to IFN- β is the down-regulation of IFN- γ production.¹⁴

Moreover, the increase in the level of IL-10 of splenocytes observed in the IF-1 group in the present study was in line with previous observations reporting that in overweight patients,¹⁵ and in experimental diabetes¹⁶ CR elevates IL-10 production. IL-10 is primarily secreted by Th2 cells, B cells and monocytes. IL-10 reduces the expression of MHC II and suppresses cytokine secretion by macrophages. This cytokine plays a unique role in recovering from EAE.¹⁷ Increase in cerebral IL-10 level was reported during the remission phase of MS.⁹ Treating Lewis rats with human recombinant IL-10 at the onset of the disease has prevented the incidence and decreased the severity of EAE.¹⁸ IL-10-deficient C57BL/6 mice are more prone to EAE development. They exhibit a more severe form of EAE.¹⁹

We also showed that prescribing mice with IF at the onset of the disease decreased the secretion of TNF- α from splenocytes. In contrast to our results, in the study

by Piccio et al. a 40% calorie restriction for 30 days did not lead to the suppression of TNF- α secretion from lymphoid tissues in EAE-induced C57BL/6 mice.⁴ TNF- α is mainly secreted by activated T-cells, NK cells, and phagocytic cells.⁹ TNF- α injection led to the exacerbation of clinical signs and prolongation of the clinical course of EAE.²⁰ TNF- α release parallels the disease course of EAE,²¹ and the maximum severity of EAE is between days 19 and 22.²² Therefore, the difference between our results and findings of Piccio et al. may in part be due to the time of assessing the cytokine profile, which was about 21 days post induction (p.i.) in the current study and 30 days p.i. in the research by Piccio et al. On the other hand, in agreement with our results, the decreasing effect of calorie restriction reduced TNF- α level in previous studies conducted on experimental diabetes¹⁵ as well as the animal model of immunity and inflammation.²

Furthermore, the following mechanisms have been proposed for the beneficial effects of CR in experimental model of MS. First, CR can improve the function of immune system. Long-term CR modifies T-cell function and delays immune senescence. Squifino et al. reported the favorable effect of 66% CR in modifying the lymph node mitogenic response to lipopolysaccharide and Concanavalin A. It also improved 24-hour rhythmicity of B, T, CD4+CD8+, and CD4+ lymph node cell subsets function.^{5,6} Moreover, CR resulted in the increment of the mean values of submaximally lymph node (SMLN) Con A response as well as the reduction of the number of B-cells.⁷ Second, CR could enhance glucocorticoids production. Glucocorticoids showed inhibitory effects on the expression of inflammatory genes.²³ Corticosteroid administration has been used as a standard therapy in MS relapse.²⁴ In rodent, treating with exogenous glucocorticoids suppressed EAE.²⁴ In Piccio et al. study, CR elevated corticosterone level.⁴ Notably, the endogenous production of corticosteroids would prevent the side-effects of exogenous administration of steroids.⁴ Adipose tissue can produce IL-6. CR decreased body fat and subsequently IL-6 level. IL-6 together with TGF- β , were reported to restrain the production of T regulatory cells (T-regs), resulting in the inhibition of Th-17 gene expression.⁴ Expression of Th-17 is known as one of the key features of MS.²⁶ We did not measure Th-17, which is one of the limitations of our study. Assessing the effect of CR on Th-17 and consequently granulocyte-macrophage colony-

stimulating factor (GM-CSF) production is warranted in future studies. In conclusion the fact that intermittent fasting after the onset of EAE attenuates disease severity, proposed the idea that fasting might dampen immune responses and alleviate EAE symptoms when applied at the early phase of the disease. Moreover, Fasting after the establishment of the disease had no unfavorable effect on EAE severity.

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