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Evaluation of IL-17 Serum Level, Brain Inflammation and Demyelination in Experimental Autoimmune Encephalomyelitis C57BL/6 Mice Model with Different Doses of Myelin Oligodendrocyte Glycoprotein

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ABSTRACT

Multiple sclerosis (MS) is an autoimmune disease that affects the central nervous system.MS creates a wide range of symptoms with lifelong debilitating consequences. The hallmark of the disease is the inflammation of the nervous system, which can lead to damage to the nerve tissue and loss of function of the neurons. IL-17 has a prominent role in the beginning of inflammatory reactions. Here, we analyzed a mouse model developed using anti-myelin antibodies. This mouse model mimics many symptoms of MS in humans.

C57BL/6 mice were randomly divided into five groups. Mice were immunized subcutaneously with 50 μg, 100 μg, 150 μg and 200 μg myelin oligodendrocyte glycoprotein in complete Freund's adjuvant containing 4 mg/Ml *Mycobacterium tuberculosis* and two injections of 800 ng of pertussis toxin intraperitoneally, on day 0 and 2 post immunization**.** Serum level of IL-17 was measured, inflammation and demyelination of brain tissue were also evaluated.

Mice with experimental autoimmune encephalomyelitis demonstrated inflammatory cell accumulation, different degrees of demyelination in the brain, and rising levels of serum IL-17 depending on the dose of the anti-myelin antibody.

Our study demonstrates that level of IL-17 production is directly associated with inflammation and demyelination. In addition, different degrees of experimental autoimmune encephalomyelitis in mice can be utilized to test a wide range of therapeutic interventions for MS treatment.

Keywords: Demyelination; Experimental autoimmune encephalomyelitis; Inflammation; Interleukin 17; Multiple sclerosis

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INTRODUCTION

Multiple sclerosis (MS) is an incapacitating autoimmune disorder indicated by inflammation and

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demyelination of the central nervous system (CNS), resulting in multiple clinical symptoms.[1,2] Experimental autoimmune encephalomyelitis (EAE), as an important mouse model of MS, shows several features of the human MS such as inflammation, demyelination, and paralysis.[3-6] The EAE is a valorous instrument for a better perception of pathological and acute phase MS-like lesions .mechanisms involved in dysfunction, injury and development in MS. Therefore, EAE is a model for recognizing modern therapeutic purposes. T helper 17 cells are a lymphocyte subset responsible for inducing autoimmune and inflammatory diseases.[7-10] For a long time, it has been thought that EAE was a Th1-mediated disease,[11,12] until some recent researches have shown that Th17 cells play a primary role in the development of this disease.[13,14] This pattern shift led to a rapid change in research from the Th1 route toward Th17 route.[15-18] O'Connor et al study showed that the entry of Th17 to the CNS is facilitated by Th1.[19] Th17 cells then invade CNS and cause demyelination.[20] Th17 produces cytokines IL-17A, IL-17F, IL-21, and IL-22.[10,21] IL-17(A) as a proinflammatory cytokine is the most outstanding member of theIL-17 family. The cytokine IL-17 as an important proinflammatory cytokine stimulates T cells and some immune cells to produce several pro-inflammatory cytokines and chemokines such as IL-1β and TNF-α.[22] Increased expression of IL-17 cytokine observed in patients with multiple sclerosis[23] and several diseases, such as rheumatoid arthritis,[24] systemic lupus erythematosus[25] and asthma[26] proposing the implication of IL-17 in the progression of these diseases. In EAE mice, Th17 cells accumulate in the brain and spinal cord, and the amount of IL-17 increases.[27,28] Several immune cells, including T-cells, B-cells, and macrophages penetrated into the CNS, causing inflammation, oligodendroglia death, and demyelination.[29,30] Some studies have indicated that the main factors for the pathogenesis of MS and EAE are activation of CD4+T-cells and an imbalance inTh1/Th2 production.[31] Nevertheless, for the progression of the disease, innate immune cells have crucial roles. Recent studies have shown that imbalance of regulatory T/Th17 is the most important factor in the neurological dysfunction of MS/EAE.[32] Immunization with myelin antigens (Ag) such as Myelin Oligodendrocyte Glycoprotein (MOG), Myelin Basic Protein (MBP) and Proteolipid Protein (PLP) peptides, or transferring of myelin-specific $CD4^+$T cells can induce EAE.[33] MOG is the most commonly used Ag in inducing EAE in mice.

There is no definitive treatment for EAE/MS and current therapies are based on clinical symptoms. At present, the most important way to diagnose this disease is apparent symptoms and adaptation of these symptoms with the degree of inflammation and demyelination.

The aim of this study is indication of the pathological characteristics, severity of the disease and its adaptation to clinical symptoms in the acute phase of the disease.

MATERIALS AND METHODS

Animals

C57BL/6 mice were purchased from the North Research Center of Pasteur Institute of Iran (Amol, Iran). All mice were 8 to 10 weeks-old female, pathogen-free and weighing 18±0.5 gr. Each group was maintained in separate cages at 20±2°C and a 12h light and dark cycle. All mice were kept and handled according to animal protocols approved by the Animal Use and Care Committee of the Golestan Medical University, Iran (N.IR.GOUMS.REC.1395.249).

EAE Model

C57BL/6 mice were immunized subcutaneously with 50 μg, 100 μg, 150 μg, and 200μg MOG (AnaSpec, Fremont, USA), in Complete Freund's Adjuvant (CFA) (Sigma-Aldrich, St. Louis, USA) containing 4 mg/mL *Mycobacterium tuberculosis* H37Ra (Fisher Scientific, Hampton, USA) and received two injections of 800 ng of pertussis toxin (PT) (Sigma-Aldrich, St. Louis, USA) intraperitoneally, on day 0 and day 2 after immunization. Clinical scores were evaluated according to a 0–5 scale as follows: 0, no abnormal signs; 0.5, partial limp tail; 1, limp tail; 2, limp tail with gentle hindlimb debility; 2.5, partial hindlimb paralysis; 3, full paralysis of hindlimb; 3.5, full paralysis of hindlimb with partial paralysis of forelimb; 4, full paralysis of forelimb; 5, dead or moribund.[34-39]

Animal Grouping and Drug Treatment

25 mice were randomly divided into 5 groups as follows: Normal control group (recipient of PBS); 50 μg of MOG recipient group; 100 μg of MOG recipient group; 150 μg of MOG recipient group, and 200 μg of MOG recipient group**.**

Cytokine Measurement

18 days after immunization with different doses of MOG/CFA, the serum concentration of IL-17A was

measured using Enzyme-linked immunosorbent assay (ELISA) kit (IBL International, Hamburg, Germany) according to the manufacturer's instructions. Every test was repeated at least once. The concentration of IL-17 was determined using a relevant standard curve.

Histopathology

For assessment of Central nervous system inflammation and demyelination, mice were sacrificed with 4% paraformaldehyde through the heart. Brains were collected from healthy and EAE mice and inserted into falcon tubes containing 10% formalin. 5-10 µm sections were prepared from embedded tissues in paraffin and stained with Hematoxylin & Eosin (H&E) and Luxol Fast Blue (LFB) (Sigma-Aldrich, St. Louis, USA) for inflammation and demyelination assessment respectively as previously described.[34,35,40] After getting microscopic images with a digital camera, Image analysis was performed by Image J software. Demyelination results were expressed based on area/diameter parameter.

Statistics

Samples were analyzed using IBM SPSS Statistics for Windows, version 22 (IBM Corp., Armonk, N.Y., USA). One-way ANOVA was used to compare the means of normally distributed groups. Normal distribution of samples was evaluated by the Kolmogorov-Smirnov test. Every group included at least 4 experimental mice. Differences of p values less than 0.05 were considered as statistically significant.

RESULT

Effects of Different MOG Dosages on EAE Score

Clinical signs were developed 18 days after mice immunization with MOG/CFA. The EAE mice showed poor feeding capability and slow movement, with a hobble, and the reduced activity. The EAE score appraised in mice reflected the severity of EAE. The onset of clinical signs for treated mice was observed in days of 7, 8, 9, and 10 for doses 200, 150, 100, and 50 µg of MOG, respectively. Base on clinical data of EAE, doses of 50, 100, 150, and 200 µg of MOG showed different scores. As figure 1 shown, the severity of EAE was related to the dose of MOG**.** When the dose of MOG increases, the severity of the disease also increases accordingly. On day 18 (acute stage), the mean clinical score of mice received50 µg of MOG was 2.5, and for mice received100, 150, and 200 MOG was 3, 3.5, and 4 respectively (Figure 1). Scores difference among the groups on day 18 were statistically significant (p<0.0005).

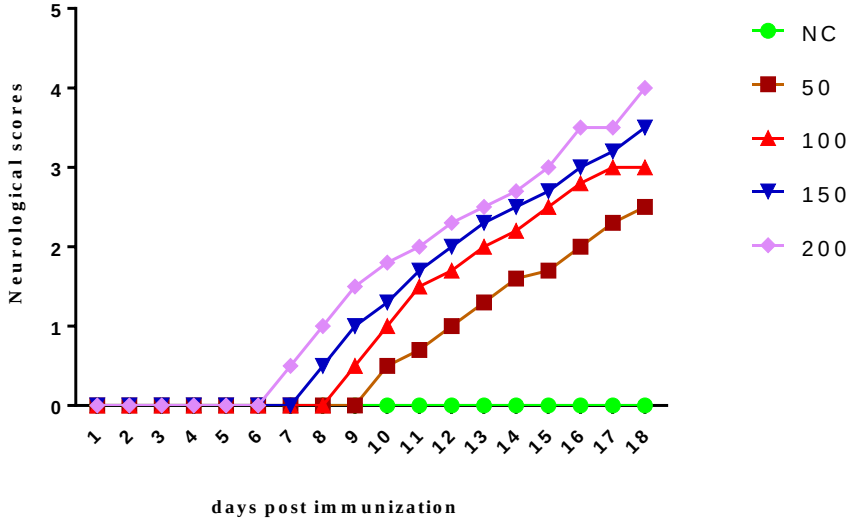


Figure1.The neurological scores. Coinjection of MOG/CFA with PTx induced EAE in mice. The graph indicate the progression of experimental autoimmune encephalomyelitis according to neurological scores in 1 out of 2 independent experiments. Data was expressed as the mean±SD for each group (n=5) and p<0.0005. NC, normal case.

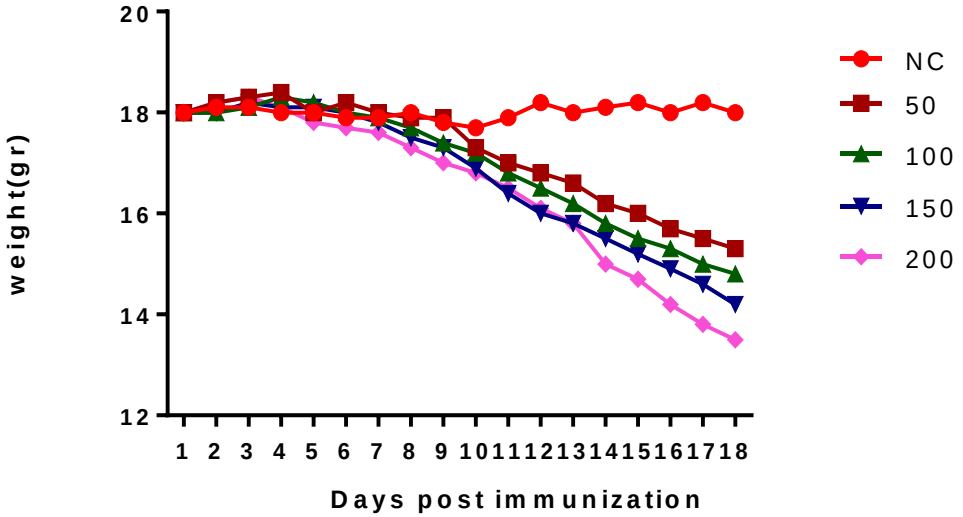


Figure 2. Induction of Experimental Autoimmune Encephalomyelitis with the injection of MOG/CFA and Pertussis Toxin lead to weight loss in mice. The highest weight loss was seen on day 18. There was a direct relation between Myelin Oligodendrocyte Glycoprotein dose and weight loss. The graph also indicates the progression of EAE according to weight loss in 1 out of 2 independent experiments. Data was expressed as the mean±SD for each group (n=5) and p<0.001.NC, normal case.

Effects of Different MOG Dosages on the Weight

After the induction of EAE in mice, a weight loss in mice was observed. The losing weight in each group started on different days. The EAE mice that received 200 µg MOG, began losing weight on the 7th day while groups that received 150, 100, and 50 µg of MOG, weight loss began on 8th, 9^{th,} and 10th respectively (Figure2). The highest weight loss was observed in the group receiving 200 µg MOG and the least weight loss was observed in the group receiving 50 µg MOG. Comparing the groups receiving the drug with the normal group showed that there is a significant difference between them (p<0.001) (Figure 2).

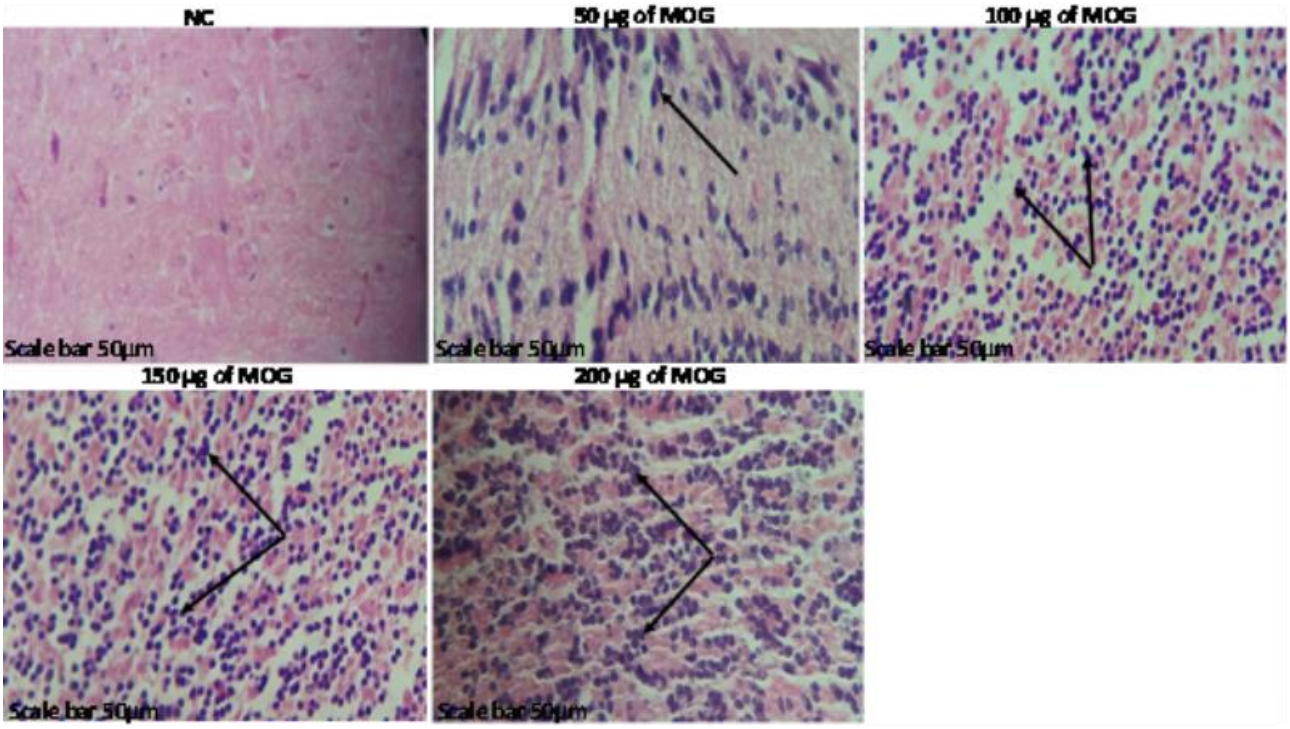
Effects of Different MOG Doses on Tissue Inflammation and Demyelination

After sacrificing mice on day 18, the brain of mice was removed and prepared as slides. Slides were then stained with H&E and LFB stains. Pathological changes of the brain were evaluated by light microscope. In H&E staining, normal mice had no infiltration of inflammatory cells while immunized mice had a large number of inflammatory cells. Mice immunized with 50 µg MOG showed low inflammatory cells infiltration, as a result of low inflammation. Reversely, the Immunized mice with 200 µg MOG represented a high inflammatory cells infiltration, as a consequence high inflammation (Figure 4).

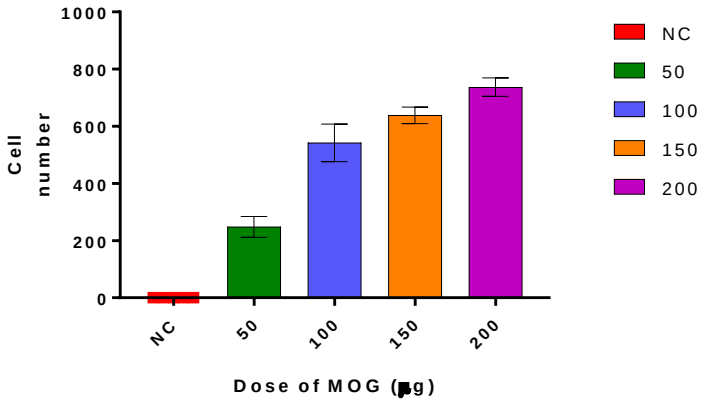
In LFB staining, mice that received PBS displayed no demyelination, while mice injected with different doses of MOG showed various degrees of demyelination (Figure 5).

Effects of Different Doses of MOG on the Serum Level of IL-17

IL17 has a crucial role in the inflammatory reaction. The results acquired by ELISA test showed, in comparison with normal control group (117.4±5.27pg/mL), the levels of IL17 in EAE mice in doses 50, 100, 150, and 200µg of MOG were 192.2±12.13 pg/mL (p<0.0001), 247.4±5.59pg/mL (p=0.0001), 281.6±5.94pg/mL (p<0.0001) and 371.8±5.80pg/mL (p<0.0001), respectively(Figure 6).

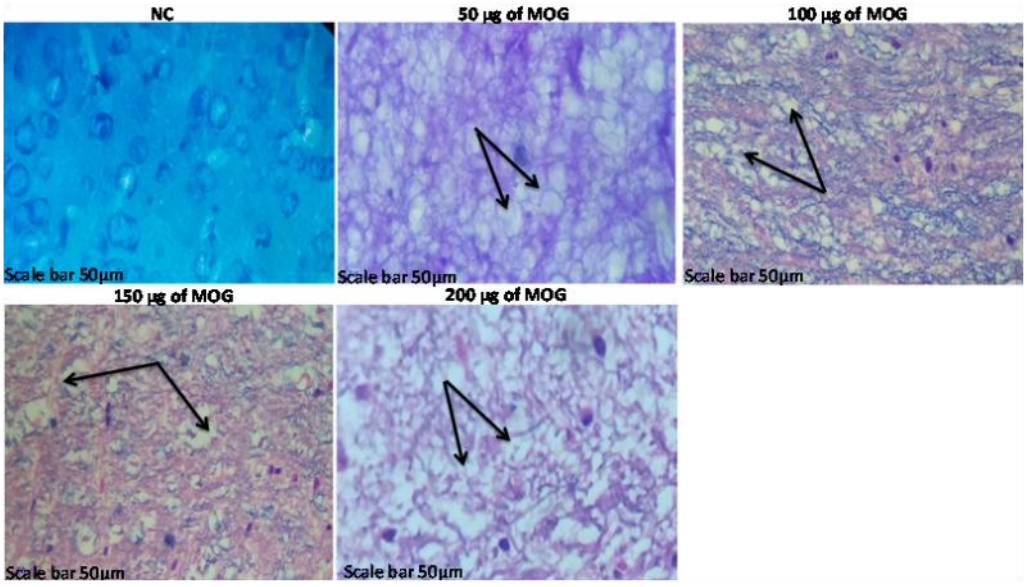


A



B

Figure 4. Pathological variations observed in the brain of mice by light microscope (400x and scale bar, 50µm) on day 18 post immunization. (A) Color of healthy mice brain tissue section stained with H&E is red and immune cells are not seen, while in mice affected with MOG, red color is reduced and background is white color. The severity of EAE base on the infiltration of inflammatory cells (shown by arrows) is different. Induction of EAE by 200µg of MOG causes more infiltration of inflammatory cells. The most severity of condition belongs to the mice that received 200 µg of MOG. The others showed less intensity. (B)Inflammatory cells in slides were counted by ImageJ software. NC mice indicated no infiltration of inflammatory cells, while cell count in EAE mice that received 50 µg of MOG was 231±12 and for mice that received 100 µg, 150 µg and 200 µg of MOG was 605±10, 651±11 and 704±9 respectively. Data was expressed as the mean±SD for each group (n=5) and p<0.0001. NC, normal case.



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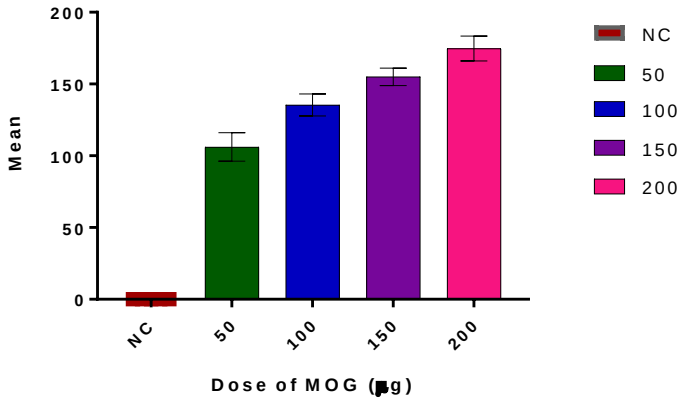

B

Figure 5. Pathological changes observed under the light microscope (400x and scale bar, 50 µm) on the 18 days post immunization. (A) Mice brain tissue section stained with Luxol Fast Blue. Demyelination of samples was measured by ImageJ software. The blue color of the background shows that the myelin of neurons is not hurt. When the intensity of the blue color is weak or when the color is changed, for instance into white (shown by arrows), demyelination happened. NC mice indicated no demyelination, while EAE mice exhibited a large area of demyelination. The results were explained as a whole area/diameter (mean) of myelin.

(B). NC mice indicated no demyelination, while the intensity of demyelination in EAE mice that received 50 µg of MOG was 120 and for mice that received 100 µg, 150 µg and 200µg of MOG was 136, 154 and 162 respectively. *p*<0.0001. NC, Normal case.

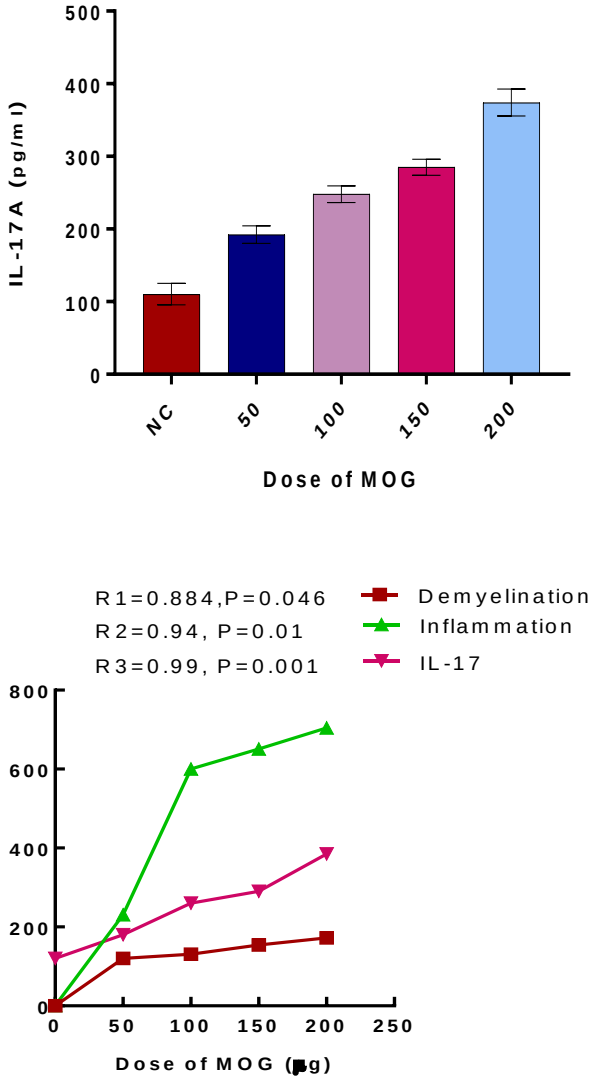

Figure 6. (A). IL-17 was measured by ELISA. The proportion of IL-17 increased in the serum of experimental autoimmune encephalomyelitis mice after myelin oligodendrocyte glycoprotein immunization. (B). Correlation between MOG dose with inflammation, demyelination and IL-17 production.

DISCUSSION

Inducing animal models of EAE is performed by MBP, PLP, and MOG peptides. The MOG as the most widely Ag used to create EAE models is mostly in doses of 50 and 200 µg. In this study, in addition to the above doses, we also applied doses of 100and 150 µg to induce EAE in C57BL/6 mice.

In the present study, we decided to evaluate the effects of different doses of MOG on EAE model of C57BL/6 mice and analyzed association of their pathological and immunological features with clinical symptoms and severity of the disease. In EAE mice, neurological symptoms and weight loss are two important clinical signs. These clinical signs were measured daily and, scored at day after sensitization to realize exact onset time of the development of EAE. Body weight loss is a general feature of EAE, and remains during the amelioration phase.[41-43] Body weight commences to elevate during the chronic phase; thus, weight loss is a significant sign during the acute phase of EAE.

Injection of MOG/CFA with PT into C57BL/6 mice stimulates the immune system to infiltrate immune cells into the CNS and demyelinate nervous cells, leading to neurological symptoms and weight loss.[44] The present study showed, when different doses of MOG were injected into mice, EAE was induced but the severity of neurological symptoms, weight loss, and infiltration of immune cells and demyelination were different. Some studies indicated that clinical signs start between days 9-11 as some of them were consistent with our study[45-47] and some were not.[48,49] We demonstrated that after injection of MOG into mice, clinical indications began at different days so that by injection of 50 µg MOG, neurological symptoms started on day 10 and weight loss on day 9. When we used more quantities of MOG, neurological symptoms and weight loss started earlier.

We showed that by using different doses of MOG, the number of inflammatory cells penetrated into the brain (Figure 4A), the degree of nerve cell demyelination (Figure 5A), the serum levels of IL-17 (Figure 6), the rate of weight loss (Figure2) and the severity of clinical signs (Figure1) are different. We also showed that by increasing the dose of MOG, production of IL-17, the introduction of inflammatory cells into the brain, and demyelination of nerve cells were increased that resulting in more intensity of clinical signs and weight loss.

IL-17 that acts as a mediator in inflammatory and allergic response is synthesized by Th17, and plays a crucial role in the development of EAE.[36,37] Our study showed that EAE was induced in mice with an increase in the level of IL-17, while some studies have shown that EAE can be induced in mice without increasing the IL-17 production.[32,36-40]

An increase in the level of IL-17 following the

increase in MOG dose suggests that there is a direct relationship between inflammation and demyelination. In this study, we presented a collection of changes in neurological symptoms, weight loss, IL-17 production and pathological features in EAE mice which can be useful for researchers who are studying on MS treatment.

Finally, by using Pearson Regression Coefficient, we showed that there is a direct correlation between MOG dose with inflammation, demyelination and IL-17 production, so that, when the dose of MOG increases, the level of demyelination, inflammation, and IL- 17 production increase (Figure 6B).

In conclusion, the current study showed that in EAE mice model the IL-17 production, weight loss, the onset of clinical signs, Infiltration of inflammatory cells and the severity of demyelination increase, following the increase in MOG dose.

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