Effect of Honey Bee venom on Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS)

Jelodar, Sh1., Zare Mirakabadi, A2 ., Oryan, Sh3 ., Mohammadnejad, L4.

1. Department of Biology, Islamic Azad University, Science and Research Branch, Tehran, Iran
2. Department of Venomous Animals and Anti venom Production, Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization, Karaj, Iran
3. Dean Faculty of Biological Sciences, Department of Biology, Kharazmi University of Tehran, Tehran, Iran
4. Biology, Basic Sciences, Azad Islamic University, Sciences and Research Branch, Tehran, Iran
Corresponding author: zareabbas83@gmail.com

Abstract

Experimental autoimmune encephalomyelitis (EAE) has been widely employed as a model to study multiple sclerosis (MS). Interleukin-27 (IL-27) has complex effects on Th17 immune responses to break the normal activity of effector T cells leading to autoimmunity. Bee venom (BV) therapy is a form of medicine originated from the ancient Greece and China. BV and BV-derived active components might have potent therapeutic effects on refractory immunological and neurodegenerative disease, such as multiple sclerosis. In this research we investigated the effect of Iranian honey bee venom on progression of EAE in mice. Experimental autoimmune encephalomyelitis was induced in twelve female C57BL/6 mice by immunization with an emulsion of myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) in complete Freund’s adjuvant (CFA), followed by administration of pertussis toxin (PTX) in Phosphate buffer (PBS). Following appearance of clinical signs, the mice were treated intraperitoneally with BV. Histopathological studies and immunological studies has been investigated. Experimental autoimmune encephalomyelitis was induced in animals within 9-14 days. Results revealed a significant reduction in Interleukin-27 following EAE induction in mice. However, Bee Venom (BV) treated group of mice showed a significant increase in IL-27 as compared to control group. Histopathology results also showed that the amount of inflammatory cells following treatment with BV were reduced in the parenchyma of brain. Based on results obtained in the present study, BV may be suitable candidate for the treatment of inflammatory disease such as MS.

Keywords: Bee venom, Interleukin-27, Multiple sclerosis, Experimental autoimmune encephalomyelitis, MOG, C57BL/6 mice
Introduction

More than 2.5 millions of people living with multiple sclerosis (MS) worldwide and women are estimated to be afflicted twice to thrice as much as men (Bove and chitnis, 2014). Multiple sclerosis (MS) with clinical symptoms including ataxia, loss of coordination, sensory impairment, cognitive dysfunction, and fatigue, is an autoimmune disease associated with chronic inflammatory demyelination of the central nervous system (McFarland and Martin, 2007). Due to disease complexity and heterogeneity effective treatments have not yet been developed and almost all the therapeutics tested in multiple sclerosis patients are based on concepts derived from experimental autoimmune encephalomyelitis (EAE) data (Croxford et al., 2011). It is established that T self-reactive cells have potential to differentiate into effector T cell subsets as Th1 and Th17 (Zorzella-Pezavento et al., 2014). Effector CD4+ T cell subsets play an important role in Multiple Sclerosis (MS) (Legroux and Arbour, 2015). Interleukin-27 (IL-27) suppresses Th (Th1, Th2 and Th17) cells and dampens autoimmunity and tissue inflammation by promoting the generation of Type 1 regulatory T cells (Tr1) (Babaloo et al., 2013).

The search for alternative therapeutic strategies for treatment of MS continues and conflicting evidence in the literature indicates that Bee Venom (BV) can exert anti-inflammatory and antinociceptive effects on inflammatory reactions (Lee et al., 2001). Although research on BV started almost a hundred years ago, its mechanism of action is still uncertain. Some research workers found that immunotherapy using BV could lead to production of some interleukins including IL10, while others reported the decreased IL-4 and IL-5 (Bellinghousen et al., 1997). The target of this research is to analyze the effectiveness of bee venom in progress cessation of EAE induced mice.

Materials and methods

Animal model
The experiments were carried out in 8-10 weeks old female C57BL/6, weighing 18-20 g, purchased from Razi Vaccine and Serum Research Institute of Iran. Mice were housed according to institutional guidelines with access to food and water. Mice were randomly divided into 3 groups. Group 1: Healthy controls (6 mice). Group 2: EAE group (6 mice, Myelin oligodendrocyte glycoprotein (MOG) 35-55-induced EAE) and finally Group 3: BV Treatment (6 mice, MOG35-55 – induced EAE and BV treated).

This animal experiments were carried out in accordance with recommendations from the declaration of Helsinki and the internationally accepted principles for the use of experimental animals.
Induction of EAE and Treatment
Mice were injected subcutaneously with 50 µg MOG\textsubscript{35-55} peptide (Sigma-Aldrich, USA) in complete Freund’s Adjuvant (CFA) containing 1 mg of killed Mycobacterium tuberculosis emulsion (Sigma-Aldrich, USA). This was followed by two intraperitoneal injections of 200 ng of pertussis toxin (Sigma-Aldrich, USA) on day 0 and second day (Stephen D, et al 2010). Bee Venom was provided by Razi Vaccine and Serum Research institute, Iran dissolved in normal saline. Pharmacological dose of BV was calculated based on its effective dose in animal studies (Stephen D, et al 2010). In group 3 of animals the BV (3µg/0.1ml per mouse) was injected intraperitoneally (Camila G.Dantas Tássia et al 2014). Bee venom treatment was performed two times in first week of experiment every 3 days once. Clinical signs and weight of each mice was monitored daily. Ten point standardized rating scale was used to evaluate motor deficit: 0= no clinical signs; 1= partially limp tail; 2= paralyzed tail; 3= hind limb paresis, uncoordinated movement; 4= one hind limb paralyzed; 5= both hind limbs paralyzed; 6= hind limbs paralyzed, weakness in forelimbs; 7= hind limbs paralyzed, one forelimb paralyzed; 8= hind limbs paralyzed, both forelimbs paralyzed; 9= moribund; 10= death.

Cytokine and Histological Analysis
The blood was collected from heart at the end of experiment (day 26\textsuperscript{th}). Serum was separated by centrifuge and preserved at -80 \degree C until analysis. The rate of serum IL-27 was determined using a mouse IL-27 ELISA Kit [CUSABIO, Cat, No. CSB-E08466m]. Reading of OD was performed at 450 nm within 5 minutes and resulting data were analyzed according to the standard curve. Histological analaysis. Mice were euthanized on day 26\textsuperscript{th} of experiment in all 3 groups. They were perfused with PBS followed by 10\% paraformaldehyde for tissue fixation. The fixed brain tissues were embedded in paraffin. Paraffin-embedded sections were cut at 6 µm thickness and mounted on silane-coated standard glass microscope slides. Histological evaluation was performed by staining with hematoxylin and eosin (H&E) for inflammatory cell infiltration analyses. The intensity of inflammatory cell infiltration was assessed according to the protocol (Evonuk et al., 2016). Statistical analysis. Data were expressed as mean ± SEM. Comparisons between groups were made by the 1-way ANOVA with Tukey test. The P values less than 0.05 was considered statistically significant. Statistical analysis was performed using the statistical software SPSS version 16.

Results
No change in Signs and symptoms of clinical disorders was observed in negative control group (group 1) of animals during the experiment. Following the immunization of mice with MOG + CFA and PTX (groups 2 and 3), the first EAE signs appeared 8 days after immunization. The maximal degree of paralysis, indicating an acute phase, occurred around day 14, when the average clinical score reached 2.5. From this time on the animals showed a decrease in the clinical
score, partially recovering from paralysis. However, the recovery was not complete and the disease stabilized around day 20 with an average score of 1.25 (fig 1). Variation in body weight showed an expected course characterized by a significant weight drop during the acute phase. However, weight recovery was observed when the animals achieved the chronic disease phase (fig 2). Mice in groups 2 and 3 from 8th days post – immunization, showed the signs of decreased activities, nutrition behavior and weight loss. Clinical analysis of EAE + BV treated mice subjected to EAE revealed that although no significant differences were found between two groups from the onset until the peak of the disease (about 14 days post – immunization) and both of them showed higher clinical score (ranging between 2.0 and 2.5), but from day 20th the reduction of clinical score started in BV treated mice (group 3) and reached to score 1 on day 25th, while in non-treated mice (group 2) with BV the clinical score remained at 1.5.

**Brain parenchymal lesions**

In an attempt to establish a correlation between clinical signs and morphological changes in the brain of mice, histological section of brain was collected on day 26th after scarifying mice and were analyzed in light microscopy. Typical mark lesions, characterized by an intense perivascular inflammatory infiltrate were observed in the brain of all the mice in group 2 (fig 3b). However a visual inspection indicated that EAE and EAE+ BV mice presented comparable degrees of inflammation. The brain parenchyma of control group showed no penetration of inflammatory cells (fig 3a). While, we noticed an inflammatory infiltration mainly composed by mononuclear leukocytes in parenchyma, the existence of inflammatory cells around the blood vessels. Likewise, mice with EAE + BV showed only mild inflammation around meninges (fig 3c). Hence the histological analysis indicate a significant decreased intensity of pathological changes in the EAE + BV mice (fig 3).

**Serum cytokine expression (IL-27)**

In the present study serum levels of IL – 27 were evaluated in all 3 groups of mice on last day of experiment. The normal level of IL-27 in serum was found to be 127.26 ± 35.72 pg/ml in negative control group of mice. The results revealed a significant decrease (P<0.05) in the serum levels of IL-27 in positive control group (EAE group) which was found to be 73 ± 22.72 pg./ml. However a highly significant (P<0.001) rise in serum level of IL-27 was observed following BV treatment in group 3 of animals and reached to 232.23±38.3 pg/ml.
Discussion

Multiple Sclerosis (MS) is an autoimmune disease of Central Nerve System (CNS) with penetration of macrophages and lymphocytes into CNS leading to demyelination. Etiology of this disease is unknown. However, it seems, T cells and B cells got significant role in attack on myelinated parts of nervous system. In this study we used the mice, C57BL/6. It is well established fact that this race of mice are susceptible to induction of EAE. Induction of EAE carried out by using MOG-CFA-PTX. MOG along with CFA as antigenic component. To enhance the EAE in mice we used PTX (Tompkins et al., 2007).

The results of present study showed a marked changes in physical activity of mice received MOG-CFA-PTX. It is reported that Pertussis toxin (PTX) is an immune-adjuvant utilized to effectively promote an inflammatory response in multiple autoimmune disease animal models and is critically involved in the pathogenesis of EAE (Zhao et al., 2008). Administration of PTX reported to be pro-inflammatory cascade of IL-6, TGF-β, and Th17, in the CNS, which recently have been identified as essential in the development of EAE (Peon et al., 2017). The first EAE signs appeared 8 days after disease induction. The maximal degree of paralysis, indicating an acute phase, occurred on day 14±1, when the average clinical score reached 2.5. The positive control group of mice as well as BV treated EAE induced mice were sacrificed on day 26 for histological studies. This is in agreement with the results obtained by Peon et al 2017. Some research workers mentioned that the first signs of neurological disease is generally weight loss with loss of activity between 10 and 17 days. However, it is mentioned that using the adoptive transfer method, signs are observed somewhat earlier, starting 5 to 7 days after cell transfer 9 (Kostic et al., 2014).

The results of our study showed lower serum levels of IL-27 in EAE group as compared to healthy controls mice suggesting IL-27 potential to be an effective response modifier for immunotherapy of autoimmune disease. Results of this study is similar to other reports that showed the reduction in serum IL-27 following EAE induction (Fitzgerald et al., 2007). However the amount of serum IL-27 increased in BV-treated mice as compared to healthy mice and EAE group suggesting the effectiveness of BV to interfere the disease progress. On the other hand in the present study the effect of BV in EAE induced mice was investigated considering inflammatory cells along with pathological changes in CNS. Experimental autoimmune encephalomyelitis is considered as a disease mediated primarily by the action of Th1 and Th17 lymphocytes, which release several pro-inflammatory cytokines and chemokines with consequent mobilization and activation of peripheral leukocytes to the CNS parenchyma (Becher and Segal, 2011). The peak of the disease is characterized by the prevalence of effector T cells (Th1 and Th17). In fact, recent studies indicate a positive correlation between the presence of regulatory T cells and the regression of EAE symptoms and IL-27 potently suppressed the expansion of encephalitogenic Th17 cells in culture (Langrish et al., 2005). Recent studies also identified different types of inflammatory infiltrates in CNS and Th1 cells have been shown to correlate with a predominantly monocyte CNS infiltrate, while Th17 cells were associated with a higher proportion of neutrophils.
in the CBS infiltrate (Kroenke et al., 2008). Additionally, the clinical symptoms of Th1- and Th17-mediated EAE were found to be different: Th1 cells induced classic EAE, whereas Th17 cells induced an EAE with a more severe clinical phenotype (Jäger et al., 2009).

Medicinal properties of Bee products have been known from ancient times and today the Bee venom is used extensively for the treatment of arthritis and other inflammatory, autoimmune and destructive diseases. Bee venom includes some kinds of peptides, enzymes, active amines and other components, which can be effective in the treatment of various diseases. For example, melitin is one of the most effective and well-known anti-inflammatory factors (Bkaily et al., 1997).

Adolapin is another effective anti-inflammatory substance that suppresses the activity of cyclooxygenase (COX) enzyme (Habermann and Reiz, 1965). Bee Venom also seems to play a role in maintaining homeostasis in our body’s immune system and nervous system, because BV therapy can regulate two immunologically opposite conditions, i.e., allergic disorders (Th2 dominant) and autoimmune diseases (Th1 dominant) (Kim, Bae, 2010). Other T cell populations, such as Th17 cells and Tregs, have emerged as a key player in BV-induced modulation of immune and nervous system (Maddur et al., 2012). Several recent studies reported that BV or bvPLPA2 could upregulate peripheral Tregs and/or suppress Th17 responses in various animal models (Lee et al., 2016).

In conclusion, based on the results obtained in the present study the treatment of EAE induced mice with Bee venom decreased symptoms and pathological changes and increase level of serum IL-27. This activity of Bee venom can be due to the anti-inflammatory effects and the immune-modulatory effects of the venom.

**Conclusion**

Based on results obtained in the present study, BV may be suitable candidates for the treatment of inflammatory disease such as MS.

**ACKNOWLEDGMENTS**

We would also like to show our gratitude to the Dr. Pejman Mortazavi (Associated professor of veterinary pathology, Science and Research Branch, Islamic Azad University) for analyzing and interpreting pathological findings that greatly improved the manuscript.

**REFERENCES**


Figure 1 Effect of treatment with BV on EAE development. C57BL/6 mice were submitted to EAE induction and then injected with Bv. Clinical score variation were daily evaluated. Data were presented by mean ± SEM of 6 mice and representative of three independent experiments.
Figure 2: Effect of treatment with BV on EAE development. C57BL/6 mice were submitted to EAE induction and then injected with Bv. Weight variation were daily evaluated. Data were presented by mean ± SEM of 6 mice and representative of three independent experiments.

Figure 3: Effect of treatment with BV on CNS inflammation. C57BL/6 mice were submitted to EAE induction and then injected with BV. Brain inflammatory infiltrates of control mice without EAE (a). Control group of mice with EAE (b) and EAE induced mice treated with BV (c). Mice were scarified on 26th day. The panel is representative of 6 animals/group.
Figure 4: Interleukin-27 levels in the serum. Mice were submitted to EAE induction and then injected with BV. IL-27 was tested 26 days after scarifying mice. ELISA was used for the determination of serum IL-27. The level of IL-27 in different groups compared to control. *p< 0.05 and **p< 0.01. ***p< 0.001.