Anti-inflammatory activity of Caspian Cobra (Naja naja oxiana) snake Venom on The serum level of IL-27 and Histopathological Changes in MOG-EAE induced mice

Mohammadnejad 1, L., Zare Mirakabadi 2 *, A., Oryan 3, Sh., Jelodar Dezfouli 1, Sh.

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 (AREEO), Karaj, Iran
3. Dean Faculty of Biological Sciences, Department of Biology, Kharazmi University of Tehran, Tehran, Iran
Corresponding Author email: Zareabbas83@gmail.com

Abstract:

Multiple sclerosis (MS) is considered as chronic disease of the Central Nervous System, with a strong neurodegenerative component. The exact mechanism of MS is not clear. However the therapeutic strategies of controlling MS are through immune modulation and inflammation control. In this regard, the present study is to elucidate the influence of snake venom on suppressing of immune system after induction of experimental autoimmune encephalomyelitis (EAE). For this purpose, C57BL/6 female mice, divided into 3 groups, were selected in order to be induced by EAE, the groups 2 and 3 of which were received flank injection emulsion of myelin oligodendrocyte glycoprotein (MOG 35-55) as well as complete Freund adjuvant, followed by administration of pertussis toxin. The treatment group, as an immune-modulator, also received cobra snake venom (CV) after EAE induction. Mice were then evaluated daily by dint of clinical symptoms, weight changes (within 26 days), histopathological analysis, and serum levels of IL-27 for neurological motor deficits. Clinical signs of MOG-EAE in C57BL/6 mice began between day 9-14 post-immunization. Histopathological results also revealed that CV-treated EAE mice, compared to the untreated EAE group, witnessed a significant reduction in the intensity of inflammatory cells in parenchymal sections. Furthermore, increased levels of IL-27 was significant in the CV-treated group (p=0.001) compared with the EAE and control groups. Based on results obtained in present study, it may be concluded that Naja naja oxiana snake venom can as a potential candidate for considering as immunomodulatory and may be employed for MS treatment.

Keywords: Multiple sclerosis; EAE; MOG 35-55; Cobra venom; Interleukin-27

Introduction:
Multiple sclerosis (MS) is a disease with inflammation in central nervous system causing destruction of oligodendrocytes and neurons leading to pathologic changes in white matter (WM) causing clinical symptoms (McFarland and Martin, 2007; Fox et al., 2006; Lopez and Weiner, 2008). It is usually diagnosed between the age of 20 to 40 years and can produce debilitating neurological impairments like muscle spasticity, muscle paralysis, and chronic pain (Rahn et al., 2014). Experimental autoimmune encephalomyelitis (EAE) used as animal model for in vivo MS studies (Raine, 1984; Steinman, 1999). Interleukin-27 which is a member of cytokines family, plays significant role in fundamental processes such as neuronal growth and immune regulation (Batten et al., 2006; Zoë et al., 2010; Villarino and Huang, 2004). Interleukin-27 affects Th17 immune responses (Zoë et al., 2010), to break the normal activity of effector T-cells leading to autoimmunity (Diveu, 2009). Several therapeutic agents are proven helpful, but they are mainly decrease the number of attacks and slowing progression of disease. Some animal venoms are reported to be able in blocking K channel including dendrotoxin I, and/or beta-Bungarotoxin. The K channel blockers can act as immunosuppressive agents with beneficial symptomatic effects in experimental model of MS (Judge and Bever, 2006; Bidard et al., 1987). The agents for MS treatment should be able inhibiting demyelinating process, thus venom therapy with the aim of symptomatic treatment can be considered in research and treatment of MS. Hence this research is undertaken to investigate the effect of *Naja naja oxiana* venom on EAE induced mice.

2. Material and methods

2.1. Animals

For the present study 18 female mice C57BL/6 aged 8 to 12 weeks, weighing 18 to 20, were purchased from Razi Vaccine and Serum Research Institute (Karaj, Iran). Mice were housed in a regulated environment (22±2°C, 12:12h light: dark cycle). Mice according to institutional guidelines, received food (pelleted diet) and water (Kilkenny et al., 2012). Animal randomly allocated to 3 experimental
groups. Group 1- called as control (6mice) didn't receive any drug except normal saline, group2- called as EAE group (6mice), they received MOG_CFA and PTX. Group 3- called as Cobra snake venom treated group (6mice), they received Naja naja oxiana snake venom on day 8\textsuperscript{th} and 16\textsuperscript{th} post MOG injection.

2.2. Induction of EAE

Experimental autoimmune encephalomyelitis (EAE) was induced in C57BL/6 mice by immunization with 50µg of myelin oligodendrocyte glycoprotein 35_55peptide (MOG\textsubscript{35_55}, Sigma Aldrich) in mixture of complete Freund's adjuvant (CFA, Sigma Aldrich). Inject antigen/CFA emulsion subcutaneously into two different sites on each hind flank. Mice also received 2 doses at 0 and 48h after immunization, of 200 ng of Bordetella pertussis toxin (PTX, Sigma Aldrich) intraperitoneally.

2.3. Disease scoring

Animal were daily inspected for any unwanted symptoms, weight changes and clinical scores. Animals were monitored as they walked across a flat plane and checked reflex after turning them over. Responses were scored according to the following clinical assessment scale. Disease intensity was graded as (Bittner, 2014): Score 0= no clinical signs, score 1= partially limb tail, score 2= paralyzed tail, score 3= hind limb paresis, uncoordinated movement, score 4=one hind limb paralyzed, score 5= both hind limbs paralyzed, score 6= hind limbs paralyzed, weakness in forelimbs, score 7= hind limbs paralyzed, no forelimb paralyzed, score 8=hind limbs paralyzed, both forelimbs paralyzed, score 9=moribund and score 10=death.

2.4. Treatment with Cobra snake venom:

Lyophilized venom from Naja naja oxiana was obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran). Pharmacological dose of CV was calculated based on its effective dose in human and animal studies(Pakmanee et al., 1997). The first dose was injected intraperitoneally, two times a week at dose of 0.5 µg/mice, 16 days after immunization of C57BL/6 mice with MOG plus CFA.

2.5. Cytokine analysis
Blood sample from all the 3 groups was collected by micro-tube from mice heart and serum was separated by centrifuging at 3000 rpm for 15 min. and stored at -80°C. Interlukin-27 levels were evaluated by ELISA method, using mouse IL_27 (CUSABIO, cat, No. CSB-EO8466m) ELISA Kit. Mice were sacrificed on day 26 after anesthetized with ketamine /xylezine Alfason, Holland) (3:1.)

2.6. Histological analysis

Brain removed and fixed in 10% formaldehyde. Tissues were dehydrated in graded ethanol and embedded in a 100% paraffin block. Serial section with 5 micrometer thickness were cut and stained with hematoxylin and eosin (H&E). Histopathological severity of inflammatory cells infiltration was evaluated by two blinded observers according to the following criteria (Okuda et al., 2002): 0= absence of infiltrates, 1= perivascular infiltration of inflammatory cells, 2= mild infiltrates, 3= the average infiltrates, 4= bold infiltrates.

2.7. Statistical analysis:

Data were analyzed as mean± SEM. Comparisons between groups were made by one-way ANOVA. Statistical analysis was performed using the statistical software SPSS16. Significance level was considered as P <0.05.

3. Results

3.1 Clinical signs and symptoms and weight changes

The immunization of mice is done by MOG 35-55 peptide emulsified in CFA plus PTX. The primary signs and symptoms appeared 7days post immunizations. Animals showed decreased activity, loss of body weight and obvious clinical signs of EAE On 12th day of immunization. Neurological impairment, including tail paralysis and hind limb paresis was observed. The signs peaked on day 14 in EAE group with average clinical score of 2.25. However the disease stabilized on day 23 with average score of 1 in EAE/MS group and 1.25 on day 24 in CV treatment group. In control group as a result no changes were observed (fig.1). However, the incidence rate of EAE showed no difference between the EAE and CV treatment mice.

3.2 Histopathology of CNS lesions in C57BL/6 mice

Histopathological evaluation of the CNS lesion was performed on day 28 after scarifying the mice. Typical lesions, characterized by an intense perivascular
inflammatory infiltrate were observed in the sections of Brain parenchymal in control group, EAE and CV treatment groups. (fig. 3a, b and c). In control group, there was lack of inflammatory leukocytes. While untreated EAE group mice showed bold inflammatory mononuclear cell infiltrate in the brain parenchymal (fig.3.b). Although in CV treated animals inflammatory cytokines infiltration was observed but the score was comparatively low (fig.3.c). Therefor the histopathological results showed significant reduction intensity of inflammatory cells in CV treated EAE mice as compared to untreated EAE group.

3.3 Serum levels of IL-27 (pg/ml)

Serum levels of IL-27 was evaluated in control, EAE and CV/EAE mice groups. The results revealed increase in the serum levels of IL-27 in CV treated group(275.62±124.64pg/ml) compared to EAE(73±22.72pg/ml) and control groups (117.35±35.72pg/ml) (fig. 4).

In our study results showed reduction of IL-27 in EAE group compared to the health control group was significant (Pv<0.05), also when the venom group was compared with EAE (Pv<0.01) and control (Pv <0.01) group was significantly.

4.Discussion:

Multiple sclerosis (MS) is immune-mediated neurodegenerative disease of the central nervous system (CNS). Its pathogenesis has not been completely understood, but categorized as a CD4+ T cell mediated autoimmune disease (Bielekova et al., 1999; Zhang et al., 1994). Animal model to study this disease is believe to be induction of EAE (Handel et al., 2011) The C57BL/6 mice are the most commonly used strain for which can responses to MOG (Bittner et al., 2012; Mendel et al., 1995). To induce EAE we used MOG. The immune system usually recognizing protein components of the myelin sheath as antigens called Myelin Oligodendrocyte Glycoprotein (MOG) (Vanderlugt and Miller, 1996). The MOG35-55 has been identified as an immunodominant epitope for T cell responses in MS and can induce chronic paralytic EAE in the C57BL/6 mice. For induction of disease MOG is not sufficient, Adjuvants such as encephalogenic peptide, CFA and PTX are necessary that activate mononuclear phagocytes (Hofstetter, 2002). Moreover, we injected PTX by intraperitoneal (IP) on first and 3rd days after immunization. Co-administration of PTX increases the permeability of blood vessel junction in the blood-brain barrier (BBB) (Ryan et al., 1998; Chen et al., 2006). Our study revealed that this mechanism largely
facilitates the immunoreactive cell transmission through BBB and helps EAE induction. The infiltrating inflammatory cells including T cells and macrophage contribute in stimulating the gelial cells to cause acute plaques and neuro inflammation (Gao et al., 2005). In the present study we evaluated for changes in weight and clinical symptoms every day (Bittner et al., 2014). Thought the weight gain in group 1 (healthy control) mice was increasing till end of experiment, EAE group of mice showed a significant reduced weight till the end of experiment. However, in group 3 (venom treated group) the weight gain increased following the venom treatment on day 14 and continued until the mice were scarified. Disease onset typically correlated with a reduction of weight which began 1 to 2 days before EAE symptoms, clinical signs of EAE observed 9 days' post immunization. The main goal in this study was to investigate Cobra snake venom.CV rehabilitation effect on induced EAE model of mice. In reality, CV was not able to decrease clinical symptoms of disease in treatment group as compared to EAE group significantly in the present experiment. This may be due to acute phase of this study. The reversal of the signs and symptoms may be seen if we continue the experiment for longer time. In future project we extend the duration of treatment for as long as the clinical score gets fix trend. However, in the present study administration of CV resulted in decrease of histological severities and increased serum levels of IL-27 in treated group significantly. The treatment of diseases and decrease clinical symptoms from nature products is increasing, and animal venom has been shown to have a wide spectrum of biological activities. The issue could also be true in the case of multiple sclerosis (Reid, 2007; Ebrahim et al., 2016). Between various type of venomous animals, snakes, because of their widespread distribution, considerable volumes of venom and several bioactive components have attracted much attention in medicine, pharmacology for the treatment of conditions like chronic pains associated with advanced cancer, infection caused by herpes viruses and retroviruses (Clamette et al., 1933; Akashdip et al., 2010).

In the present study, we have studied histopathology of the brain lesion development in EAE-induced C57BL/6 female mice and compared to control group. The results clearly showed a prominent area of perivascular with mononuclear inflammatory cells, with extension of lesion into the parenchyma in EAE group (figure.3 b). On the other hand the brain of CV treated mice after induced EAE clearly indicate the reduction of mononuclear inflammatory cells in parenchyma. This is the first time we report that Caspian cobra snake venom, Naja naja oxian is able to reduce EAE severity and intensity of inflammatory leukocyte in brain parenchyma in treated mice (fig.3 c).
Some cells and cytokines are reported to be effective in inflammatory process such as T lymphocytes, IL-6, TGF-β, IFN-γ, IL-17, IL-21, IL-23 and IL-27 play an essential role in multiple sclerosis, that among which Th17, Th1, IL-17 have vital role (Pot et al., 2010). Evidence now indicates that T cells characterized by the production of IL-17 have critical role in induction of disease. Many reports indicate that IL-17 expression can be detected in the target tissue in human autoimmune diseases. (Bettelli et al., 2007; Sospedra and Martin, 2005; Ferber et al., 1996; Komiyama et al., 2006).

Our study clearly revealed the significant rise in IL27 following CV treatment in EAE induced mice. Interlukin-27 has broad inhibitory effects on T cells in mice induced autoimmune inflammation (Yoshimoto et al., 2007; Wang et al., 2007). It is believed that IL-27 can block early Th17 development (Zoe et al., 2010; Babaloo et al., 2013). It is reported that IL-27 clearly plays a role in acute models of autoimmunity, and hence treatment with IL-27 can reduce symptoms after onset of disease (Happel et al., 2003). Results of the present study suggest that CV positively can regulates the reduction of infiltration of immune inflammatory cells into CNS. These findings are in agreement with previous studies by other research workers that used cobra venom (Newitt et al., 1991; Nishio et al., 1998). The mechanism of these positive results is still unclear. However, it has been reported that neurotoxins from venom of African Elapidae snake (black mamba), dendrotoxin I (DTX-I), can act as potassium channel blocker (Newitt et al., 1991; Nishio et al., 1998; Rehm et al., 1988). In addition, β-Bungarotoxin (β-Butx), a presynaptically active neurotoxin, is thought to bind to a subtype of voltage-gated K+ channels (Schmidt and Betz, 1989; Herkert et al., 2001). On the other hand autoimmune diseases are along with tissue injury, caused by autoantigen-specific T-cells and memory T cells (TEM) encounter self-antigens activates, Kv1.3 channels significantly (Cahalan et al., 2001). The potassium channel Kv1.3 participate in modulating T-cell proliferation, immune activation and cytokine production (Cahalan et al., 2001; Zhao et al., 2015). Hence it seems peptides that can target Kv1.3 can selectively impact TEM cell function and may be useful for treatment of autoimmune diseases (Cahalan et al., 2001). Some reports reveals that the Kv1.1 and K1.2 channels of myelinated axons are located under the myelin sheath (Chiu et al., 1999; Rasband et al.,
1998). Hence it seems some peptides in the venom of cobra snake, can easily enter into the CNS after the Blood Brain Barrier (BBB) has been broken down by inflammatory cells and influence the physiological functions of neurons.

5. Conclusion:

The results of our study confirm previous reports on induction of acute EAE in mice by MOG-CFA-PTX and Cobra Venom has inhibitory effects on clinical symptoms and histopathological changes via increasing serum levels of IL-27 and may act as immunomodulator.

Reference:

Akashdip C. Dhanak, Dinesh D. Rishipathak, Dr. Paraag S., 2010. Multiple Sclerosis & its Treatment with Alpha-Cobratoxin, Gide MET’s Institute of Pharmacy, Bhujbal Knowledge City, Adgaon, Nashik; 422 003, India


Bittner, S. et al., 2012. The TASK1 channel inhibitor A293 shows efficacy in a mouse model of multiple sclerosis. Exp. Neurol. 238, 149-


Rehm, H. Bidard J.N. Schweitz, H. Lazdunski, M. The receptor site for the bee venom mast cell degranulating peptide. Affinity labeling and evidence for a
common molecular target for mast cell degranulating peptide and dendrotoxin, a snake toxin active on K⁺ channels. Biochemistry, 1988, 27:1827-1832


Figure 1. Weight changes on EAE/MS and EAE/CV (A). Clinical score changes on EAE/MS and EAE/CV (B). C57BL/6 mice were submitted to EAE and then CV group, treated with cobra snake venom 2 doses. Weight variation (a) and clinical score (b) were daily evaluated. Data were presented by mean ± SEM.
**Figure 2.** Immuno histochemical examination of the CNS inflammatory cell infiltration in brain parenchymal of C57BL/6 mice. Animals were submitted to EAE and then treated with cobra venom. (a) Healthy control mice (b) Positive control group with EAE (c) Induced EAE Mice treated with venom.

**Figure 3.** Variation in Serum levels of IL-27 was evaluated in control, EAE and CV/EAE mice groups. The level of IL-27 in different groups compared to control, *=p< 0.05 and **=p<0.01. The level of IL-27 in CV-EAE compared to EAE, ***=p<0.001.