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Original Article

Anti-inflammatory Activity of Caspian Cobra (*Naja naja oxiana*) Snake Venom on the Serum Level of Interleukin-27 and Histopathological Changes in Myelin Oligodendrocyte Glycoprotein-experimental Autoimmune Encephalomyelitis-induced Mice

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

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, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
3. Dean Faculty of Biological Sciences, Department of Biology, Kharazmi University of Tehran, Tehran, Iran

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Corresponding Author: zareabbas83@gmail.com

Abstract

Multiple sclerosis (MS) is considered a chronic disease of the central nervous system, with a strong neurodegenerative component. The exact mechanism of MS is not clear. However, the therapeutic strategies for controlling MS are based on immune modulation and inflammation control. Regarding this, the present study was conducted to investigate the influence of snake venom on the suppression of the immune system after the induction of experimental autoimmune encephalomyelitis (EAE) in mice. For this purpose, C57BL/6 female mice, divided into three groups, were selected to be induced by EAE. Groups 2 and 3 received flank injection with the emulsion of myelin oligodendrocyte glycoprotein (MOG 35-55), as well as complete Freund adjuvant, followed by the administration of pertussis toxin. Furthermore, the treatment group, as an immune-modulator, received cobra venom (CV) after EAE induction. The mice were then evaluated daily based on clinical symptoms, weight changes (within 26 days), histopathological analysis, and serum levels of interleukin 27 (IL-27) for neurological motor deficits. The clinical signs of MOG-EAE in C57BL/6 mice began 9-14 days 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, the increase of IL-27 levels was significant in the CV-treated group (P=0.001), compared with those in the EAE and control groups. Based on results obtained in the present study, it may be concluded that *Naja naja oxiana* snake venom is a potential immunomodulatory agent that can be effective in the treatment of MS.

Keywords: Multiple sclerosis; EAE; MOG $_{35-55}$; Cobra venom; Interleukin-27

Activité Anti-inflammatoire du Venin de Cobra (Serpent) de la Caspienne (*Naja naja oxiana*) sur le Taux Sérique de l'interleukine-27 et les Changements Histopathologiques Après l'induction d'une Encéphalomyélite Auto-immune Expérimentale par Injection dela Glycoprotéine des Oligodendrocytes de Myéline Chez la Souris

Résumé: La sclérose en plaques (SEP) est considérée comme une maladie chronique du système nerveux central, avec une forte composante neurodégénérative. Le mécanisme exact de la SEP n'est pas clair. Cependant, les stratégies thérapeutiques pour contrôler la SEP sont basées sur la modulation immunitaire et le contrôle de

l'inflammation. Le but de cette étude était d'étudier l'influence du venin de serpent sur la suppression du système immunitaire après l'induction de l'encéphalomyélite auto-immune expérimentale (EAE) chez la souris. A cet effet, des souris femelles C57BL/6ont été sélectionnées et divisées en trois groupes. Les groupes 2 et 3 ont reçu une injection avec une émulsion de glycoprotéine oligodendrocyte de myéline (MOG 35-55), ainsi qu'un adjuvant de Freund complet, suivi de l'administration de la toxine de la coqueluche. De plus, le groupe de traitement, en tant que modulateur immunitaire, a reçu du venin de cobra (VC) après induction de l'EAE. Les souris ont ensuite été évaluées quotidiennement sur la base de leurs symptômes cliniques, des changements de poids (dans les 26 jours), de l'analyse histopathologique et des taux sériques d'interleukine 27 (IL-27) pour les déficits moteurs neurologiques. Les signes cliniques de MOG-EAE chez les souris C57BL/6 ont commencé 9 à 14 jours après l'immunisation. Les résultats histopathologiques ont également révélé que les souris EAE traitées par VC, comparées au groupe EAE non traité, ont été témoins d'une réduction significative de l'intensité des cellules inflammatoires dans les coupes parenchymateuses. En outre, l'augmentation des niveaux d'IL-27 était significative dans le groupe traité par VC (P = 0,001), par rapport à ceux observés dans les groupes EAE et témoin. Sur la base des résultats obtenus dans cetteétude, on peut conclure que le venin de serpent *Naja naja oxiana* est un agent immunomodulateur potentiellement efficace dans le traitement de la SEP.

Mots-clés: Sclérose en plaques; EAE; MOG_{35-55}; venin de Cobra; Interleukine-27

Introduction

Multiple sclerosis (MS) is a disease with inflammation in the central nervous system (CNS) causing the destruction of oligodendrocytes and neurons leading to pathologic changes in the white matter (WM) and inducing clinical symptoms (Fox et al., 2006; McFarland and Martin, 2007; Lopez-Diego and Weiner, 2008). This disease is usually diagnosed between the age of 20 and 40 years and can produce debilitating neurological impairments, such as muscle spasticity, muscle paralysis, and chronic pain (Rahn et al., 2014). Experimental autoimmune encephalomyelitis (EAE) is used as an animal model for in vivo MS studies (Raine, 1984; Steinman, 1999).

Interleukin-27 (IL-27), which is a member of cytokines family, plays a significant role in fundamental processes, such as neuronal growth and immune regulation (Villarino et al., 2004; Batten et al., 2006; Zoë et al., 2010). Interleukin-27 affects Th17 immune responses (Zoë et al., 2010) to break the normal activity of effector T cells leading to autoimmunity (Diveu et al., 2009). Several therapeutic agents are helpful in the management of this disease; however, they mainly decrease the number of attacks and slow down disease progression.

Some animal venoms are reported to be able to block K channel, including dendrotoxin I and/or beta-bungarotoxin. The K channel blockers can act as immunosuppressive agents with beneficial symptomatic effects on the experimental model of MS (Bidard et al., 1987; Judge and Bever, 2006). The treatment agents for MS should be able to inhibit demyelinating process. Therefore, venom therapy with the aim of symptomatic treatment can be considered in MS investigation and treatment. With this background in mind, the present research was performed to investigate the effect of *Naja naja oxiana* venom on EAE-induced mice.

Material and Methods

Animals. The present study was conducted on 18 female C57BL/6 mice with the age of 8-12 weeks and weight of 18-20 g, which were purchased from the Razi Vaccine and Serum Research Institute (Karaj, Iran). The mice were housed in a regulated environment (22±2°C, 12:12 h light:dark cycle). According to the institutional guidelines, they received food (pelleted diet) and water (Kilkenny et al., 2010). The animals were randomly allocated into three experimental groups. Group 1 or the control group (n=6) did not receive any drug, except for normal saline. Group 2 or

EAE group (n=6) was administered with myelin oligodendrocyte glycoprotein (MOG)-complete Freund's adjuvant (CFA) and pertussis toxin (PTX). Furthermore, group 3 or cobra snake venom-treated group (n=6) was subjected to *Naja naja oxiana* snake venom on days 8th and 16th post-MOG injection.

Induction of experimental autoimmune encephalomyelitis. Experimental autoimmune encephalomyelitis was induced in C57BL/6 mice by immunization with 50 µg MOG 35-55 peptide (MOG_{35-55}, Sigma Aldrich) in a mixture of CFA (Sigma Aldrich). The mice were subjected to a subcutaneous injection of antigen/CFA emulsion into two different sites on each hind flank. They also received two doses of 200 ng *Bordetella* PTX (Sigma Aldrich) intraperitoneally at the baseline and 48 h after immunization.

Disease scoring. The animals were daily inspected for any unwanted symptoms, weight changes, and clinical scores. They were monitored as they walked across a flat plane and checked for reflex after being turned over. Responses were scored based on a clinical assessment scale. In this regard, disease intensity was graded on a score range of 10 (0=no clinical signs, 1=partially limb tail, 2=paralyzed tail, 3=hind limb paresis and uncoordinated movement, 4=one hind limb paralysis, 5=both hind limbs paralyzed, 6=hind limbs paralysis and weakness in forelimbs, 7=hind limbs paralysis and no forelimb paralysis, 8=hind limbs paralysis and both forelimbs paralysis, 9=moribund, and 10=death) (Bittner et al., 2014).

Treatment with cobra snake venom. Lyophilized venom from *Naja naja oxiana* was obtained from the Razi Vaccine and Serum Research Institute (Karaj, Iran). The pharmacological dose of cobra venom (CV) was calculated based on the effective dose reported in human and animal studies (Pakmanee et al., 1998). The first dose was injected intraperitoneally, two times a week at a dose of 0.5 µg/mice, 16 days after the immunization of C57BL/6 mice with MOG plus CFA.

Cytokine analysis. Blood samples were collected from the mice heart in all three groups using microtubes. Subsequently, the serum was separated by centrifuging at 3,000 rpm for 15 min and stored at -80°C. Interleukin-27 levels were evaluated by the enzyme-linked immunosorbent assay (ELISA) method, using mouse IL-27 ELISA Kit (CUSABIO, cat, No. CSB-EO8466m). The mice were sacrificed on day 26 after being anesthetized with ketamine/xylazine (3:1; Alfason, Holland).

Histological analysis. For the purpose of histological analysis, the brain was removed and fixed in 10% formaldehyde. The tissues were then dehydrated in graded ethanol and embedded in a 100% paraffin block. Serial sections with 5-µm thickness were cut and stained with hematoxylin and eosin. The histopathological severity of inflammatory cell infiltration was evaluated by two blinded observers on a 5-point scale (0=absence of infiltrates, 1=perivascular infiltration of inflammatory cells, 2=mild infiltrates, 3=average infiltrates, and 4= bold infiltrates) following Okuda et al. (2002).

Statistical analysis. The data were analyzed in SPSS software (version 16), using descriptive (mean±SEM) and inferential statistics. The research groups were compared using one-way ANOVA. A p-value less than 0.05 was considered statistically significant.

Results

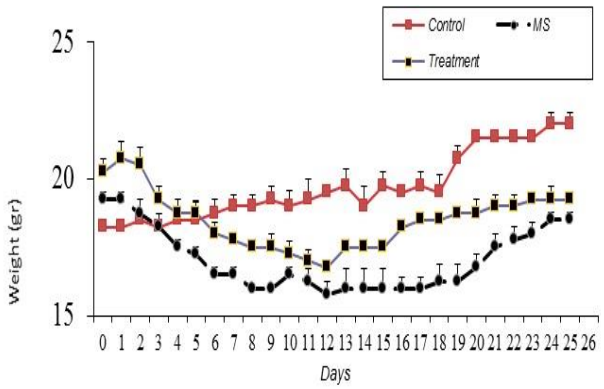
Clinical signs and symptoms and weight changes. The immunization of mice was performed using MOG_{35-55} peptide emulsified in CFA plus PTX. The primary signs and symptoms appeared 7 days post-immunization. The animals showed decreased activity, loss of body weight, and obvious clinical signs of EAE on the 12th day of immunization. Neurological impairments, including tail paralysis and hind limb paresis, were observed in the animals. On day 14, the signs peaked in the EAE group with an average clinical score of 2.25. However, the disease stabilized on day 23 with an average score of 1 in the EAE/MS group and on day 24 with an average score of 1.25 in the CV treatment group. However, no changes were observed in the control group

(Figure 1). The results revealed no difference between the EAE and CV treatment mice in terms of the incidence rate of EAE.

Histopathology of central nervous system lesions in C57BL/6 mice. The 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 brain parenchymal sections in the control, EAE, and CV groups (Figures 2a, b, and c). In the control group, there was a lack of inflammatory leukocytes. However, the untreated EAE group showed bold inflammatory mononuclear cell infiltrate in the brain parenchyma (Figure 2b). Although in the CV-treated animals, inflammatory cytokines infiltration was observed, the score was comparatively low (Figure 2c). Therefore, the histopathological results showed a significant reduction in the intensity of inflammatory cells in the CV-treated EAE mice as compared to that in the untreated EAE group.

Serum levels of interleukin-27. The serum levels of IL-27 was evaluated in the control, EAE, and CV/EAE groups. The results revealed an increase in the serum levels of IL-27 in the CV-treated group (275.62±124.64 pg/ml), compared to those in the EAE (73±22.72 pg/ml) and control (117.35±35.72 pg/ml) groups (Figure 3). The results of the present study were indicative of a significant decrease in IL-27 level in the EAE group, compared to that in the control group ($P<0.05$). The venom group also showed a significant difference with the EAE ($P<0.01$) and control ($P<0.01$) groups in this regard.

a)



b)

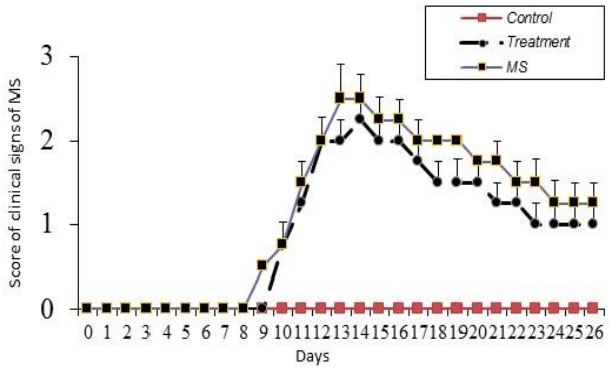


Figure 1. Weight changes in EAE/MS and EAE/CV (A), clinical score changes in EAE /MS and EAE/CV (B) (C57BL/6 mice were subjected to EAE; then, the CV group was treated with cobra snake venom at two doses. Weight variation (a) and clinical score (b) were daily evaluated. Data are presented by mean ±SEM.)

EAE: experimental autoimmune encephalomyelitis, MS: multiple sclerosis, CV: cobra ven

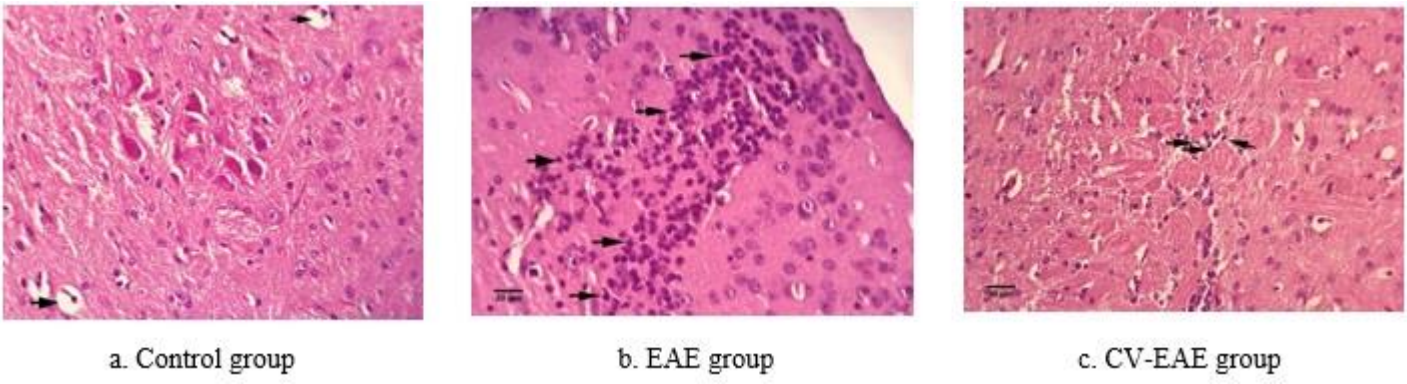


Figure 2. Immunohistochemical examination of the CNS inflammatory cell infiltration in the brain parenchyma in C57BL/6 mice (Animals were submitted to EAE and then treated with cobra venom); (a) healthy control mice, (b) positive control group with EAE, and (c) EAE-induced mice treated with venom

CNS: central nervous system, EAE: experimental autoimmune encephalomyelitis

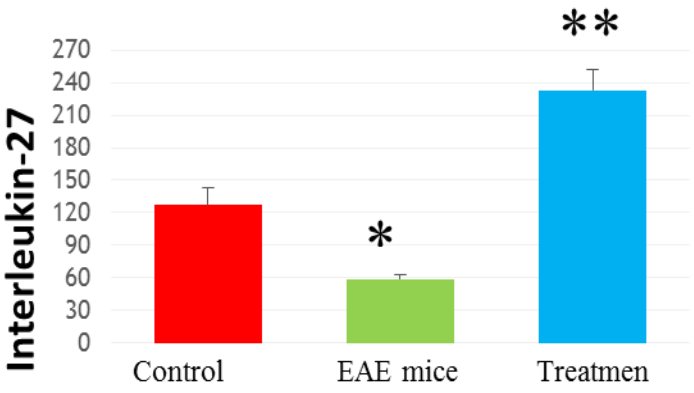


Figure 3. Variation in the serum levels of IL-27 in the control, EAE, and CV/EAE groups (The level of IL-27 in different groups, compared to those in the control group [*=P<0.05 and **=P<0.01]. The level of IL-27 in CV-EAE group, compared to that in the EAE group [***=P<0.001])

IL-27: interleukin-27, EAE: experimental autoimmune encephalomyelitis, CV: cobra venom

Discussion

Multiple sclerosis is an immune-mediated neurodegenerative disease of the CNS. The pathogenesis of this disease has not been completely understood; however, it is categorized as a $CD4^+$T cell-mediated autoimmune disease (Zhang et al., 1994; Bielekova et al., 1999). An animal model to study this disease is believed to be the induction of EAE (Handel et al., 2011). The C57BL/6 mice are the most commonly used strain for responding to MOG (Mendel et al., 1995; Bittner et al., 2012).

In the present study, MOG was used for the induction of EAE. The immune system usually recognizes the protein components of the myelin sheath as antigens called MOG (Vanderlugt and Miller, 1996). The MOG_{35-55} has been identified as an immunodominant epitope for T cell responses in MS and can induce chronic paralytic EAE in C57BL/6 mice. For the induction of disease, MOG is not sufficient. To this end, it is required to use such adjuvants as encephalitogenic peptide, CFA, and PTX, which activate mononuclear phagocytes (Hofstetter et al., 2002). Moreover, in the present study, the mice were subjected to PTX intraperitoneal injection 1 and 3 days after immunization. The 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 to the stimulation of the glial cells to cause acute plaques and neuroinflammation (Gao et al., 2005). In the present study, the mice were screened for changes in weight and clinical symptoms on a daily basis (Bittner et al., 2014). Though the weight gain in group 1 (i.e., healthy control) was increasing until the end of the experiment, the EAE group showed a significant decrease in weight till the end of the intervention. However, in group 3 (i.e., venom-treated group), the weight gain increased following the venom treatment on day 14 and continued until sacrificing the mice.

In the current research, disease onset was typically correlated with weight reduction, which began 1-2 days before the emergence of EAE symptoms. The clinical signs of EAE were observed 9 days post-immunization. The main goal of this study was to investigate the rehabilitation effect of CV on the induced-EAE model of mice. In the present research, the CV was not able to significantly decrease the clinical symptoms of the disease in the treatment group as compared to those in the EAE group. This may be due to the acute phase of this study. The reversal of the signs and symptoms could possibly be seen if the experiment was continued for a longer time.

In a future project, we will extend the duration of the treatment as long as the clinical score obtains a fixed trend. However, in the present study, the administration of CV resulted in the significant mitigation of histological severities and elevation of serum IL-27 in the treated group. The use of natural products for the treatment of diseases and reduction of clinical symptoms is on an increasing trend. Accordingly, animal venom has been shown to be useful in this domain because of its wide spectrum of biological activities. This issue could be also true in the case of MS (Reid, 2007; Ebrahim et al., 2016). Between various types of venomous animals, snakes have attracted much attention in medicine and pharmacology for the treatment of conditions like chronic pains associated with advanced cancer and infection caused by herpes viruses and retroviruses mainly because of their widespread distribution, considerable volume of venom, and several bioactive components (Calmette et al., 1933; Akashdip et al., 2010).

In the present study, the histopathology of brain lesion development in EAE-induced C57BL/6 female mice was studied and compared to that of a control group. The results clearly showed a prominent perivascular area with mononuclear inflammatory cells, with the extension of lesions into the parenchyma in the

EAE group (Figure 3b). On the other hand, the brain of the CV-treated mice after EAE induction showed the reduction of mononuclear inflammatory cells in parenchyma. This is the first report on the potentiality of Caspian cobra snake venom (*Naja naja oxiana*) to reduce the severity of EAE and intensity of inflammatory leukocyte in the brain parenchyma in the treated mice (Figure 3c).

Some cells and cytokines are reported to be effective in the inflammatory process (e.g., T lymphocytes, IL-6, TGF-β, IFN-γ, IL-17, IL-21, IL-23, and IL-27) and play an essential role in MS. Among these, Th17, Th1, and IL-17 have a vital role (Pot et al., 2010). Evidence now indicates that T cells characterized by the production of IL-17 have a critical role in the induction of diseases. Based on multiple reports, IL-17 expression can be detected in the target tissue in human autoimmune diseases (Ferber et al., 1996; Sospedra and Martin, 2005; Komiyama et al., 2006; Bettelli et al., 2007).

Our study revealed a significant rise in IL-27 following CV treatment in EAE-induced mice. Interleukin-27 has broad inhibitory effects on T cells in mice induced with autoimmune inflammation (Wang et al., 2007; Yoshimoto et al., 2007). It is believed that IL-27 can block early Th17 development (Zoë et al., 2010; Babaloo et al., 2013). The IL-27 plays a role in the acute models of autoimmunity; therefore, treatment with IL-27 can reduce symptoms after the onset of a disease (Happel et al., 2003). The results of the present study suggested that CV can positively regulate the reduction of the infiltration of immune-inflammatory cells into the CNS. These findings are in agreement with those of the previous studies examining CV (Newitt et al., 1991; Nishio et al., 1998). However, the mechanism of these positive results is still unclear.

Based on the evidence, neurotoxins from the venom of African Elapidae snake (black mamba), dendrotoxin I, can act as a potassium channel blocker (Rehm et al., 1988; Newitt et al., 1991; Nishio et al., 1998). In addition, ß-bungarotoxin, 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, along with tissue injuries caused by autoantigen-specific T-cells and memory T cells (TEM), lead to a significant increase in the activities of Kv1.3 channels (Cahalan et al., 2001). The potassium channel Kv1.3 participates in modulating T-cell proliferation, immune activation, and cytokine production (Cahalan et al., 2001; Zhao et al., 2015). Hence, it seems that peptides can target Kv1.3 can selectively impact T_{EM} cell function and may be useful for the treatment of autoimmune diseases (Cahalan et al., 2001). Some reports reveal that the Kv1.1 and K1.2 channels of myelinated axons are located under the myelin sheath (Rasband et al., 1998; Chiu et al., 1999). Therefore, it seems that some peptides in the venom of cobra snake can easily enter the CNS after the BBB is broken down by inflammatory cells and influence the physiological functions of the neurons.

Conclusion

The results of our study are in line with previous reports on the induction of acute EAE in mice by MOG-CFA-PTX. It was revealed that CV has inhibitory effects against clinical symptoms and histopathological changes via increasing the serum levels of IL-27. Therefore, CV was concluded to have the potential to act as an immunomodulator.

Authors' Contribution

Research student for M. Sc.: L. M
Research supervisor and designer of the study: A. Z. M.
University supervisor: Sh. O.
Co-worker of the project: Sh. J. D.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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