Combined Effect of Honey Bee Venom and Vitamin B12 on Lewis Rats with Experimental Allergic Encephalomyelitis Induced by Guinea Pig Spinal Cord Homogenates

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ABSTRACT

Multiple sclerosis (MS) is a progressive, neurodegenerative disease of central nervous system (CNS). Experimental allergic encephalomyelitis (EAE) is a widely accepted animal model for MS. Honey bee venom (Apis mellifera) contains a variety of low and high molecular weight peptides and proteins including melittin, apamin, adolapin, mast cell degranulating peptide and phospholipase A2. Bee venom (BV) could exert anti-inflammatory and antinociceptive effects on the inflammatory reactions. Chronic inflammation is often accompanied by oxidative stresses. New observations suggest that cobolamins (derivatives of vitamin B12) may modulate the responses of oxidative stress. Regarding the role of vitamin B12 in the formation of myelin sheet, modulation of tumor necrosis factor alpha (TNF-α) and its role in the regulating the immune reactions and anti-oxidant role; this compound is used in the present study as an accessory and accompanying agent with the bee venom for studying their combined impact on rat model EAE. The hematoxylin, eosin and luxol fast blue methods were used in the analyses of inflammation and demyelination detection, respectively. While, immunohistochemistry, ELISA and HPLC were used for the data assessment. In this study, we showed that the treatment of EAE with a combination of bee venom and vitamin B12 decreased the symptoms of disorder, pathological changes, level of serum TNF-α, gliosis and the nitric oxide (NO) production.

KEY WORDS: Bee venom; vitamin B12; experimental allergic encephalomyelitis; TNF-α; gliosis; nitric oxide.

1. INTRODUCTION

Multiple sclerosis (MS) is a progressive, neurodegenerative disease of central nervous system (CNS). This disease is recognized by symptoms like inflammation, demyelination and the destruction of neurological actions (Urbach-Ross and Kusnecov, 2007). Experimental allergic encephalomyelitis (EAE) is considered as a valuable animal model for MS research and the scientists who use it both for the evaluation of the process and treatment of diseases (Hedruel et al., 2009). EAE is created in animals by injecting the tissue of myelin basic protein (MBP), CNS, or Myelin oligodendrocyte glycoprotein (MOG) along with the adjuvant. EAE and MS are similar diseases. In EAE, certain symptoms are observed such as paralysis, inflammation, ataxia, elevated interferon-γ (IFN-γ), brain-blood blood-brain barrier damage and the penetration of CD4⁺ T-cells and macrophages to the central nervous system (Ferguson et al., 1990; Mao et al., 2007).

The venom of honey bee (Apis mellifera) consists of different types of light and heavy chain peptides and the proteins such as; melittin, apamin, adolapin, phospholipase A2 (Mirshafiey, 2007). The healing bee poison is largely effective in treating chronic anti-inflammatory diseases (Han et al., 2007). Moreover, anti-inflammatory specifications of bee venom in rat model, having induced arthritis, have been reported, and it is observed that the injection of bee venom suppresses leukocyte migration and reduces the level of TNF-α (Mirshafiey, 2007). Bee venom contains several bioamines, such as apamin, histamine, procaine, serotonin, and nor-epinephrine, which facilitate nerve transmission and healing in a variety of nerve disorders. This gives BV the ability to travel along the neural pathways from the spine to various trigger points and injury areas to help repair nerve damage and restore the mobility (Son et al., 2007).

Chronic inflammation is often accompanied by oxidative stresses and many diseases depending on age such as cancer and atherosclerosis, diseases accompanied by the destruction of nervous system and
arthrosis. New observations suggest that cobalams may modulate the response of oxidative stress. Inflammatory diseases are accompanied by an increase in the level of transcobalamins. The density of cobalams modulates the level of TNF-α in the cerebrospinal fluid and it is suggested that the increase of cobalamin can be used as a complement in the cellular response to inflammation (Brich et al., 2009).

Decrease in vitamin B12 results in the defects of myelin sheath formation and methylation of myelin basic protein (MBP), which is a main constituent of CNS. The activities of demyelination and inflammation in MS patients is accompanied by the revival of myelin and results in the consumption of vitamin B12 (Miller et al., 2005). Regarding the role of vitamin B12 in myelin sheath formation, TNF-α modulation, regulation of immune reactions and antioxidant activity; this compound is used in the present study as an accessory and accompanying agent with bee venom for studying their impacts on rat model EAE.

2. MATERIALS AND METHODS

After anesthetizing guinea pigs, their spinal cords were extracted and mixed with equal volume of water at 4 °C to acquire a homogeneous mixture. The guinea pig spinal cord homogenate (GPSCH) was emulsified in 1:1 ratio of complete freund’s adjuvant (CFA), consisting of 1 mg/ml Mycobacterium tuberculosis (Sigma-Aldrich, F5881).

EAE was created by subcutaneous injection of 0.2 ml GPSCH-CFA to the adult female Lewis rats (weight, 180-200 g; Laboratory of Animal Center, Darupakhsh Pharmaceutical Company, Tehran, Iran).

The animals used in this research were kept under standard conditions and fed with water and food ad libitum. The experimental procedures were done in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Academy Press, which was accepted by the ethic committee of the AUSR in Iran (Washington, D.C. 1996).

EAE was induced in 40 rats, randomly placed in 4 groups of 10:
- Group 1: Named E-S, received normal saline (0.2 ml) everyday.
- Group 2: Named E-B12, received 15 mg/kg vitamin B12 (Sigma-Aldrich) every other day.
- Group 3: Named E-BV, received 0.5 mg/kg honey bee venom everyday.
- Group 4: Named E-BBV, received 0.5 mg/kg honey bee venom everyday and 15 mg/kg of vitamin B12 every other day. The treatments started from the first day post immunization by GPSH-CFA and lasted till the tenth day.

The day of GPSH-CFA injection was considered as the zero day post immunization (dpi). Rats were evaluated daily for the purpose of any unwanted symptoms and weight changes. Then they were scored daily by the following degrees:
- Score zero: normal and without symptoms, score one: tail without natural stretch, score two: paralysis of the tail, score three: partial paralysis in hind legs, score four: complete paralysis of hind legs, score five: tetraplegia, score six: death.

Rats were anesthetized after injection of the combination of ketamine and xylazine (Alfasan, Holand). Then, their brain and spinal cords were removed. They were kept under the process of histotechnique for 24 hours in 10% formalin as a fixative. The sections of brain and spinal cord were then stained with hematoxylin and eosin (H & E) for inflammatory cell infiltration and luxol fast blue (LFB) for demyelination analyses.

The intensity of inflammatory cell infiltration was assessed according to the protocol of Okuda et al., (1999) and classified according to the obtained scores as follows; score zero: the absence of inflammation, score one: the penetration of cells around blood vessels and meninges, score two: subtle penetration of cells in parenchyma (1- 10/section), score three: average penetration of inflammatory cells in parenchyma (1-100/section; Okuda et al., 1999).

The rate of serum TNF-α was specified by using rat TNF-α ELISA KIT (Abcam, UK). The rate of serum nitrite and nitrate was specified by the method of high performance liquid chromatography (HPLC) according to Xia et al., 2003.

Five micrometer sections of brain, after deparaffinization and rehydration, were transmitted to 850 W microwaves for antigen retrieval. The slices were placed in Tris-EDTA. In the microwave, the temperature reached to high temperature. Then, it decreased up to 40 °C and was kept for 15 minutes. The slices were washed with cold water and Tris-balanced salt (TBS). For aborting the endogenous peroxide activity, 1% H2O2 was used as a blocker. In the next step, the sections were incubated with rabbit anti-rat GFAP antibody (a marker of astrocytes, Abcam, UK, 1/500 dilution) for 24 hours at 4 °C. The sections were washed in TBS and incubated with HRP anti-rabbit IgG antibody (Abcam, UK) for 30 minutes in 37 °C. Diaminobenzidin (DAB) technique was used for visualization of the samples. All the above
procedures, without using primary antibody, were carried out for the control experiment. The number of GFAP+ cells in each section was counted by a person who was not aware of the treatment groups and the mean number was calculated randomly in five high power fields (× 400) (Wang et al., 2009).

Data were analyzed using the SPSS statistical program (version 17 for Windows). In all of the cases for comparison between different groups, Mann-Whitney U test was used. Significance level was set at p<0.05 throughout the experiment.

3. RESULTS

Following the immunization of the rats with GPSCH-CFA, some of them in different groups from 9 dpi showed the signs of decreased search activities, nutrition behavior and weight loss. On 11 dpi, the signs like tail stretch loss, partial paralysis of tail in the groups E-S and E-B12 were seen. These signs were seen in group E-BV on 12 dpi and in group E-BB with 13 dpi, and these signs increased day by day, which finally resulted in the complete paralysis of the hind legs. It should be noted that these signs were observed in great amounts within the group E-S (Figure 1). The average intensity of diseases in group E-BB and E-BV decreased considerably in comparison to the group E-S. Results show that the combination of honey bee venom and vitamin B12 can meaningfully decrease the clinical symptoms and effects of immunization of Lewis rats with GPSH-CFA.

![Figure 1: Combined effect of bee venom and vitamin B12 on the clinical score in EAE rats induced by GPSCH-CFA.](image)

The onset of clinical signs of EAE was seen at 11 dpi. After five days, the average of clinical scores reached the maximum and then it reduced. Combination of bee venom and vitamin B12 caused a considerable reduction in the maximum rate of the average clinical scores as compared to the group receiving normal saline.

Following the staining sections and analyzing the samples with microscope, no penetration of inflammatory cells to brain parenchyma and spinal cord was observed in the control group tissue samples. While, in the samples containing the signs of penetration of mononuclear inflammatory cells in parenchyma, the existence of inflammatory cells around the blood vessels and meninges belonged to four-labeled groups. These signs were observed with varying intensity in the specified groups. The intensity of pathological changes and the penetration of inflammatory cells both in the brain tissue and spinal cord of group E-S was noticeable. The intensity of pathological changes in the groups of E-BB and E-BV showed a significant decrease. This decrease was seen both in brain parenchyma and spinal cord tissue of group E-B12 but it was non-significant (Figures 2 and 3). The findings of received scores in four labeled groups were in accordance with the results of clinical investigations. In this investigation, the combination of bee venom and vitamin B12 caused a decrease in penetration of inflammatory cells with observed pathological changes.
Along with the results of the inflammatory cell penetration, demyelination showed a decrease for treatment groups. While, demyelination was not observed in the control group but this process was considerably increased in group E-S. Demyelination in group E-BB showed a significant decrease as compared to the group E-S (Figure 4). These results have shown that the combination of bee venom and vitamin B12 can considerably decrease the demyelination process, which is caused by the administration of GPSCH-CFA vaccine to the rats.
Figure 4: Decreased demyelination of the CNS in EAE rats, induced by GPSCH-CFA, after treatment with the combination of bee venom and vitamin B12. Where, A-D displays the slices of spinal cord and their demyelination process within different experimental groups: (A) E-S group, (B) E-B12 group, (C) E-BV group and (D) E-BB group (Scale bar A-D = 125 µm).

Immunohistochemical analysis of the effect of honey bee venom and vitamin B12 was evaluated for the astrocyte activity of rat brain tissue by the use of GFAP marker (a determinant of the astrocytes). The number of GFAP + cells in the group E-BB containing the brain stem had decreased significantly as compared to the group E-S. This reduction process, but not significant, was seen in E-BV and E-B12 groups (Figure 5). The results have shown that the combined treatment of bee venom and vitamin B12 causes a reduction in the number of GFAP + cells and finally results in the reduced activity of astrocytes and gliosis in rats, which were induced with GPSCH-CFA administration.
Figure 5: Decreased activity of astrocytes and gliosis in the brainstem of EAE rats, induced by GPSCH-CFA, after the combined administration of bee venom and vitamin B12. (A-E) Representative brain sections showing GFAP-positive cells from the brainstem of the EAE rats in different treatment groups: (A) E-S group, (B) E-B12 group, (C) E-BV group, (D) E-BB group and (E) The number of GFAP+ cells of brainstem sections in random 5 high power fields (×400) (*p<0.05) as compared to E-S group (Scale bar A-D = 62.5 µm).

The amount of TNF-α in the serum of rats was measured by ELISA method for different treatment groups. The amount of TNF-α had decreased in the treatment groups as compared to E-S and this reduction process in groups E-BB and E-BV was significant (Figure 6). These results have demonstrated that the combination of bee venom and vitamin B12 causes a decrease in the amount of serum TNF-α, which was considerably affected by GPSCH-CFA.

Figure 6: Reduction of the serum TNF-α and nitrate levels in the rats, induced by GPSCH-CFA, after treatment of brain cells with a combination of bee venom and vitamin B12. ELISA and HPLC were used for the determination of TNF-α nitrate. (A) The level of TNF-α in different groups as compared to E-S. (B) The level of nitrate in different groups as compared to E-S, where *p<0.05 in all of the treatment groups.
The amount of serum nitrite and nitrate was evaluated for the treatment groups with HPLC method in order to consider the antioxidant and anti-inflammatory effects of bee venom and vitamin B12. The amount of serum nitrate in group E-S had increased considerably as compared to the control group. While, this amount showed a significant decrease in the groups E-B12, E-BV and E-BB as compared to the E-S group (Figure 6). The serum nitrite was not detected in most of the samples as a large amount of it changed into the nitrate, so its evaluation was disregarded. The results have shown that the combination of bee venom and vitamin B12 can decrease the amount of serum nitrate, which were induced by GPSCH-CFA.

4. DISCUSSION

Multiple sclerosis (MS) is an autoimmune disease of CNS, which shows pathological characteristics like the penetration of macrophages and lymphocytes into the CNS, demyelination and axonic damage and etc. Etiology of this disease is unknown, but in this disorder the myelinated parts of NS are attacked by T and B cells (Chen et al., 2010).

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 (Han et al., 2007). 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 (main substrate of bee venom) is one of the most effective and well-known anti-inflammatory factors. Adolapin is another effective anti-inflammatory substance that suppresses the activity of cyclooxygenase (COX) enzyme (Son et al., 2007). It is reported that bee venom prevents the production of Interleukin -1ß (IL-1ß) from macrophages in rats in response to the inner stimulation by bacterial lipopolysaccharides (Kwon et al., 2002).

Primary allergic compounds of bee venom such as histamine and phospholipase A2 induce the production of Interleukin-10 (IL-10) by the T-helper 2 (Th2) cells, suppress T-cell proliferation and can be effective in the reduction of inflammation and demyelination (Jutel et al., 2007). Bee venom causes a reduced expression of matrix metalloproteinase 2 and 9 (MMP2 and MMP9) (Hamedani et al., 2005). MMP2 and MMP9 are related to MS disease and their amount increases in EAE condition (Dong et al., 2009).

The role of bee venom in increasing the interferon-ß (IFN-ß) level is a well understood phenomenon (Hamedani et al., 2005). According to the study carried out by Mastronardi et al., (2004), it was seen that the combination of IFN-ß and vitamin B12 led to a significant reduction of clinical and pathological conditions in EAE and non immune demyelinated models. (Mastronardi et al., 2004). The immune suppression and anti-inflammatory effects of bee venom have been reported in MS disease, rheumatoid arthritis and their laboratory models (Mirshafiey, 2007). The present study has also evaluated the results similar to those of the studies on the effects of bee venom on inflammatory autoimmune diseases and its anti-inflammatory and immune suppressing activities (Mirshafiey 2007; Son et al., 2007).

TNF-α and interferon-γ are the members of pro-inflammatory cytokines, which are mainly secreted by autoimmune T-cells, directly destruct blood-brain barrier and induce apoptosis of oligodendrocytes. These cytokines have also been considered as the demyelination factor (Akassoglou et al, 1998). Plasma level of TNF-α is related to the severity of EAE and MS, which explains the immune status (Polka et al., 2003).

In EAE rat model, the serum level of TNF-α increase in the beginning of disease, which shows that TNF-α has an important role in the distribution of disease (Schneider et al., 2009). During the present study, we have observed that TNF-α decreases in a group treated with the combination of bee venom and vitamin B12. Bee venom can prevent the production of pro-inflammatory cytokines like TNF-α (Nam et al., 2003). Previous studies support the importance of immune-regulatory effects of vitamin B12, which include the modulation of TNF-α activity (Miller et al., 2005).

The present study shows that the combination of bee venom and vitamin B12 decreases the rate of glial fibrillary acidic protein (GFAP), which is an astrocyte marker. Astrocyte activation occurs during injury, inflammation, disease, genetic disorder and the exposure to chemicals, which causes astrogliosis – a condition characterized by rapid synthesis of GFAP (Eng et al., 2000). Our results are in accordance with those of Mastronardi et al., (2004), which state that the combined administration of IFN-ß and vitamin B12 causes a decrease in GFAP in EAE models (Mastronardi et al., 2004).

Nitric oxide (NO) is one of the most important mediators of inflammation during the inflammatory disorders. It is produced from nitric oxide synthase (NOS). There are four members of the NOS family: neuronal NOS (nNOS), endothelial NOS (eNOS), inducible NOS (iNOS) and mitochondrial NOS (mtNOS). iNOS is expressed following the immunological or inflammatory stimulation in macrophages, astrocytes, microglial cells (Guix et al., 2005). NO is quickly metabolized into nitrite and nitrate. How to diagnose nitrite or nitrate in plasma or urine can be helpful in diagnosing the
inflammation process and the treatment of immune disorders (Moshage, 1997). Our investigations are in accordance with those of Han et al., (2007), who have proved that bee venom stops the production of NO in microglia, activated by lipopolysaccharides (Han et al., 2007). Bee venom acts as an anti-inflammatory agent through the prevention of NOS activity and COX production (Jang et al., 2005). Bee venom is a preventing factor as it results in the decrease of iNOS activity in Rat C6 glioma cells (Lee et al., 2009).

Cobalamin acts as an antioxidant, which can be because of its direct and indirect effects. These effects include: an increased methionine synthase activity, direct reaction with reactive oxygen and nitrogen species, protective effect of cobalamin for glutathione and the modification of signaling molecules in inducing the stress responses. Finally, the cobalamin protect the cells from oxidative stress and are used as antioxidants in pharmacology (Birech et al., 2009). It can be said that the combined administration of honey bee venom and vitamin B12, during the present study, has caused a significant reduction in the clinical symptoms of EAE rats, which can be linked directly to the antiproliferative effect of the honey bee venom. So, the present results are in line with those of the Mastronardi et al., (2004), who demonstrated the same results by the administration of vitamin B12 and interferon-β for the EAE disorder.

As a conclusion, the treatment of EAE with a combination of bee venom and vitamin B12 decreases the disease symptoms and pathological changes, level of serum TNF-α, gliosis and the NO production. This activity of a combination of bee venom and vitamin B12 may be caused by the anti-inflammatory effects of these substances and the immuno-modulatory and anti oxidant effects of vitamin B12 as well.

REFERENCES


