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Ameliorative Effect of Melittin Encoded DNA Plasmid in an Ovalbumin-induced Murine Model of Allergy

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ABSTRACT

Melittin is a natural toxin used in traditional medicine as an anti-inflammatory drug. It seems that the anti-inflammatory properties of melittin are caused by suppressing the expression of inflammatory genes and inhibiting signaling pathways. However, the use of melittin is limited due to instability, rapid degradation, and impurity. The aim of this study was to investigate the intranasal administration of a melittin-encoded plasmid as a new melittin delivery method for allergic diseases.

After the induction of a mouse model of allergic rhinitis, mice received intranasal melittin plasmid. After the final challenge with allergen and allergic symptom assessment, the required samples were collected, and transforming growth factor-beta (TGF-β), interferon-gamma (IFN-γ), and interleukin-4 (IL-4) cytokine levels, serum levels of ovalbumin-specific immunoglobulin (Ig) E and histopathological changes were assessed. In addition to investigating the immune response, the effect of melittin on the expression level of genes involved in apoptosis was also investigated.

The melittin plasmid significantly improved nasal symptoms and decreased eosinophil infiltration into the nasal mucosa. Moreover, melittin decreased the expression levels of IL-4 and TGF-β in nasal lavage fluid, while IFN-γ expression was increased. Regarding the expression level of genes involved in apoptosis, melittin led to an increase in *BAX* mRNA expression.

These results suggest intranasal administration of a plasmid encoding melittin can suppress nasal symptoms, eosinophil infiltration, and immunomodulation of the immune response, which can be considered a promising approach in the treatment of inflammatory diseases.

Keywords: Allergic rhinitis; Intranasal; Melittin; Plasmid

INTRODUCTION

Type I hypersensitivity is one of the most prevalent diseases caused by the immune response to allergens. The prevalence of allergic diseases is increasing

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worldwide, and an estimated 40% of people have specific immunoglobulin (Ig) E against environmental proteins.[1] Hypersensitivity reactions in allergic individuals are induced by exposure to allergens on mucosal surfaces and skin. Allergic reactions are associated with the induction of a Th2-based cellular immune response, which leads to the release of cytokines such as interleukin (IL)-5, IL-4, and IL-13 and the production of allergen-specific IgE.[2] Innate immunity also plays a vital role in the hypersensitivity reaction, such as the secretion of IL-33 and thymic stromal lymphopoietin by damaged epithelial cells and mast cells.[3,4] The activation of mast cells by allergen-specific IgE leads to the release of mediators such as histamine that cause clinical symptoms of hypersensitivity. Currently, the most effective way to deal with allergic diseases is to avoid contact with allergens. However, corticosteroids are used in the treatment of type 1 hypersensitivity, and the side effects of these drugs are a concern.[5,6] Bee venom is a poison secreted by honey bees (*Apis mellifera*) and has been used in traditional medicine for a long time.[7] Bee venom consists of various components, including melittin, adolapin, phospholipase A2, apamin, and mast cell degranulating peptide.[8,9] Bee venom has broad pharmacological effects, and many studies have investigated its anticancer,[10] antinociceptive,[11-13] antimutagenic,[14] radioprotective,[15] antibacterial,[16] antiviral,[17] and anti-inflammatory activity.[18,19] The effects of bee venom depend mainly on melittin, which is approximately 50% of the dry weight of bee venom.[19] Melittin is a cationic peptide with a linear structure consisting of 26 amino acids (NH2-GIGAVLKVLTTGLPALISWIKRKRQQ-CONH2).[20]
Previous studies have shown the anti-inflammatory effects of melittin in neurodegenerative diseases,[21,22] atherosclerosis,[23-25] liver inflammation,[26,27] arthritis,[28,29] and allergy.[5,18] Although the anti-inflammatory mechanism of melittin is not fully defined, inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) translocation to the nucleus seems to be the primary mechanism. However, the in vivo administration of melittin is limited due to instability, rapid degradation, impurity, and side effects such as skin damage and hemolysis. The difference in melittin dose and delivery method also affects the anti-inflammatory effects of melittin.[5] In this study, a new method of melittin delivery has been investigated to induce anti-inflammatory properties.

MATERIALS AND METHODS

Animals

Thirty female BALB/c mice (specific pathogen-free and 6 to 8 weeks old) were purchased from the Animal Care and Breeding Center of Shahrekord Azad University and kept in standard conditions. Animal handling and experimental procedures were approved by the Baqiyatallah University of Medical Sciences (BMSU) Animal Experiments Ethics Committee (IR.BMSU.AEC.1402.003).

Allergic Rhinitis Mouse Model and Melittin Plasmid Administration

The allergic rhinitis mouse model was induced in the sensitization and challenge phases, based on previous studies with slight changes.[30] In the sensitization phase, mice received 3 intraperitoneal injections of ovalbumin (OVA) (Sigma Aldrich, grade V) (25 µg) and adjuvant alum (2 mg) on days 0, 7, and 14. Seven days after the last intraperitoneal injection, the mice were intranasally challenged with 20 µL of OVA for 5 consecutive days (Figure 1). On days 25 and 32, mice in groups C and D received melittin plasmid or empty plasmid intranasally (20 µg per 20 µL of phosphate-buffered saline [PBS]). One week after the last plasmid administration, mice in groups A, B, and C were rechallenged with OVA. On day 42, the mice were sacrificed, and the required samples were collected (Figure 1). The mouse model induction was confirmed by examining the serum level of OVA-specific IgE and clinical manifestations after the challenge phase.[30]

Alum-adsorbed OVA Preparation

To prepare alum-adsorbed OVA, 1.89 g of aluminum potassium sulfate ($Kal[SO_4]_2 \cdot 12H_2O$) (Merck) was dissolved in 10 mL of distilled water and adjusted with NaOH to a pH of 6.5. After incubation at room temperature and centrifugation, the precipitate was dissolved in 5 mL of distilled water; every 10.5 µL of this solution contained 2 mg of alum. Next, 10.5 µL of the prepared solution and 20 µg of OVA were mixed and incubated for 15 minutes at room temperature and centrifuged to form an alum-adsorbed OVA precipitate. The precipitate was dissolved in 100 µL of cold PBS for each intraperitoneal injection. This step was done immediately before injection due to the solution's instability.[30]

Plasmid Construction

The recombinant pcDNA3.1+ plasmid encoding melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) was synthesized by Generay Biotech Company (Generay Biotech, China). Sanger sequencing method and enzymatic digestion (*BamH*I/*EcoR*V) were performed to confirm the construction (Figure 2). In order to multiply the plasmid, pcDNA3.1+ encoding melittin and empty pcDNA3.1+ were transformed into *Escherichia coli Top 10F'* using heat shock and calcium chloride method. The transformed bacteria were cultured in LB-Ampicillin broth, and the amplified plasmid was isolated by the Maxi Prep plasmid extraction kit (Favorgen, Taiwan).

Assessment of Melittin mRNA Expression

The nasal mucosa of the mice was collected after sacrifice and total RNA was isolated using the RNX Plus RNA extraction kit (SinaColon, Iran, Co., Iran). DNA contamination was removed from the extracted RNA by DNase I (Yekta Tajhiz Azma, Iran). Then complementary DNA (cDNA) was synthesized using the first strand cDNA synthesis kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's protocol. The polymerase chain reaction (PCR) was performed using melittin-specific primers, and the expression of melittin mRNA in the nasal mucosa was investigated (Figure 2).

Evaluation of Allergic Symptoms

To investigate changes in allergic symptoms, mice were immediately placed in the observation cage after the last OVA challenge on day 41. Investigators blinded to the treatment counted scratching and sneezing movements for 10 minutes.

Histopathologic Assessment

After sacrificing the mice on day 42, we separated the mice's heads to investigate histopathological changes in the nasal mucosa. The upper jaw was fixed in 10% neutral buffered formalin. After decalcification (10% EDTA at 4°C for 10 days), we prepared 5-µm paraffin-embedded sections, performed H&E staining, and compared the number of eosinophils counted in 10 random nonoverlapping high-power fields (×400) in different groups.[31]

Enzyme-linked Immunosorbent Assay

Nasal lavage fluid (NALF) was collected[32] and the levels of IL-4, IFN-γ, and TGF-β in NALF were measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Karmania Pars Gene, Iran). To collect NALF, 1 mL of cold PBS was gently instilled from the trachea to the nasopharynx, and NALF was collected from the nostrils. After centrifugation (6000 rpm for 4 minutes), the supernatant was kept at −70°C to measure the level of cytokines. The serum level of OVA-specific IgE (sIgE) was also measured using the OVA-sIgE kit (East Biopharm, China).

Quantitative RT-PCR (qRT-PCR)

Total RNA from the nasal mucosa of mice was isolated using the RNX Plus RNA extraction kit (SinaColon, Iran), and then complementary DNA (cDNA) was synthesized using the first strand cDNA synthesis kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's protocol. The relative mRNA expression of *BAX* and *BCL2* in nasal mucosa was assessed by the PCR using a Rotor-Gene Real-Time PCR Machine (Qiagen AG, Germany). Target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative mRNA expression level. PCR was performed with the following primer pairs: *BAX* F: 5′-AGGTCTTTTTCCGAGTGGCAGC-3′, *BAX* R: 5′-GCGTCCCAAAGTAGGAGAGGAG-3′, *BCL2* F: 5′-GTGGATGACTGAGTACCT-3′, *BCL2* R: 5′-CCAGGAGAAATCAAACAGAG-3′, *GAPDH-F*: 5′-TCCCGTAGACAAAATGGTGAAGG-3′, *GAPDH-R*: 5′-ATGTTAGTGGGGTCTCGCTCCTG-3′.

Statistical Analysis

Statistical analysis of data was performed using IBM SPSS 22 software (SPSS Inc., Chicago, IL, USA). The comparison between the variables in different groups was done by the analysis of variance (ANOVA) test followed by Tukey's post hoc. All the data are displayed as mean ± standard error of the mean (SEM), and a *p* value<0.05 was considered significant.

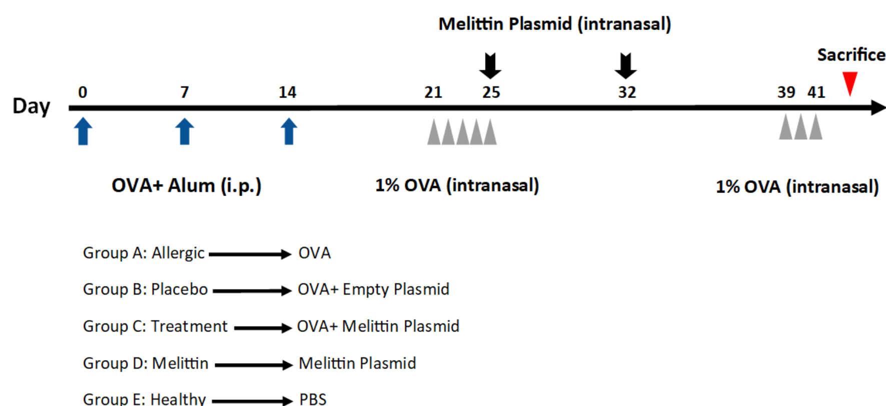

Figure 1. Schematic diagram of the experimental protocol. Thirty mice were randomly divided into 5 groups. In the sensitization phase, mice received 3 intraperitoneal injections of ovalbumin (OVA); then, the mice were intranasally challenged with 20 µL of OVA for 5 days. On days 25 and 32, mice in groups C and D received melittin plasmid or empty plasmid intranasally. The final challenge with OVA was also done on days 39 to 41. PBS: phosphate-buffered saline.

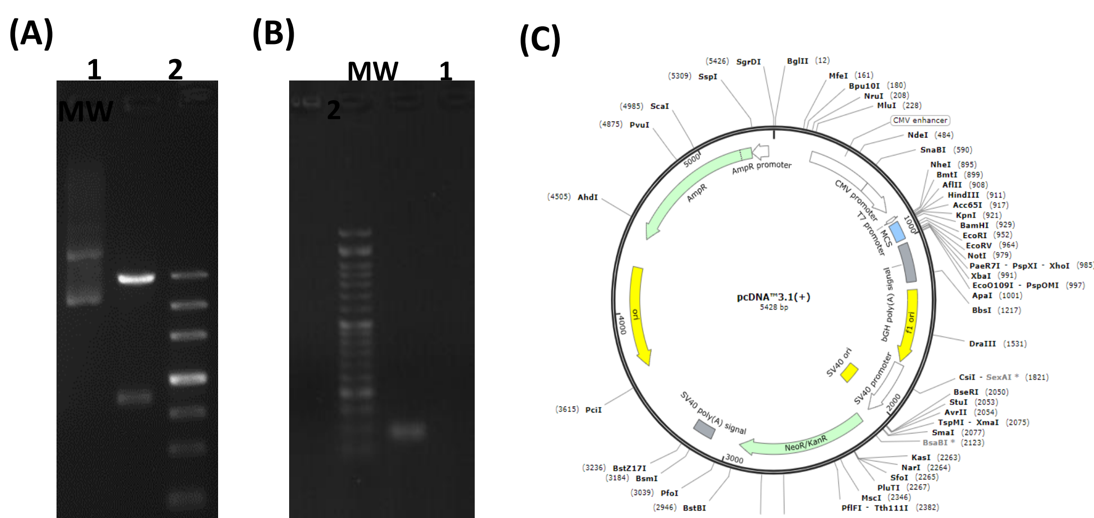

Figure 2. (A) Agarose gel electrophoresis. Lane 1, undigested pcDNA3.1+ Melittin plasmid. Lane 2, pcDNA3.1+ Melittin plasmid digested with BamHI/EcoRV restriction enzymes. Molecular weight (MW), DNA molecular weight marker III. (B) Melittin mRNA expression. Melittin mRNA was assessed in melittin (lane 1) and control (lane 2) groups. MW, DNA molecular weight marker (50 bp) (TYT company, Iran). (C) pcDNA3.1+ gene map (mammalian expression plasmid with the cytomegalovirus (CMV) promoter. The multiple cloning site is in the forward orientation [+]).

RESULTS

Intranasal Melittin Plasmid Improved Nasal Symptoms

To investigate the recombinant plasmid encoding melittin's effect on nasal symptoms, we counted the number of nose rubs and sneezes immediately after the last intranasal OVA administration. As shown in Figure 3, scratching and sneezing movements in the allergic group increased significantly compared with the healthy control group. Intranasal melittin plasmid administration significantly reduced nasal symptoms. Additionally, as shown in Figure 3, the nonallergic group receiving melittin (group E) did not show allergic symptoms.

Examining Histopathological Changes Showed a Decrease in Infiltrated Eosinophils into the Nasal Mucosa

We investigated histopathological changes and the number of infiltrated eosinophils in the nasal mucosa to explore the effect of intranasal administration of the recombinant melittin-encoded plasmid. As shown in Figure 4, the number of eosinophils of the nasal mucosa in the allergic and allergic groups receiving an empty plasmid (placebo) increased significantly compared with the healthy control group. In the allergic group treated with melittin, the number of eosinophils was significantly reduced compared with the allergic groups, indicating the antiallergic effect of melittin. Examination of other inflammatory cells in the nasal mucosa did not show significant changes in neutrophil or monocyte numbers in the tissue (Figure 4).

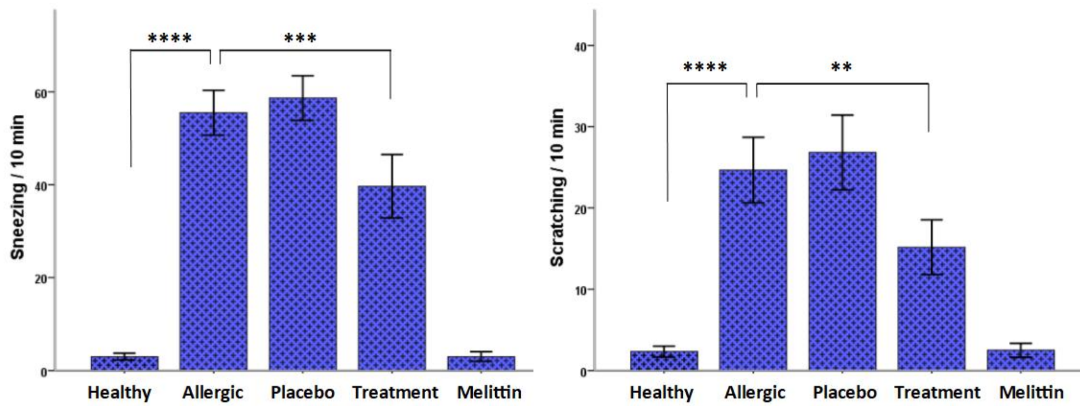

Figure 3. Effect of pcDNA3.1+ Melittin plasmid on sneezing and scratching movements. The data are shown as the mean ± SEM, **p<0.01; ***p<0.001; and ****p<0.0001, n=6 per group.

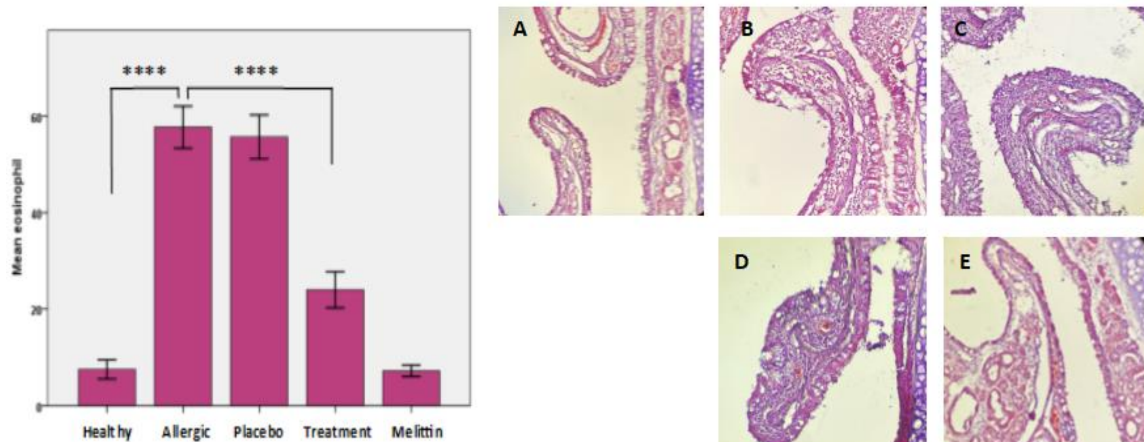

Figure 4. Histopathological analysis. The number of infiltrated eosinophils in the allergic mice group increased (p<0.0001) compared to the healthy control group and eosinophil infiltration in the melittin group was remarkably reduced (p<0.0001). The data are shown as the mean±SEM (**p<0.0001). The nasal cavity (nasal turbinate and maxillary turbinate) of the mice in different groups A to E (original magnification×200).**

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Intranasal Melittin Plasmid Did Not Affect the Serum Level of Anti-ovalbumin IgE

As expected, the serum level of anti-OVA IgE in the allergic and placebo groups significantly increased compared with the healthy control group, indicating the successful induction of the allergic rhinitis mouse model. As shown in Figure 5, melittin did not affect the serum level of OVA sIgE.

Melittin Modulates the Cytokine Response in NALF

To investigate the effect of the melittin-encoded plasmid on the cellular immune response, we measured the level of NALF cytokines. As shown in Figure 6, intranasal melittin plasmid administration in nonallergic mice (group E) led to a significant increase in TGF-β and IFN-γ and a nonsignificant decrease in IL-4 compared with the healthy control group, indicating that melittin administration promotes a Treg/Th1 cell-based response. In the treatment group, IL-4 significantly decreased, and TGF-β and IFN-γ significantly increased compared with the allergic and placebo groups. As expected, in the allergic group, IL-4 levels increased and IFN-γ levels decreased compared with the healthy control group.

The Effect of Melittin on *BAX* and *BCL2* Genes in Nasal Mucosa

As shown in Figure 7, we measured the relative expression levels of *BAX* and *BCL2* mRNA in mucosal tissue. The level of *BCL2* mRNA expression in the melittin (E) and treatment (D) groups decreased significantly compared with the healthy group. However, the *BCL2* expression level showed a nonsignificant decrease in the treatment group compared with the allergic group. In the melittin group (group E), the *BAX* mRNA expression level significantly increased compared with the healthy control group. Also, the *BAX* mRNA expression level significantly increased in the treatment group compared with the allergic group.

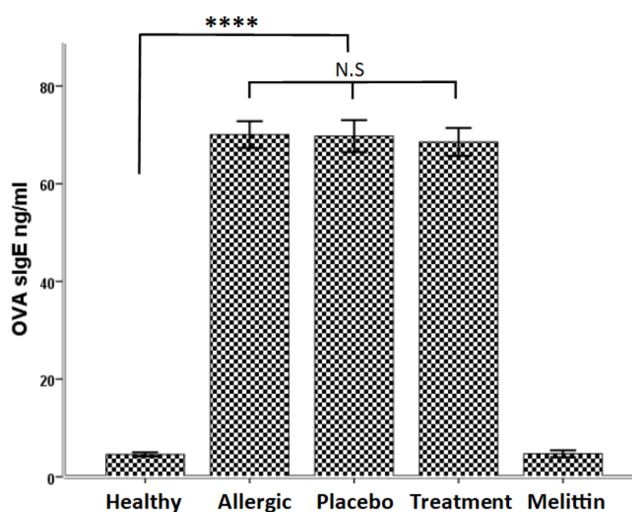

Figure 5. Levels of serum OVA-specific IgE. Levels of OVA-specific IgE after the last OVA intranasal challenge. The data are shown as the mean±SEM. (**p<0.0001)**

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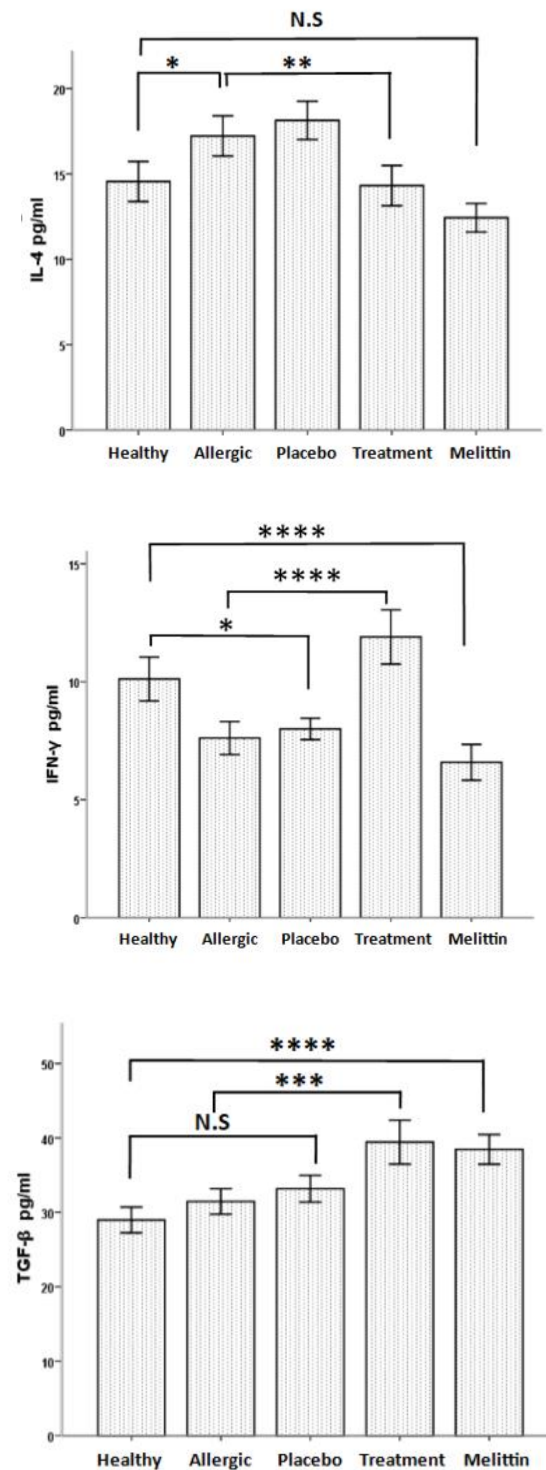

Figure 6. Modulation of cellular immune response in melittin-treated mice. The level of IL-4, IFN-γ, and TGF-β in nasal fluid lavage. Data are shown as mean ± SEM. (*$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$).

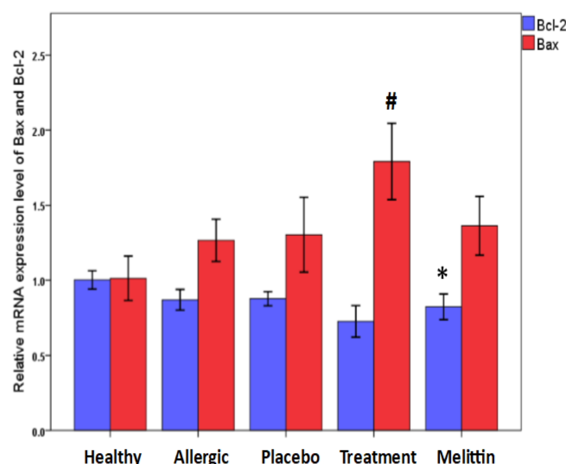

Figure 7. Effect of pcDNA3.1+ Melittin plasmid on *BAX* and *BCL2* mRNA expression level. The relative expression level of *Bax* was significantly increased in the treatment group compared to the allergic group (p=0.01), but the expression level of *BCL2* in the treatment group was not significantly different compared to the allergic group. In comparing the non-allergic group receiving melittin (group E) with the healthy control group (group A), the expression level of *BCL2* has decreased significantly (p=0.02).

DISCUSSION

As a natural peptide, melittin has several properties, including anti-inflammatory effects on allergic and autoimmune diseases.[19] It seems that the mechanism of melittin's anti-inflammatory action suppresses the signaling pathway and inhibits inflammatory gene expression. Melittin prevents NF-κB transfer to the nucleus and reduces p38, ERK1/2, and AKT activation. However, melittin's beneficial function in inflammatory diseases is not fully understood.[10,19,33,34] Several studies have investigated the antiallergic effects of melittin. Melittin improves atopic dermatitis by reducing the expression of chemokines and proinflammatory genes *CCL17* and *CCL22* chemokine and proinflammatory gene expression and inhibiting the STAT and NF-κB signaling pathways.[35] Bee venom has also been shown to inhibit histamine release. Melittin suppresses dendritic cell activation and maturation in the lymph nodes by limiting RelB activity. Additionally, it inhibits allergen-specific T-cell proliferation and activation and limits Th2-type cytokine release.[36] However, melittin use is limited due to its instability and rapid degradation.[37] In this study, we investigated a novel melittin delivery method to create anti-inflammatory effects. Today, DNA plasmids are an alternative method for allergen-specific immunotherapy and therapeutic peptide and protein delivery.[38,39] Compared with melittin peptide administration, DNA plasmids encoding melittin have advantages such as stability, cheap production and maintenance, high purity, and the absence of other allergens. However, intranasal naked plasmid administration is limited in inducing an optimal immune response in allergy immunotherapy.[38-40] In the present study, we aimed to transfer the melittin plasmid to the nasal mucosa to express the melittin peptide. It should be mentioned that many previous studies have mentioned the effect of melittin dose on its anti-inflammatory effects.[18,21,28,35] Plasmid DNA encoding melittin was successfully expressed in the nasal mucosa and showed antiallergic effects, although more studies are needed to select the optimal dose and investigate side effects. In addition to determining the optimal dose, another limitation of the study is the lack of comparison of melittin with common allergy drugs such as corticosteroids, which should be considered in future studies. To investigate plasmid DNA melittin's effect on the cellular immune system response, we examined the levels of IL-4, IFN-γ, and TGF-β cytokines in NALF. IL-4 plays a vital role in Th2 cell differentiation, induction of IgE production, VCAM expression, eosinophil migration, and mucus secretion.[41] The expression level of IL-4 increased in the allergic and placebo groups compared with the healthy group, characteristic of allergic inflammation. On the other hand, previous studies have shown that melittin decreases IL-4,[28,35,36,41] which agrees with this research. IFN-γ, an indicator cytokine of Th1 cell, increased in allergic mice receiving melittin

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compared with the allergic group. However, IFN-γ decreased in the nonallergic group receiving melittin compared with the healthy group. It is speculated that melittin reduces IFN-γ expression by suppressing signaling pathways.[28,41] However, increased IFN-γ expression in the treatment group may be influenced by other factors, such as reduced Th2 cell and IL-4 activity and melittin expression level.[41] TGF-β, an anti-inflammatory cytokine and an indicator of T-regulatory cell activity,[42] increased under the influence of melittin and may play an essential role in melittin's antiallergic properties. A study investigating melittin's effect on an allergic dermatitis model found that the Th2 cytokine response was suppressed. The Th1 cytokine response was also controlled and IFN-γ expression increased. This study's results in reducing allergic and inflammatory manifestations and modulating cytokine response agree with the present study.[36] Another study investigated melittin's anti-inflammatory effects in a mouse model of OVA-induced allergic dermatitis. In this study, mast cell infiltration and OVA-specific IgE decreased under the influence of melittin. Furthermore, the expression of inflammatory genes, especially interleukin-4, decreased, consistent with the results of our study. The decrease in OVA-specific antibody, which contrasts with our study, may be due to the difference in melittin's systemic delivery and the intraperitoneal injection of melittin in the mentioned study.[18]

In general, according to melittin's effect of melittin on the immune response in diseases such as cancer[43] and inflammatory diseases,[19] melittin seems to play an immunomodulatory role, and its effects depend on the characteristics of the inflammation, the delivery method and the dose administered. We measured OVA sIgE levels to investigate the effect of melittin plasmid on humoral immunity. Contrary to a previous study44 that showed melittin's decreasing effect of melittin on IgE in allergic diseases, the serum antibody level did not change in the present study, which may be due to the difference in the delivery system and IgE half-life. Plasmid melittin increased the Bax/Bcl2 ratio, probably indicating melittin overexpression in the nasal mucosa. One problem with intranasal administration of naked plasmid DNA in allergy immunotherapy is low transfection and lack of a proper immune response. In the present study, the melittin plasmid successfully transfected nasal mucosa cells, although more studies are needed to select the optimal melittin plasmid dose for optimal anti-inflammatory function. Although the Bax/Bcl2 ratio increased in this study, nasal symptoms and eosinophil infiltration improved. It is suggested that apoptotic cells also play a role in creating anti-inflammatory properties.[45-47] Macrophages that phagocytose apoptotic cells with TGF-β and IL-10 secretion can suppress inflammation and play an immunomodulatory role.[45-47] Here, the question arises: what effect does apoptosis have on inflammation in mucosal tissue? More studies should be directed to this issue. In general, intranasal administration of a plasmid encoding melittin can suppress nasal symptoms, eosinophil infiltration, and immunomodulation, which can be a promising method for treating inflammatory diseases.

STATEMENT OF ETHICS

The Animal Experiments Ethics Committee of the Baqiyatallah University of Medical Sciences (BMSU) approved this research (IR.BMSU.AEC.1402.003).

FUNDING

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

AI Assistance Disclosure

Not applicable.

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