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Evaluation of the efficacy of nicotine in treatment of allergic asthma in BALB/c mice

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ABSTRACT

Nicotine, an nAChR agonist, shows prominent anti-inflammatory properties, and some studies have illustrated its suppressive effects on inflammation. Here, we have examined whether nicotine as a medicine may have beneficial effects on the treatment of asthma in a mouse model of allergic asthma. BALB/c mice were sensitized with OVA and alum. Two weeks later, the mice received nicotine with concentrations of 1 and 10 mg/kg three times every other day. After 10 days, the mice were challenged with OVA (5%) using an ultrasonic nebulizer and died the next day. Our results showed that the administration of nicotine reduced lung-tissue inflammation, the number of eosinophils in bronchoalveolar fluid, allergen-specific IgE and IL-4 production, while it increased the TGF-β/IL-4 ratio and the number of Treg cells. Our results showed that nicotine applies its suppressive effects in a dose-dependent manner: administration of 10 mg/kg of nicotine showed more suppressive effects than 1 mg/kg. Such data suggested that nicotine might be a good candidate to be used as a medicine in the treatment of allergic asthma by decreasing allergic inflammation severity and potentiating Treg cells proliferation against the allergen.

1. Introduction

Asthma is a significant worldwide health challenge, which has prevalence and severity among all age groups and within all regions [1]. Asthma not only affects the patient's lifestyle, but also sometimes becomes a life-threatening illness [2].

Now, the main strategy for the treatment of asthma is to use a combination of inhaled drugs to make a rapid symptomatic relief of asthma exacerbation, to reduce bronchoconstriction, and to decrease airway inflammation. However, this therapy combination cannot result in a beneficial effect in 5–10% of the patients, while other patients have undesirable side effects or low adherence upon continued use [3–6]. Therefore, it seems that finding new drugs for the treatment of asthma is necessary.

The main characteristics of asthma, as a chronic inflammatory airway disease, are eosinophilic inflammation, mucus hyperproduction, and airway hyper-responsiveness [7,8]. Inflammation plays an important role in the pathophysiology of asthma and thus its tight regulation is a valuable step in its treatment [9]. Different pathways of asthma inflammation are mediated at multiple points by various cytokines including interleukin-4 (IL-4), IL-5, IL-13, IL-17, IL-25, IL-33, thymic stromal lymphopoietin [1]. Although asthma is more than just a Th2-type disease, Th2 cells are the most important players in the inflammation of asthma as they are the main producers of cytokines like IL-4, IL-5 and IL-13 whose tight linkage to the pathogenesis of asthma has been shown [8]. So, as expected, efforts have been made to decrease undesired Th2 immune responses to ameliorate asthma inflammation.

Regulatory T cells (Treg) play important roles in self-tolerance and in the regulation of inflammatory responses. The importance of Treg cells in many diseases, including asthma, has been investigated. It has

Abbreviations: nAChR, nicotinic acetylcholine receptor; OVA, Ovalbumin; Treg, Regulatory T cell; TGF-β, transforming growth factor beta; IL-4, interleukin 4; Th2, T helper 2; H & E method, hematoxylin and eosin method; BAL fluid, bronchoalveolar lavage fluid; FBS, fetal bovine serum; BSA, bovine serum albumin; PBST, PBS + 0.05% Tween 20; Nic10, the group who received nicotine in concentration of 10 mg/kg; Nic1, the group who received nicotine in concentration of 1 mg/kg

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been shown that there is a negative correlation between Treg population and severity of asthma. Thus, it may be desirable to increase the population of Treg cells in the treatment of asthma [10–13].

The cholinergic anti-inflammatory pathway is an interaction between the central nervous system and the immune system by which the CNS regulates uncontrolled inflammation caused by the immune system. The pathway uses the vagus nerve and its stimulation results in the inhibition of pro-inflammatory cytokines release and subsequent suppression of the inflammation. Two types of receptors have been described for the pathway: muscarinic and nicotinic receptors. Researchers have verified the existence of both types of receptors on cytokine-producing immune and non-immune cells [14,15]. Nicotine shows anti-inflammatory properties. Studies have shown the anti-inflammatory functions of nicotine mediated by nicotinic receptors (mainly by alpha 7 subunit) [16,17]. So, even though nicotine is the major addictive component of tobacco, its beneficial effects have been shown in various inflammation-related disorders [18,19].

In this study, we have examined the efficacy of nicotine as a treatment, in modulation of inflammation, reducing Th2 cytokines and allergen-specific IgE and increasing Treg cells population in a mouse model of allergic asthma.

2. Material and method

2.1. Animals

Six-to-eight-week-old healthy male BALB/c mice were used for the experiments. The mice purchased from Razi Vaccine and Serum Research Institute (Karaj, Iran) and were housed one week before the experiments with free access to food and water. All the experiments were conducted in accordance with the Animal Care and Use Protocol of Urmia University of Medical Sciences, Urmia, Iran.

2.2. Allergy induction, treatment, and challenge protocol

The mice were sensitized on days 0 and 7 using 10 μg of OVA (Grade V, Sigma, USA) and 100 μl of alum intraperitoneally. Different groups (5 mice per group) received different doses of nicotine (Santa Cruz Biotechnology, USA) on days 21, 23, and 25 subcutaneously. To find out which dose of nicotine may show appropriate results in allergic asthma treatment, we examined 3 different doses of nicotine (1, 10, and 32 mg/kg) but finally chose 1 and 10 mg/kg doses. A group received nicotine by concentration of 10 mg/kg in 100 μl sterile saline (Nic10). Another group was treated with 1 mg/kg of nicotine in 100 μl sterile saline (Nic1). The control group was injected only with 100 μl sterile saline. The groups received their treatment subcutaneously from day 21 to 25 every other day. The mice were challenged with OVA (5% in pyrogen-free saline) aerosols on days 35, 38 and 41 for 20 min using an ultrasonic nebulizer (Omron NE-U17, Japan). Twenty-four hours after the last challenge, the mice were killed to perform different experiments [20–22].

2.3. Pathologic evaluation of lung tissue

Twenty-four hours after the last challenge, the mice (5 mice per group) were anesthetized deeply using ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture [23]. Their tracheas were exposed by removing the tissues above the trachea and then punctured by a tracheal cannula carefully. The trachea and tracheal cannula were tied by a surgical suture. Blood samples were collected for allergen-specific IgE measurement and then formalin 10% in volume of 1 ml was injected into the lung through the tracheal cannula slowly. To ensure that the lung was filled with formalin, inflation of the lung was monitored. The tracheal cannula was pulled out and immediately the suture was tied more firmly in order to prevent formalin leakage. The lung was harvested and allowed to fix in 50 ml formalin 10% for at least 24 h. Then, it was embedded in paraffin and used for sectioning. The sections were stained with hematoxylin–eosin (H & E) method. Determination and analysis of the tissues inflammation and its severity were performed by a pathologist who was not aware of the groups. In addition, digital images were obtained from the lung sections after the analyzing. A semi-quantitative scoring system was used to evaluate and grade the lung inflammation, where +5 was considered as the highest inflammation degree (more than three cells deep) and accumulation of inflammatory cells around the majority of vessels and bronchioles and +1 reflected the lowest inflammation rate with a small number of inflammatory foci [24].

2.4. Bronchoalveolar lavage (BAL) fluid analyzing

The mice (5 mice per group) were killed on day 42; their tracheas were punctured using a tracheal cannula and fixed by a surgical suture. The airways of the mice were lavaged twice with 0.9 ml cold PBS containing 5% BSA (Merck, Germany) and transferred into 15-ml polystyrene tubes and kept on ice. The BALF thus obtained was centrifuged at 2000 RPM (4 °C) for 5 min and the cell pellet re-suspended in 100 μl of PBS and then spread on the surface of glass slides which were left to dry in laboratory temperature. After that, the slides were fixed in methanol for 5 min. Methanol-fixed slides were stained with Wright-Giemsa stain. Eosinophil counting was performed by considering 20 random fields per slide (at least 200 cells per slide) [25,26].

2.5. IL-4 and TGF-β measurement

Splenocytes were cultured to compare the cytokines production. The spleen was removed aseptically and single-cell suspensions were prepared in RPMI 1640 medium (Gibco, UK). Red blood cells were lysed osmotically using ammonium chloride buffer (0.9%). The cells were washed twice by RPMI 1640 and after the final washing, the cell pellet were re-suspended in RPMI enriched by 10% FBS (Gibco, UK). The viable cells were counted using trypan blue (0.4%, w/v). The cells by concentration of 1×10^5 per well were cultured in V-Bottom Microplates (96-well) using RPMI 1640 that were supplemented with 10% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin. The cells were stimulated with 40 μg/ml OVA and incubated at 37 °C in 5% carbon dioxide (CO_2). Seventy-two hours later, the plates were centrifuged (10 min, 2000 rpm, 4 °C) and the supernatant was collected and stored at −70 °C. The concentration of IL-4 and TGF-β in the supernatants was estimated using a commercial enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, USA) [27,28]. Briefly, test samples were diluted 2 folds and added to the wells that were precoated with anti-mouse IL-4 antibody. Then, biotin-conjugated anti-mouse IL-4 antibody were added to all wells and incubated in room temperature for 2 h. The wells were washed 3 times and then streptavidin-HRP was added to all wells and incubated for 1 h. Afterwards, the wells were washed 3 times and TMB substrate solution was added to the wells and then incubated for 10 min in a dark place. Stop solution used to terminate the reaction and followed by reading the absorbance with a microplate reader device. Almost, a similar protocol has been used for TGF-β assay except an additional preparation step for test samples and streptavidin-HRP and TMB substrate solution incubation sections that required more incubation time. According to the information presented by the manufacture, the sensitivity of ELISA kits was 2 pg/ml for IL-4 and 12 pg/ml for TGF-β. To draw a standard curve for IL-4 measurement, lyophilized standard powder was reconstituted with sample diluent solution and labeled as Standard 1 (Concentration = 250 pg/ml) and then it was diluted 2× with sample diluent solution and labeled as Standard 2. The procedure was repeated five more times to prepare other standard dilutions (Standards 1–7); somehow, the concentration of the last standard dilution was 3.9 pg/ml. All samples including standards, blank and tests have been prepared in duplicate. Absorbance of the samples was measured in 450 nm wavelengths and obtained ODs