Survey the Immuno-pharmacology Effect of Botulinum Toxin in Cell Signaling of Bronchial Smooth Muscle Cells of the Allergic Asthma

Chao Cheng
Suzhou University
Entezar Mehrabi Nasab
Tehran Heart Center
seyyed shamsadin athari (ss.athari@gmail.com)
Zanjan University

Research

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Abstract

Background

Asthma is the lung disease that influenced more than 350 million people in worldwide. ASM spasm leads to AHR and bronchial obstruction that are acute symptoms of asthma attack. BTX is bacteria toxin that acts as muscle relaxant and may have therapeutic effect on AHR and asthma. Therefore, the effect of BTX on the AHR and related gene expression was evaluated.

Methods

After producing of asthma mice model, which were treated with BTX in two ways; IN and N (0.01, 0.1, 1 and 10 U/ml). AHR was measured on day 24, 26, 28, 30 and gene expression of TrkA, TrkB, M1-M5, α7nAChR, TNF-α and ERK2 were evaluated. At least, in lung histopathology, perivascular and peribronchial inflammation, mucus production and goblet cell hyperplasia were studied.

Results

On day 24, treatment with BTX for all dosages had no significant effect on AHR but, on day 26 and 28, AHR was decreased and it was continued on day 30 for all treated groups. Treatment with BTX had no significant effect on the expressions of TrkA, TrkB, M1, M2, M3, M4, M5, α7nAChR, TNF-α and ERK2 genes, perivascular inflammation, peribronchial inflammation, hyperplasia of the goblet cell and production of mucus. Also, the mice have been received BTX 10 mg/ml, were died.

Conclusions

BTX therapy controlled asthma attacks via decreasing of AHR and induction of relaxation in the ASMs. But, it has no significant effect on inflammation and mucus production. In using of BTX, attention to the safe dose and prevention of dangerous side effects are necessary.

Introduction

Asthma is one of the main diseases of lung and influenced more than 350 million people in worldwide, which leads to huge number of people die especially in developed countries. Spasm of smooth muscle cells and airway inflammation are the main problems in asthma and the main symptoms of this disease include wheezing, shortness of breath, cough and chest tightness. Airway inflammation leads to remodeling of bronchi in long time but airway smooth muscle (ASM) spasm leads to airway hyperresponsiveness (AHR) and bronchial obstruction that are acute symptoms of asthma attack. Asthma is controllable and new treatments are more effective than ever before at preventing asthma attacks or stopping symptoms. There are two groups of anti-asthma drugs include short acting and long acting drugs. The short acting drugs have quick effect and mainly inhibit ASM spasm and airway obstruction. The long acting drugs have continues effect and mainly inhibit airway inflammation and obstruction (1–3).

AHR is the hallmark feature of asthma and with ASM contraction leads to excessive airway narrowing of in response to stimulators and asthma attack symptoms (cough, wheezing and breathlessness) are beginning. However, severe acute asthma remains difficult to control and, there is an urgent need to accelerate a novel therapeutic approach to control and treat severe acute asthma and main goal of this achievement is AHR inhibition (4, 5). AHR as an asthma hallmark, is important to recognize the severity of asthma. The seasonal allergen exposure can alter the severity of AHR. In addition, anti-asthma therapy deeply improves AHR. The exaggerated airway narrowing in asthma brings attention to the role of ASM in the manifestation of AHR. Bronchoconstriction at least in part, is due to constriction of the ASM surrounding the airway. So, increased contractility of the ASM has been touted as a AHR principal cause (6, 7).

the botulinum toxin (BTX) as bacteria exotoxin is produced by Clostridium botulinum and is a valuable therapeutic tool for the treatment of smooth muscles overactive, and hypersecretory. BTX has a heavy and light chain linked by a disulphide bond and after accesses to the target tissue, the heavy chain binds to glycoprotein structures on cholinergic nerve terminals, and then is internalized. The light chain binds to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein complex with high specificity, after which it proteolytically cleaves specific target proteins, prevents the acetylcholine vesicle docking on the inner surface of the presynaptic membrane, paresis skeletal or smooth muscle (8). Therefore, updating with new generation of therapies and certain treatments are necessary. There are some bio-toxins such as BTX can be used as muscle paralyzing and relaxant and may be a therapeutic agent in some problems such as asthma. Therefore, in this study, the effect of BTX on the AHR and ASM relaxing and also, related gene expression was evaluated.

Material And Methods

Modeling and Treatment schedule

Male BALB/c mice (n = 100) were acclimatized under the standard laboratory conditions. The mice were allocated in 10 groups and in 9 groups, allergic asthma was induced by ovalbumin (OVA) according to Fig. 1. Briefly, for sensitization, the mice were injected by intraperitoneal of OVA with alum and for challenging, were inhaled by OVA aerosols for 30 min/day. The tenth group was sensitized and challenged by PBS. Eight of the nine OVA received groups were treated with BTX. BTX was administered in two ways; it was diluted in normal saline and administered intra-nasally and aerosolized as a nebulized solution [0.01, 0.1, 1 and 10 U/ml via intra-nasal (IN) and nebulizing (N) form]. On day 24, 26, 28 and 30, AHR measurements were done and on day 31, lung tissue sampling was done.

MCh challenge test for AHR measurement

To the AHR determination, methacholine (MCh) challenge test was used by determining enhanced pause (Penh value). After anesthetizing, the mice were tracheotomized, then exposed to aerosolized methacholine with a series of doubling concentrations (0, 1, 2, 4, 8, 16 and 32 mg/ml).

Real time-PCR

In the lung cells, total RNA was extracted and reverse transcribed to cDNA. Quantitative real time-PCR was done for the expression of target genes. GAPDH was used as internal control gene. The used primers sequences were (5'-3'); GAPDH F: TGTTCCTACCCCCAATGTGT, R: GGTCCTCAGTGTAGCCCAAG; TrkA F: TCCTTCTCGCCAGTGGACGGTAA, R: AGTGCCTTGACAGCCACGAGCAT; TrkB F: TGACGCAGTCGCAGATGCTG, R: TTTCCTGTACATGATGCTCTCTGG; M1 F: TCCCTCACATCCTCCGAAGGTG, R: CTTTCTTGGTGGGCCTCTTGACTG; M2 F: CTGGAGCACAACAAGATCCAGAAT, R: CCCCCTGAACGCAGTTTTCAGT; M3 F: GCAAGACCTCTGACACCAACT, R: AGCAAACCTCTTAGCCAGCG; M4 F: CGGCTACTGGCTCTGCTACGTCAA, R: CTGTGCCGATGTTCCGATACTGG; M5 F: TAGCATGGCTGGTCTCCTTCA, R: CGCTTCCCGACCAAGTACTG; α7nAChR F: GTCGTGTGTGGTCGTTTG3', R: ATCACCTCACTCTCATCCTG; TNF-α F: GCCTCTTCTCATTCCTGCTTG, R: CTGATGAGAGGGAGGCCATT; ERK2 F: GGAGCAGTATTATGACCCAAGTGA, R: TCGTCCACTCCATGTCAAACT.

Histopathology

lung tissues (on the day 31) were isolated and after producing slide sections, were stained with H&E, PAS and AB-PAS. Then slides were evaluated for perivascular and peribronchial eosinophilic inflammation, mucus production and goblet cell hyperplasia.

Statistical analysis

The result was shown as means ± SD. The SPSS (ver. 20) was used and the paired t-test was used to analyze the differences between treated and non-treated groups. *the p value* less than 0.05 was supposed to be significant. The graphs were performed by GraphPad Prism (ver. 6).

Result

Survival

All mice of the two asthma groups that were received BTX 10 mg/ml via IN and N form, when administrated with the first dose, were died. On day 24, IN 1 group (received 1 mg/ml via IN) and on day 29, N 1(received 1 mg/ml via N) group were died.

AHR

The penh value was significantly ($p < 0.05$) increased in the non-treated asthma group compared with PBS group for all MCh concentrations. Treatment with BTX for all dosages via IN and N had no significant effect ($p > 0.05$) on day 24 for all concentrations of methacholine (in the 32 mg/ml of MCh on day 24: OVA: 15.5 ± 1.5, IN 0.01: 13.5 ± 1, IN 0.1: 12.5 ± 1.3, IN 1: 12.5 ± 1.5, N 0.01: 13.5 ± 1.2, N 0.1: 12 ± 1.3, N 1: 11.7 ± 2) (Fig. 2). All treated groups showed significant ($P < 0.05$) decreasing of AHR for concentrations of MCh on day 26 and 28 (Fig. 2). AHR decreasing was continued on day 30 for all treated

groups and has significant (P < 0.05) decreasing compared to non-treat group for concentrations of MCh (in the 32 mg/ml of MCh on day 30: OVA: 15.5 ± 0.5, IN 0.01: 5.3 ± 0.4, IN 0.1: 5 ± 0.5, N 0.01: 2.7 ± 0.1, N 0.1: 2.5 ± 0.2) (Fig. 2).

Real-time PCR

The gene expression of TrkA, TrkB, M1, M2, M3, M4, M5, α7nAChR, TNF-α and ERK2 were significantly (p < 0.05) increased in asthma group compared to the PBS challenged group. Treatment with BTX with several doses via IN and N had no significant effect (p > 0.05) on the expressions of these genes compared to the non-treated asthma group (Fig. 3).

Histopathology

In the no treated asthma group, eosinophilic inflammation in around of vascular (3.2 ± 0.4) and bronchial (3.7 ± 0.2), production of mucus (3.3 ± 0.3) and the goblet cell hyperplasia (3.4 ± 0.5) were significantly (p < 0.05) increased compared to PBS group (0.5 ± 0.2, 0.5 ± 0.1, 0.6 ± 0.1 and 0.5 ± 0.2 respectively). Treatment with BTX with 0.01, 0.1 and 1 mg/ml via IN and also N had no significant (p > 0.05) effect on the perivascular and peribronchial inflammation, hyperplasia of the goblet cell and production of mucus compared to non-treated asthma group (Fig. 4).

Discussion

Asthma attack is developed by two essential factors, inflammation and bronchoconstriction, that is mediated by smooth muscle contraction. Inflammation is beginning slowly in airway but, quick attack is initiated by contraction of the ASM and inhibition of the bronchoconstriction help to decrease morbidity and mortality from the asthma attack. Initiated bronchoconstriction from ASM contraction in asthma attack reduced air flow and decreasing responsiveness of the ASM in airways will attenuate the bronchoconstrictor response in asthma and can be achieved effective pharmacotherapy (3, 8). In asthma, ASMs are key of bronchoconstriction, which have muscarinic receptors while nicotinic acetylcholine receptors (nAChRs) are only on airway neurons. It was showed that α7 subunit of nAChR is expressed in ASM cells in asthma and by exposure to pro-inflammatory cytokines TNFα and IL-13. Classically, nicotine acts via nAChRs. In ASMs, contractility involves muscarinic receptors. Allergy in asthma increases ASMs Ca^{2+} and response to bronchoconstrictor agonists [such as acetylcholine (Ach)]. Effect of ACh on ASM is usually associated with muscarinic receptors, while nAChR is generally considered to acts as Ca^{2+} channels in pre-ganglionic neurons (9–11). On the other hand, TNF-α and IL-13 attribute to the increased CD38-mediated calcium release (12). Also, TNF-a induced muscarinic receptor density, increases the activity, as well as the amount, of G-proteins in ASM, and may also modulate b2-adrenergic function (13). On the day 24, Treatment with BTX for all dosages via IN and N had no significant effect on AHR, but on day 26 and 28, could significantly decrease AHR for concentrations of MCh. AHR decreasing was continued on day 30 for all treated groups and has significant decreasing compared to non-treat group

for concentrations of MCh. Also, decreasing of AHR on day 30, had significant difference compared to BTX-treat groups on day 26 and 28.

In normal airways, ASMs regulate the airway caliber, bronchomotor tone and assist to the mucus and in airways, β-2-adrenoceptor agonists or muscarinic antagonists can modulate the contractile function of the ASM (13). AHR as the airways predisposition to narrow excessively in response to stimuli, is considered an asthma cardinal feature. The AHR presence is associated with lung function decline and increases risk for the asthma development and persistence of wheeze. However, increased contractility of the ASM is a principal cause of AHR and the β2-adrenergic receptor genotype influences AHR. On the other hand, in asthma, ASM contraction is formed by actin-myosin cross-bridges and the activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). Hypertrophy of the constructed ASM and changes in extracellular matrix composition lead to airway remodeling and therefore, AHR has correlation with airway wall thickening and bronchoconstriction (14–16). Prevention of bronchoconstriction potentially reduces the asthma attacks severity and BTX allows chemical denervation transient of the ASM. BTX acts to weaken muscle by preventing the acetylcholine vesicle docking of the presynaptic membrane (on the inner surface), which causes chemical denervation and paresis of smooth or skeletal muscle. Duration of BTX effect was estimated at 3–5 months for skeletal muscle. Use of BTX to treat asthma would be worth exploring. BTX can be administrated to treat asthma with three ways: one, intranasally, trans-tracheally or intratracheally via bronchoscopy, two, injection to the smooth muscle and three, aerosolized as a nebulized solution (8). After the first dose of BTX, AHR (on day 24) had no significant changes, because 1 day passed and in the measurement time, BTX had not effected to the ASMs, therefore, AHR was not significantly decreased. On the day 30, the most doses had effect on the AMSc, and therefore, the best effect of BTX on AHR was observed on day 30. In all treated groups, when BTX`s doses were increased, the AHR was decreased more than lower dose.

Vocal Cord Dysfunction is a respiratory problem in which vocal cord restricts airflow by closing during inspiration that leads to coughing, shortness of breath, wheezing and chest tightness. This problem is often misdiagnosed as asthma and treated as such. It was shown that steroids used to asthma therapy are not beneficial in the treatment of Vocal Cord Dysfunction, and are therefore unnecessary. It is suggested that BTX can relax the thyroarythenoid muscles surrounding vocal cords resulting in improves airflow (17, 18). BTX is a neurotoxin that has a heavy and a light chain. The heavy chain binds to specific neuronal ecto-acceptor, the light chain cleaves SNAP-25, inhibits synaptic exocytosis, disables neural transmission and blocks the acetylcholine releasing (19). BTX has therapeutic effect on allergic rhinitis symptoms and is useful in patients whose symptoms are non-control with standard treatment. The BTX has the greatest change in rhinorrhea compared with other symptoms. The BTX effects on the nasal cavity occurs through; acetylcholine release inhibition from the cholinergic nerve in the nasal mucosa, from preganglionic cholinergic nerve in the sphenopalatine ganglion, and apoptosis induction in the nasal gland. It acts by cleaving the synaptosomal-associated protein with a molecular weight of 25 kD (SNAP-25) and after damages SNAP-5, regulates exocytosis (20, 21). We administrated BTX via intra-nasal and nebulizing form. It was observed that in the same dose of BTX with different routs of

administration, when BTX was used via nebulizing form, had strong effect on control of AHR compared to intra-nasal using form for all concentration.

Mild dose of the purified BTX attenuates chronic pneumonia, dyspnea, cough, acute respiratory failure, and neurological deficits that were recognized as the clinical symptoms of COVID-19. Therapeutic BTX improves oxygen supply thereby improving the survival rate, which can also be considered as a potent treatment for COVID-19 patients (22). In our study, all asthmatic mice that were received the first dose of BTX (10 mg/ml) via IN and N, and some groups after receiving 1 mg/ml were died. It may be happen for the effect of BTX on respiratory system and breathing related muscles that leads to paralysis and relaxation of muscles. Therefore, using of BTX for treatment of asthma and controlling of AHR, should be used carefully and the safe dose of BTX should be determined. It is a strong treatment to control of AHR and asthma attack, but can lead to respiratory paresis and death of asthmatic patients.

The nAChRs are muscle and neuronal types (23, 24) and the ERK as effector kinase is involved signaling pathway of the multiple essential cell processes such as survival, proliferation and differentiation (25, 26). TrkA is a receptor tyrosine kinase, and activated by Nerve growth factor (NGF) and auto-phosphorylation. Since TrkA receptor is expressed on the ASMs, it may be importance in asthma. However, NGF expression is increased in various allergic diseases, and TrkA receptor activation by NGF causes its internalization mediated by clathrin-dependent pathways, followed by lysosomal degradation. TrkA activation in the ASMs leads to increased TrkA expression and increased pathophysiological consequences. Therefore the TrkA receptor as an interesting target in the development of new therapeutic approaches for allergic asthma (27). Treatment with BTX via IN and N in asthma mice had no significant effect on the expressions of TrkA, TrkB, M1, M2, M3, M4, M5, α7nAChR, TNF-α and genes, perivascular inflammation, peribronchial inflammation, hyperplasia of the goblet cell and production of mucus compared to the non-treated asthma group.

Conclusions

BTX therapy for treatment of asthma is applicable and could control asthma attacks via decreasing of AHR and induction of relaxation in the ASMs. It could be used via nebulizing to have local effect and relaxes AMSs, but it has no significant effect on inflammation and mucus production. Also, in using of BTX, attention to the safe dose and prevention of dangerous side effects are necessary. At least, long term using of BTX may lead to producing of anti-BTX Antibodies and clinical resistance that should be note.

Declarations

Ethical Approval

All methods and protocols of this study were approved by the ethic committee of animal house of ix.med.vet.dep, 2021 (No. IX.MED.VET.DEP.REC.2021.3200002.2).

Availability of data and material

N/A

Competing interests

There is no competing interest.

Funding

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Informed consent

N/A

Authors' contributions

CC, EMN, SSA, participated in the study, design, evaluation, result analysis. CC and SSA supervised the study. All authors approved the submitted version.

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Figures

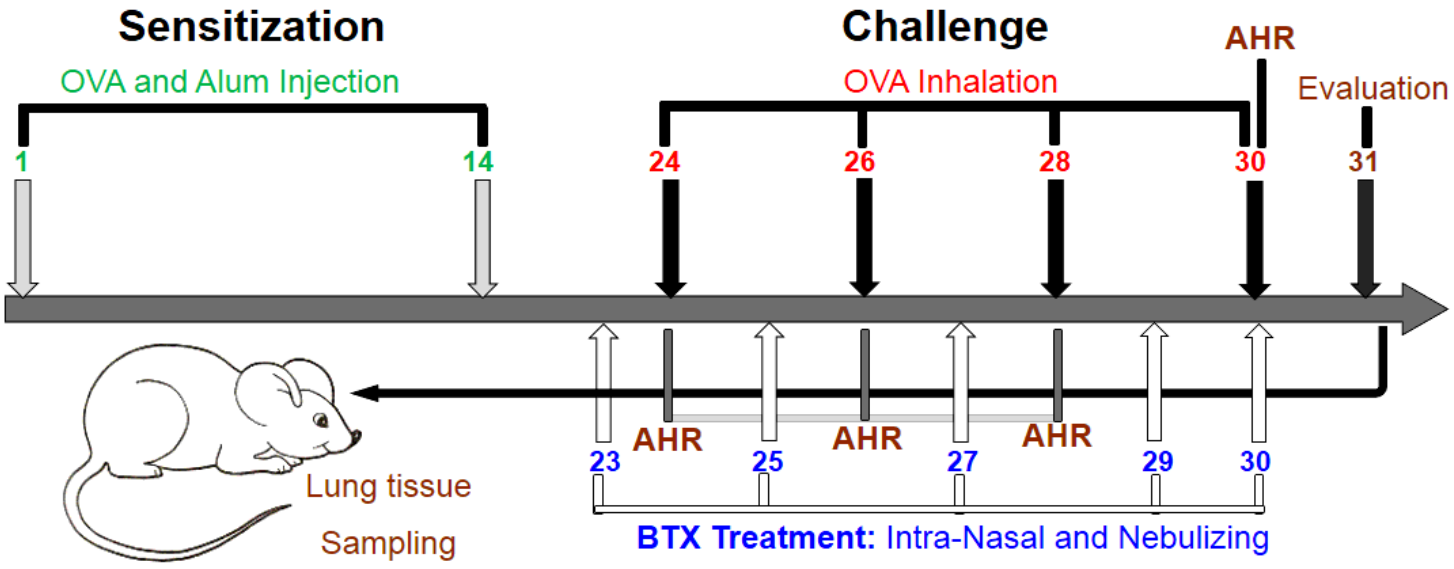

Figure 1

For animal asthma model producing, BALB/c mice were sensitized by intraperitoneal injection of OVA with alum and then challenged by OVA aerosols inhalation for 30 min/day. On days 23, 25, 27, 29 and 30, the mice were treated with BTX in N and IN form.

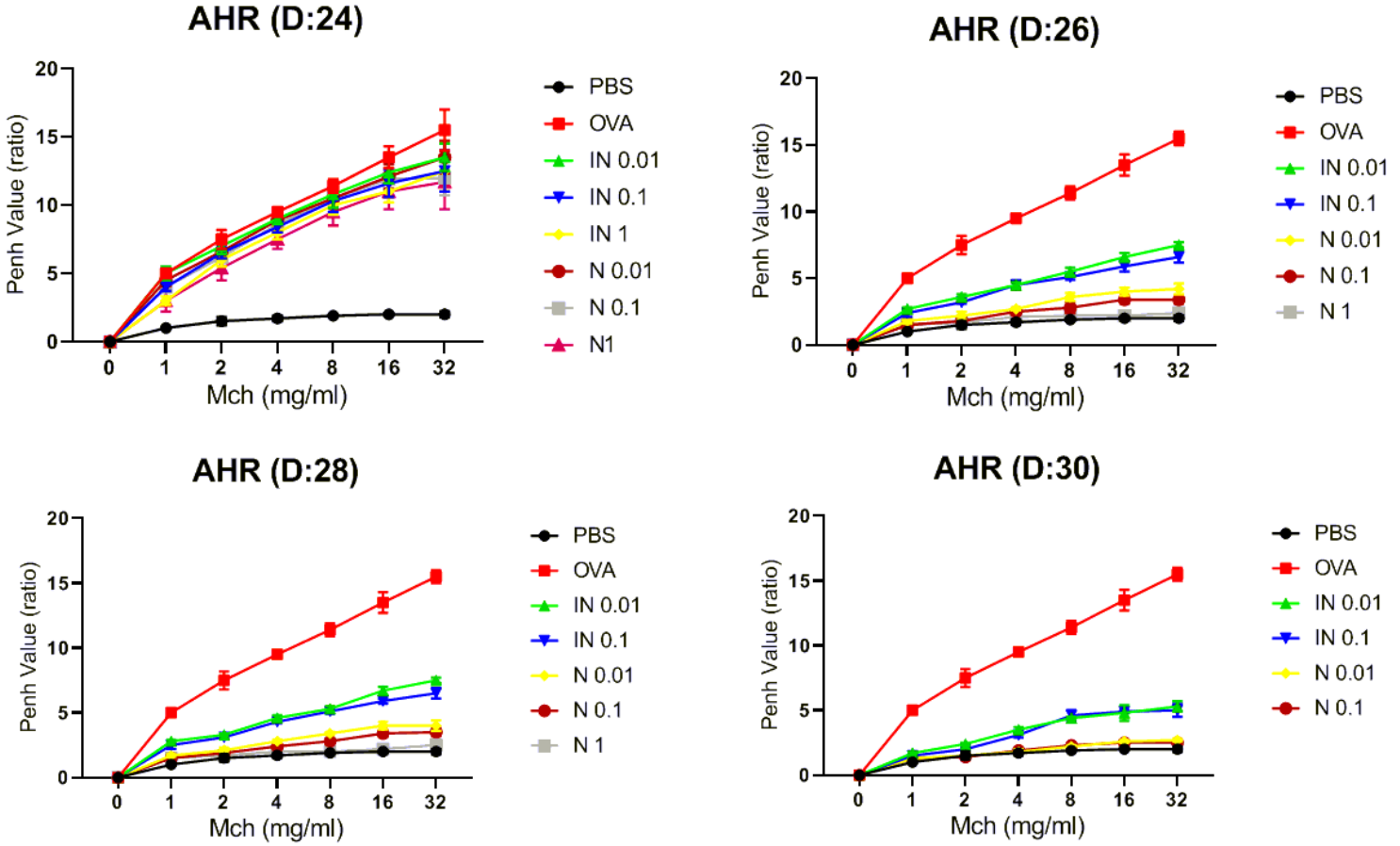

Figure 2

To determination of the AHR, MCh challenge test was done by determining Penh value on days 24, 26, 28 and 30. The mice were tracheotomized, then exposed to methacholine with a series of doubling concentrations (0, 1, 2, 4, 8, 16 and 32 mg/ml).

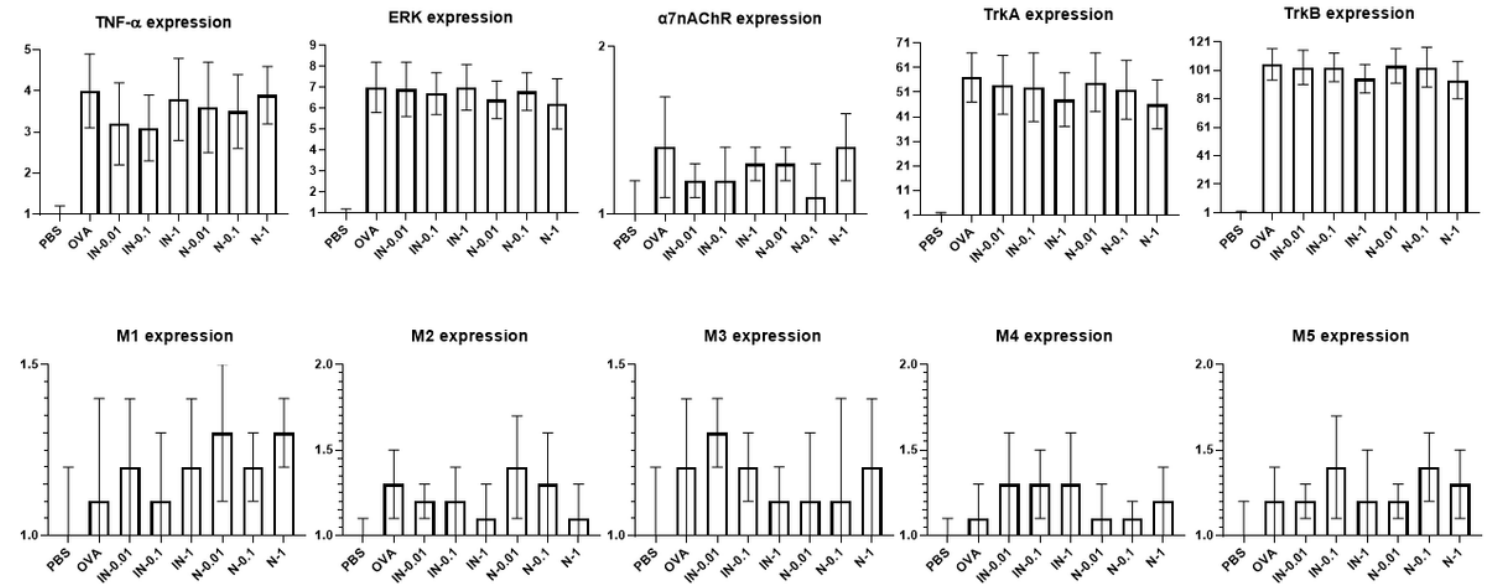

Figure 3

To determination of gene expression, after extraction of the total RNA, the cDNA was synthesized and quantitative real time-PCR was done for the expression of TrkA, TrkB, M1-M5, α7nAChR, TNF-α and ERK2.

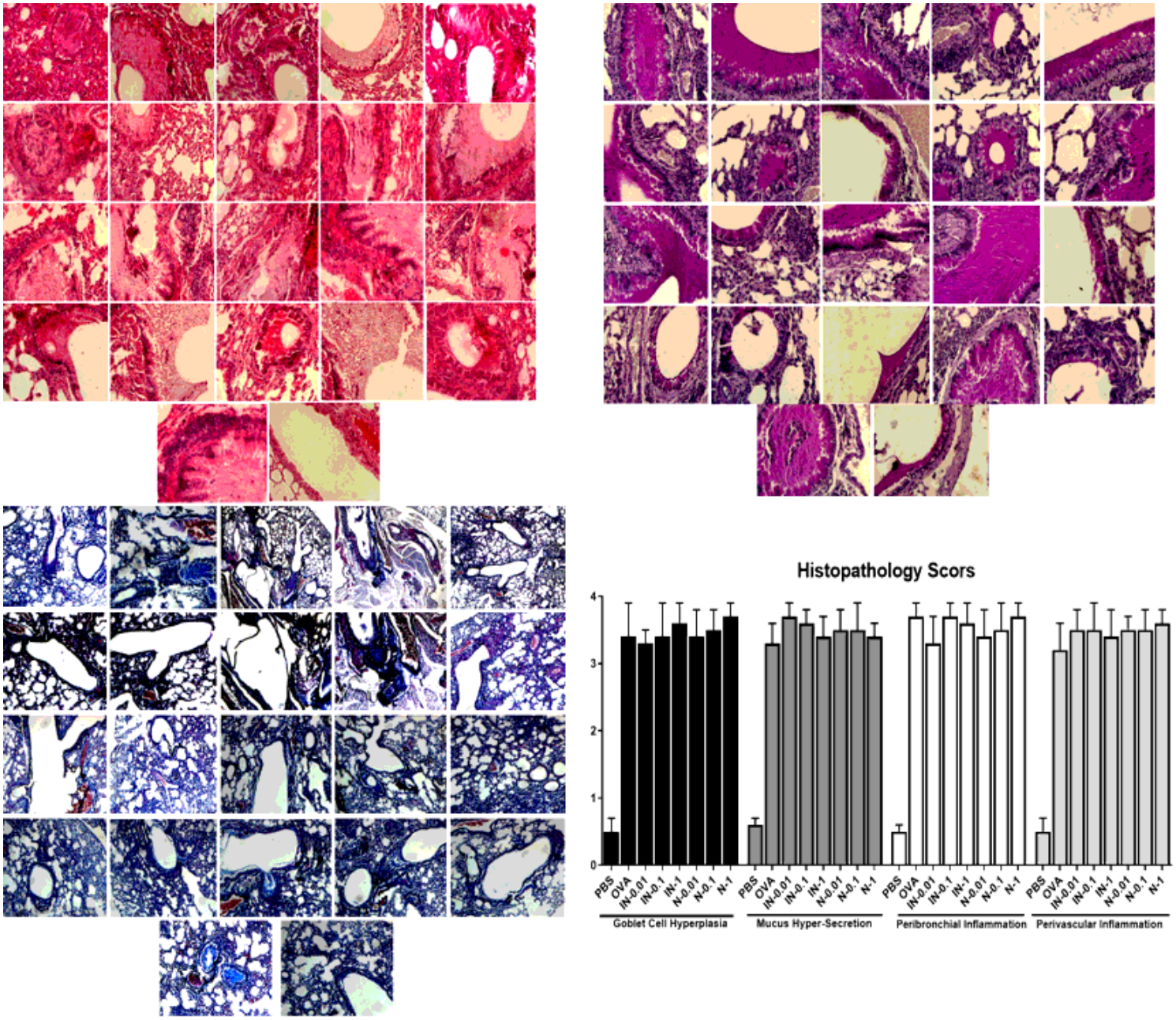

Figure 4

lung tissues were isolated and slide sections were produced. then were stained with H&E, PAS and AB-PAS and evaluated for perivascular and peribronchial eosinophilic inflammation, mucus production and goblet cell hyperplasia.

Supplementary Files

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