Bronchodilatory effect of higenamine as antiallergic asthma treatment

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ABSTRACT

Asthma is a complex airway disease that affects more than 350 million humans worldwide. Allergic asthma symptoms are induced by Th2 immune response with the release of cytokines and allegro-inflammatory mediators that amplify the inflammatory response, airway hyper-responsiveness (AHR) and hyperproduction of mucus. Higenamine, as a chemical compound, is a β_2 adrenoreceptor agonist and can be used as bronchodilator in allergic asthma.

BALB/c mice were allocated in four groups and then allergic asthma was induced in three groups. One of the asthmatic groups was treated with albuterol and other one was treated with higenamine. At least, methacholine challenge to determine the AHR, measurement of cytokines, total immunoglobulin E (IgE), LTB4 and LTC4 levels, evaluation of gene expression of Muc5ac, Muc5b, Agr2 and Arg1, and histopathological study were done.

Higenamine treatment reduced AHR, interleukin (IL)-4, IL-13 levels, mRNA expression of MUC5ac, MUC5b, Arg1 and Agr2, goblet cell hyperplasia and mucus hypersecretion. Higenamine had no significant effect on IL-5, interferon-γ (INF-γ), IgE, LTB4, LTC4 levels and eosinophilic inflammation in lung tissue.

Higenamine treatment controls asthma acute attack and breathlessness and can be used as asthma treatment with control of AHR and decrease of airflow obstruction and mucus hypersecretion and had allegro-immune-regulatory effect. But higenamine treatment had no notable effect on the inflammation and inflammatory factors.

Significance of this study

What is already known about this subject?

⇒ Higenamine controls Th2 cytokines.
⇒ Higenamine controls airway obstruction.
⇒ Higenamine controls airway hyper-responsiveness.
⇒ Higenamine controls airway hyper-responsiveness (AHR), goblet cell hyperplasia and mucus hypersecretion.

What are the new findings?

⇒ Higenamine reduces interleukin (IL)-4, IL-13 levels, and gene expression of MUC5ac, MUC5b, Arg1 and Agr2.
⇒ Higenamine regulated gene expression of cytokines.
⇒ Higenamine modulates immune-inflammatory factors.
⇒ Higenamine controls asthma attack.

How might these results change the focus of research or clinical practice?

⇒ Higenamine controls asthma attack and breathlessness that can be used as anti-asthma treatment.

INTRODUCTION

Asthma is a lung complex disease that is characterized by airway inflammation, airway hyper-responsiveness (AHR), and mucus hypersecretion. Allergic asthma as a common respiratory disease affects more than 350 million people worldwide, causing approximately 250,000 deaths every year. At present, the pathogenesis of asthma has not been fully elucidated. Allergic asthma exacerbates and its symptoms are induced by Th2 immune response with the release of cytokines and allegro-inflammatory mediators that amplify the inflammatory response, triggering AHR and production of mucus hypersecretion.[1 2]

Mucus in the airway is mainly composed of water, lipids, ions, and various macromolecules. The mucus and some proteins cover the airways' luminal surface as a thin layer to protect the respiratory epithelium. However, during asthma attack, mucus dysfunction occurs and also hypersecretion is related to obstruction of the airway. On the other hand, smooth muscle spasm around the airway leads to more obstruction of the airway and airway hyper-responsiveness begins. Also, airway inflammation increases the severity and maintenance of attack.[3 4] Therefore, to control allergic asthma attack and to cure pathological symptoms in asthma, anti-inflammatory, mucolytic effects and relaxation of smooth muscle spasm are necessary.

Higenamine (norcoclaurine) as a chemical compound is found in many parts of some plants (fruit, root, stem and seeds). It is also used in sport activity as food supplement for weight management, but along with many other β_2 agonists, higenamine is prohibited for use in sports by the World Anti-Doping Agency. Since higenamine is a β_2 adrenoreceptor agonist, and

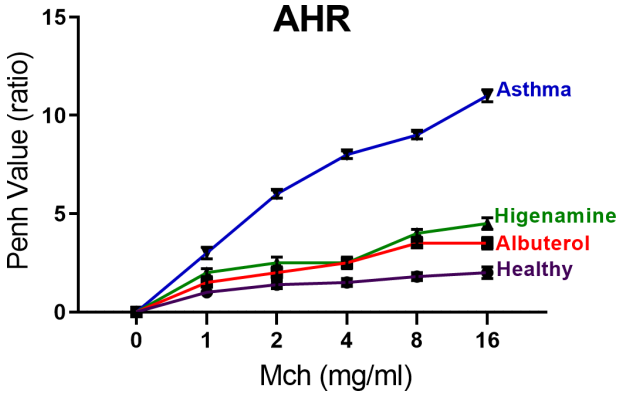

Figure 1 Determining Penh value. To study airway hyperresponsiveness (AHR), after anesthetizing, the mice were tracheotomized and then exposed to doubling concentration series of methacholine (MCh). The Penh value was increased in the asthmatic mice compared with non-asthmatic mice for all concentrations of MCh. Treatment with higenamine could reduce AHR significantly ($p<0.05$) compared with the asthmatic group.

with activation of adenylate cyclase enzyme, it is responsible for boosting the cellular messenger, cAMP.[5 6] Smooth muscles spasm is controlled by β_2 agonist and short-acting beta-agonists can provide quick relief of asthma symptoms and prevent allergen-induced bronchoconstriction and are approved for clinical use in asthma. Most of beta-agonists cannot be used more than two times per week for shortness of breath and long-acting beta-agonists are used in combination with a corticosteroid.[7 8] Therefore, a new applicable drug design is necessary and higenamine can be used as bronchodilator and β_2 adrenoreceptor agonist that was used and introduced as effective treatment for allergic asthma.

MATERIAL AND METHODS

Animal groups and treatment

Male mice (BALB/c, 6–7 weeks old) were acclimatized 1 week under the standard conditions for adaptation. Forty-eight mice were divided into four groups (n=12). In three groups, the airway allergic inflammation and asthma were induced by ovalbumin (OVA) according to a previously described protocol.[3] Briefly, sensitization of the mice was done by intraperitoneal injection of OVA with aluminum hydroxide dissolved in 1 mL normal saline on day 1 and repeated on day 14. Challenging of the mice was done by OVA solution via inhalation that was aerosolized for 30 min/day by a nebulizer on days 24, 26, 28 and 30. The fourth group was sensitized and challenged only by phosphate-buffered saline (PBS) (healthy normal group). Two of the OVA received groups were treated with albuterol (standard anti-asthma beta-agonist drug) and higenamine (inhalation form; 30 min/day) on days 25, 27 and 29. On day 30, in each group, six mice were used for AHR measurements and the other six mice in each group were used in the sampling on day 31 (bronchoalveolar lavage (BAL) fluid, blood and lung tissue). For BAL fluid sampling, after anesthetization, the mice were tracheotomized and the lavage of the lung was sampled. For lung tissue sampling, after euthanasia by CO_2, the lungs were taken and fixed in formalin buffer.

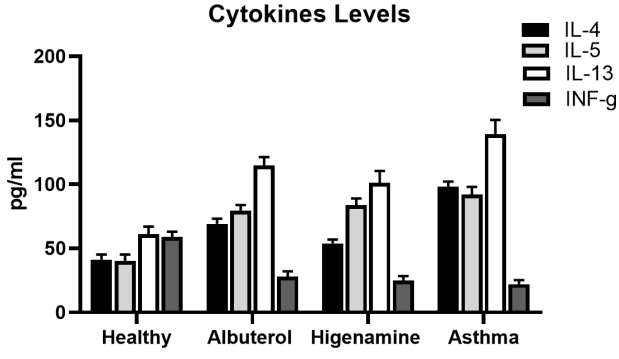

Figure 2 The levels of bronchoalveolar lavage fluid (BALf) cytokines. The levels of interleukin (IL)-13, IL-5, IL-4 and interferon-γ (INF-γ) in the BAL fluid were measured. Treatment with higenamine could decrease IL-13 and IL-4 levels significantly ($p<0.05$) compared with the asthmatic group.

AHR measurement (MCh challenge test)

To determine the AHR, methacholine (MCh) challenge test was done on day 30. After anesthetizing, AHR was assessed by determining enhanced pause (Penh value) and the mice were tracheotomized, then connected to a ventilator. Healthy control mice were exposed to PBS to obtain the baseline Penh value, whereas three asthmatic mice were exposed to aerosolized methacholine (0, 1, 2, 4, 8 and 16 mg/mL).

Cytokine level measurement

BAL fluid was sampled from the mice via intubation. The samples were centrifuged, and then the supernatant was stored to determine the cytokine levels. The IL-4, IL-5, IL-13 and INF-γ levels in BAL fluid were determined by using specific ELISA kit.

IgE level

The blood was sampled and the serum was separated. The total IgE level in the serum were measured by ELISA kit.

Serum LT levels

The serum samples were assayed in duplicate for LTB4 and LTC4 using specific ELISA kits.

Real-time PCR

In the BAL fluid cells, after RNA extraction using the TRI reagent, it was reverse transcribed to first-strand cDNA using a cDNA synthesis kit. Quantitative PCR for the expression of target genes (Muc5ac, Muc5b, Arg1 and Agr2) was done by using SYBR Green Master Mix. GAPDH was used as internal housekeeping gene. The used specific primers sequence were as follows: GAPDH 5′−3′ Forward: TGTTCCTACCCCCAATGTGT, 5′−3′ Reverse: GGTCCTCAGTGTAGCCCAAG; Muc5ac 5′−3′ Forward: CTACTGACTGCACCAACACAT, 5′−3′ Reverse: GTGCAGTCCCCATGTACTGT; Muc5b 5′−3′ Forward: TAGCATGAGCGCCTTACACC, 5′−3′ Reverse: CACGACGCAGTTGGATGTTG; Agr2 5′−3′ Forward: AAGCACCTTTCTCCTGATGGC, 5′−3′ Reverse: CGTAGAGCCGGTTTGAGTATCG; Arg1 5′−3′ Forward: CATTGGCTTGCGAGACGTAGA, 5′−3′ Reverse: TTGCCAATCCCCAGCTTGT.

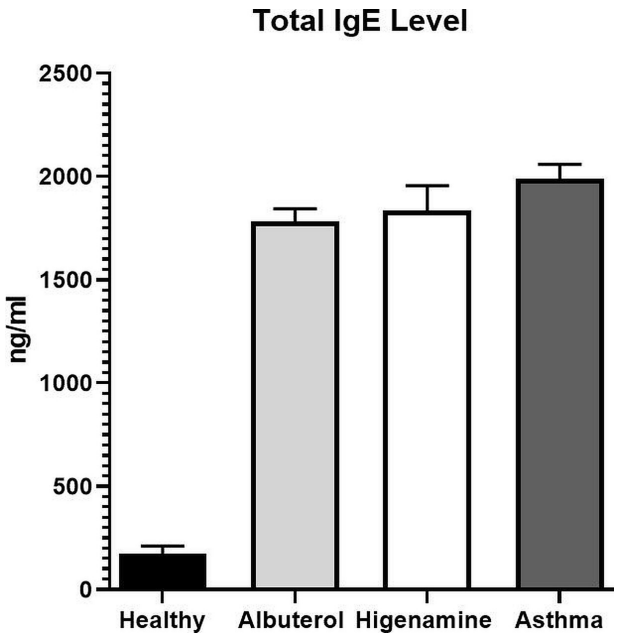

Figure 3 The total immunoglobulin E (IgE) level. In the collected blood samples, total IgE level was measured. Treatment with higenamine could decrease total IgE level compared with the asthmatic group, but the decrease was not significant (p>0.05).

Histopathology

On day 31, lung tissues after isolation were fixation in formalin solution, trimmed and paraffin-embedded, and then slide sections were produced. The slides were stained with alcian blue-periodic acid–Schiff stain (AB-PAS), PAS and hematoxylin and eosin (H&E) stain. The sections were evaluated with light microscopy for survey perivascular and peribronchial eosinophilic inflammation, mucus overproduction and hyperplasia of the goblet cell.

Statistical analysis

The experiment was repeated three times and results have been shown as means±SD. The SPSS software (V.20) has been used for analyses and were performed by using GraphPad Prism. The paired t-test was used to analyze the differences between treated and non-treated asthmatic groups. Less than 0.05 of the p value was supposed as significant.

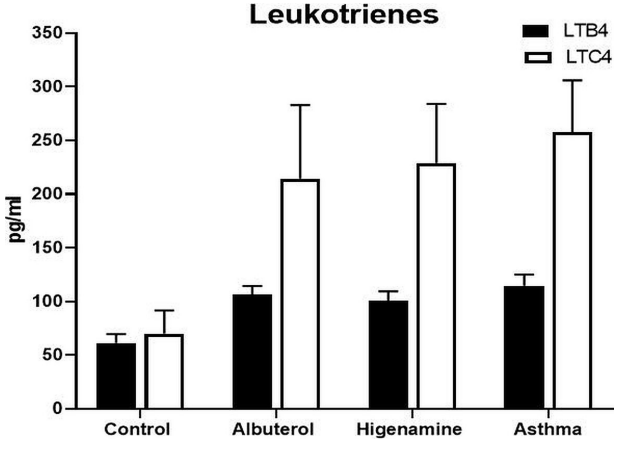

Figure 4 The leukotriene levels. The two main leukotrienes (LTB4 and LTC4) levels were measured. Treatment with higenamine could decrease LTB4 and LTC4 levels compared with the non-treated asthmatic group, but the decrease was not significant (p>0.05).

RESULT

Airway hyper-responsiveness

The Penh value was significantly ($p<0.05$) increased in the asthmatic group (1 mg/mL: 3±0.3, 16 mg/mL: 11±0.3) compared with the healthy group (1 mg/mL: 1±0.1, 16 mg/mL: 2±0.3) for all MCh concentrations (figure 1). Albuterol (1 mg/mL: 1.5±0.2, 16 mg/mL: 3.5±0.3) and higenamine (1 mg/mL: 2±0.2, 16 mg/mL: 4.5±0.2) treated groups displayed a reduced Penh value compared with the response of the non-treated group ($p<0.05$) for all concentrations of methacholine.

Cytokines

The levels of IL-4 (98.45±3.74 pg/mL), IL-5 (92.03±6.01 pg/mL), and IL-13 (139.02±11.38 pg/mL) were increased in the asthmatic group as compared with the healthy group (IL-4: 41.19±4.11, IL-5: 40.01±5.32, and IL-13: 61.32±5.84 pg/mL) and a reverse trend was found in IFN-γ (asthmatic group: 22.15±3.12 and healthy group: 58.94±4.12 pg/mL) (figure 2) ($p<0.05$). In the two treated groups, albuterol and higenamine had no significant effect ($p>0.05$) on IFN-γ and IL-5 levels, but could significantly ($p<0.05$) decrease IL-4 level. The IL-13 level was significantly decreased in the higenamine-treated asthmatic group ($p<0.05$).

IgE

The total IgE level was significantly increased in the asthmatic group (1989.4±63.32 ng/mL) compared with the negative healthy group (173.2±37.11 ng/mL) ($p<0.05$). However, treatment with albuterol and higenamine had no significant effect ($p>0.05$) on the IgE level (1784±59.4 and 1835.5±119.3 ng/mL, respectively) (figure 3).

Eicosanoids

The levels of LTB4 and LTC4 in the serum of the asthmatic group were significantly ($p<0.05$) increased (114.6±10.3 and 257.6±48.3 pg/mL, respectively) compared with the healthy group (61.4±8.2 and 70.1±21.5 pg/mL, respectively). Treatment with albuterol (106.5±7.8 and 214.5±68.3 pg/mL, respectively) and higenamine (100.3±9.1 and 228.9±55.1 pg/mL, respectively) had no significant effect ($p>0.05$) on the LTB4 and LTC4 levels (figure 4).

Real-time PCR

The mRNA expressions of MUC5ac, MUC5b, Arg1 and Agr2 were significantly ($p<0.05$) increased in the asthmatic group (8.0±1.0, 15.0±3.0, 9.0±3.0 and 14.0±2.0, respectively) compared with the healthy group. Treatment with albuterol had no significant effect ($p>0.05$) on the decrease of these four gene expressions compared with the asthmatic group. Treatment with higenamine significantly ($p<0.05$) decreased the mRNA expression of MUC5ac, MUC5b,

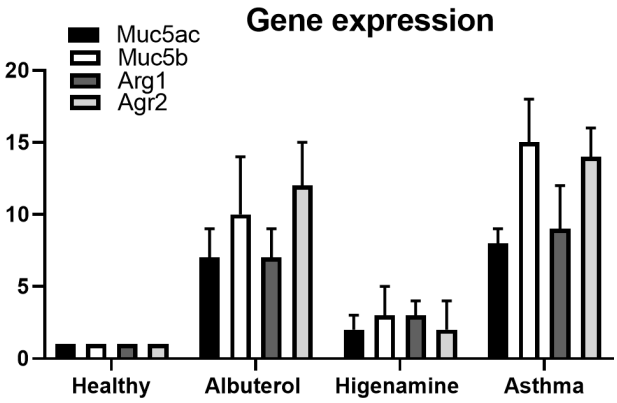

Figure 5 The gene expression analysis. The gene expressions of MUC5ac, MUC5b, Arg1 and Agr2 were studied in all groups by real-time PCR. Treatment with higenamine could control gene expression of MUC5ac, MUC5b, Arg1 and Agr2 significantly ($p<0.05$) compared with the asthmatic group.

Arg1 and Agr2 (2.0±1.0, 3.0±2.0, 3.0±1.0 and 2.0±2.0, respectively) compared with the asthmatic group (figure 5).

Histopathology

The eosinophilic inflammation in perivascular and peribronchial, hyperplasia of the goblet cell and mucus hyperproduction were significantly increased in the asthmatic groups compared with the healthy group ($p<0.05$). Treatment with albuterol had no significant ($p>0.05$) effect on the eosinophilic inflammation in perivascular and peribronchial, hyperplasia of the goblet cell and hyperproduction of mucus compared with the asthmatic group. Treatment with higenamine had no significant ($p>0.05$) effect on the perivascular (2.9±0.4) and peribronchial (3.30±0.10) eosinophilic inflammation, but could significantly ($p<0.05$) decreased goblet cell hyperplasia (1.1±0.1) and mucus hypersecretion (1.3±0.3) compared with the asthmatic group (figure 6).

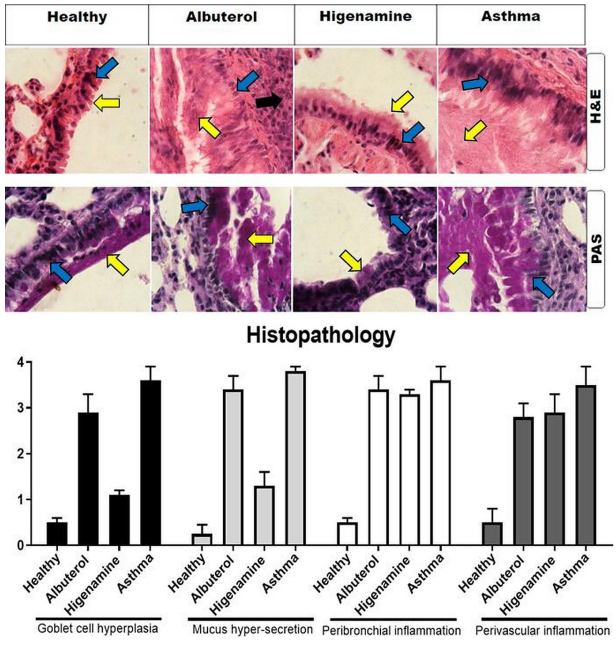

Figure 6 Histopathology. After isolation and fixation of lung tissues, lung sections were stained with two hematoxylin and eosin. (H&E) andperiodic acid–Schiff stain (PAS) stains. Afterward, the inflammation of the perivascular and the peribronchiolar, hyperplasia of the goblet cell and mucus hyperproduction were evaluated. Treatment with higenamine could control goblet cell hyperplasia and mucus hypersecretion significantly ($p<0.05$) compared with the asthmatic group. Yellow arrows show mucus overproduction, blue arrows show goblet cell hyperplasia, and black arrow shows peribronchiolar inflammation.

DISCUSSION

The β-agonists, cognate ligand of the β2-adrenoceptors (β2-ARs) located on smooth muscles of the airway, have been used as bronchodilators for the treatment of asthma. The long-acting β-agonists are used to prophylactically manage bronchoconstriction, while short-acting β-agonists are used to relieve acute exacerbations. The β2-AR has effect via cAMP that in turn activates the cAMP-dependent protein kinase (aka PKA).[9] The PKA phosphorylates numerous intracellular substrates to effect on the various functions. In the airway smooth muscles, phospholipase C, Gq-coupled receptors, IP3 receptors, K-Ca channels, myosin light chain kinase (MLCK), HSP20 are involved in inhibitory signaling of airway smooth muscle contraction and prevent airflow obstruction in patients with asthma.[9] The β2-AR activates PI3K-AKT cascade stimulation and this activation is critical for survival of the myocyte.[10 11] In this study, treatment with higenamine could reduce the Penh values, which was increased in the asthmatic mice. The effect of the higenamine on controlling AHR was similar to the effect of albuterol (one of the main anti-asthma drugs) for all concentrations of methacholine. Higenamine could affect as ligand of the β2-adrenoceptor and had bronchodilatory effect.

According to Wu *et al*'s study in 2016, higenamine has antiapoptotic effects in cardiomyocytes through activation of β2-AR. Furthermore, antiapoptotic effect of higenamine in cardiomyocytes was abolished by β2-AR but not β1-AR antagonism. In particular, higenamine stimulates AKT phosphorylation, and the required PI3K activation for the cardiac protective and anti-apoptotic effect of higenamine is mediated by the β2-AR/PI3K/AKT cascade.[12] AKT/PKB or PI3K/AKT/mTOR or PI3K-PKB/AKT signaling pathway as a serine/threonine kinase is involved in the many processes of the cells and disease regulation. PI3K/AKT pathway is an upstream activator of the NF-κB cascade that has important role in the cytokines releasing and immune responses in asthma. In addition, suppression infiltration of inflammatory cell, mucus secretion and AHR in asthma through inhibition of AKT and/or PI3K activation was revealed. On the other hand, HO-1 protects the host against oxidative stress by modulating PI3K/AKT signaling pathway and PI3K/AKT is the upstream of HO-1, and PI3K/AKT inhibition can inhibit the expression of HO-1.[12 13] Alpinetin, a flavonoid compound, possesses anti-inflammatory and antioxidant effects. It was reported that alpinetin inhibited asthma-induced phosphorylation of p65, PI3K, IκB, and AKT, and the activity of HO-1 and also exhibited a potent anti-inflammatory activity in allergic asthma through PI3K/

AKT/NF-κB and HO-1 signal modulation, which may be used as a promising therapy for allergic asthma.[13] Alpinetin relieves airway inflammatory of allergic asthma by regulating the NF-κB activation.[13] In our study, treatment with higenamine had no significant effect on the LTB4 and LTC4 levels in the asthmatic group. Also, treatment with higenamine had no significant effect on the perivascular and peribronchial eosinophilic inflammation. Therefore, higenamine may not have anti-inflammatory effect in allergic asthma. It was presented that higenamine has been traditionally used as an anti-inflammatory agent and has many pharmacological effects like vascular and tracheal relaxation, antioxidative effect, antiapoptotic, anti-inflammatory and immunomodulatory effect.[12 14 15] But we did not observe anti-inflammatory effect of the higenamine in this study. Also, IL-5 as main eosinophilic inflammatory cytokine was not decreased by higenamine treatment and therefore did not show reduction in the perivascular and peribronchial eosinophilic inflammation.

It was reported that higenamine treatment could decrease the numbers of T cells (CD4+ and CD8+), neutrophils and macrophages (CD11b+), and increase the IL-4 and IL-10 expression.[16] In the current study, treatment with higenamine had no significant effect on total IgE level that was increased in the asthmatic group and INF-γ level. However, higenamine treatment had a significant effect on decreasing the elevated IL-4 level in the asthmatic group.

MUC5ac and MUC5b are the mucin principal forms that were found in airway mucus. MUC5ac is highly expressed in goblet cells' superficial. Furthermore, the MUC5ac level and mucociliary-beating frequency are closely related to airway inflammation.[17–19] Mucin overproduction is a hallmark of asthma, and the endoplasmic reticulum protein anterior gradient homolog 2 (AGR2) is required for mucin MUC2 production. AGR2 has a main role in the all mucins production of the intestinal and airway. The AGR2 has a role in MUC5ac expression in asthma and localizes MUC5ac and MUC5b to the ER of airway cells. AGR2 increases overproduction of the mucin in asthma and loss of AGR2 impairs overproduction of allergen-induced MUC5ac and MUC5b.[20] According to histopathological study, we observed that treatment with higenamine could control goblet cell hyperplasia in allergic asthma and mucus hypersecretion was decreased in allergic asthma by higenamine treatment. Also, treatment with higenamine decreased the mRNA gene expression of MUC5ac and MUC5b in the asthmatic group and the IL-13 level was significantly decreased in the higenamine-treated asthmatic group. IL-13 is the main cytokine in mucus hypersecretion and higenamine treatment could control IL-13 level and mucus hypersecretion.

Higenamine has antioxidant activity along with inhibitory action of inducible nitric oxide synthase (iNOS) expression.[21] Oxidative stress and inflammation are critical risk factors for the cells and may cause cell death and various diseases. It was shown that higenamine has anti-inflammatory effect on lipopolysaccharides (LPS)-activated BV2 microglia and inhibits the TNF-α, IL-6, ROS as well as iNOS-mediated NO and PGE2 (mediated by COX2) production in LPS activated BV2 cells. Higenamine suppresses NF-κB signaling pathway by nuclear translocation of NF-κB/p65 subunit inhibition as well as IκBα phosphorylation in cytoplasm. Furthermore, heme oxygenase-1 (HO-1) is one of the stress protein superfamily that is also known as Hsp-32, which is regulated by Nrf2, and Nrf2 is an antioxidant transcription factor that binds to the antioxidant response element (ARE) as anti-inflammatory proteins encoding.[21 22] It was found that the anti-inflammatory effect of higenamine was accompanied by the promotion of expression of the HO-1 and nuclear factor erythroid 2-related factor-2 (Nrf2). Higenamine expresses antioxidative and anti-inflammatory effects by inhibiting NF-κB and activating Nrf2/HO-1 signaling pathways.[21 22] L-arginine is an inducible nitric oxide synthase (iNOS and cNOS) substrate that yields NO. In allergic asthma, Th2 cytokines (IL-4 and IL-13) upregulate arginases, reduce cNOS-derived NO production and increase proinflammatory peroxynitrite production by particularly inflammation-induced iNOS, by L-arginine bioavailability reduction.[23] Moreover, arginase activity increases the L-ornithine production and its downstream products polyamines and l-proline may be involved in remodeling and fibrosis of the airway. In the airways, arginase activity is increased after allergen challenge and causes AHR reaction and specific arginase inhibitor and arginase inhibition, reduce airway responsiveness by increasing NO production. Therefore, arginase inhibitors have a unique anti-allergic, anti-inflammatory, bronchoprotective, and anti-remodeling profile, which may be effective in the treatment of asthma.[23] The arginase *ARG1* and *ARG2* genes are associated with asthma risk and high arginase activity results in a low level of L-arginine in plasma and in a decrease in nitric oxide and increase airway inflammation. The *ARG1* and *ARG2* genes involve in beta-2-agonists metabolism and the *ARG1* gene may be a marker of increased risk of asthma development.[24] Treatment with higenamine reduced the gene expression of Arg1 and Agr2 that were increased in the non-treated asthmatic group. Therefore, higenamine treatment may control asthma acute attack and breathlessness with the other way and change the related gene expression. At least, higenamine can be used as asthma treatment with control of AHR and acts as β2-agonist in harnessing airflow obstruction and leads to opening of airways with controlling of smooth muscle cells spasm. Also, it can control mucus hypersecretion and decrease airway obstruction. On the other hand, in allergic asthma conditions, higenamine can manipulate gene expression and with allegro-immune-regulatory mechanism, be an anti-asthma treatment in acute phase. But higenamine treatment is not suitable for chronic inflammatory phase of asthma, because it had no notable effect on the inflammation and inflammatory factors.

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Competing interests None declared.

Patient consent for publication Not applicable.

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