Abstract: Asthma is a typical chronic inflammatory disease with a high prevalence. In recent years, studies have found that the formation of NETs is associated with the incidence of neutrophilic asthma. Ephedra-Almond herbal pair (EAHP) is a traditional Chinese medicine compatibility, which was used to relieve respiratory symptoms. The purpose of this research was to explore the potential role of EAHP in the inhibition of neutrophil proliferation and NETs formation. In this study, cells were divided into four groups, negative control (NC), lipopolysaccharides (LPS), EAHP and LPS+EAHP groups. MTT assay showed that EAHP effectively inhibited neutrophil proliferation. Western blot demonstrated that expression of anti-apoptotic protein Bcl-2 was significantly decreased in EAHP-treated neutrophils, while pro-apoptotic protein cleaved-caspase 9 was increased. Furthermore, immunofluorescence demonstrated that EAHP reduced the formation of NETs. ELISA assay showed that EAHP effectively reduced LPS-induced release of inflammatory factors such as TNF-α and IL-8. In conclusion, the present finding suggests that EAHP is a potent Chinese medicine mixture against the progress of neutrophilic asthma.

Keywords: Ephedra-Almond herbal pair, asthma, NETs, proliferation

Introduction

Bronchial asthma is a chronic allergic disease characterized by the high-sensitivity of airway [1]. The mechanism of the asthma pathogenesis is complicated and remains not fully understood [2]. So far, a variety of immunocytes have been proven to participate in asthma pathogenesis, including mast cells, eosinophils, and neutrophils [3]. With climate alteration and the increase of air pollution problems, the incidence of neutrophilic asthma has increased yearly [4]. Epidemiologically, the morbidity of neutrophilic asthma is positively correlated with the content of allergens such as particulates and bacterial endotoxin in the air [4, 5]. The symptoms of neutrophil-type asthma are commonly acute and severe, followed with poor efficacy of hormone therapy [6]. Therefore, exploring the pathogenesis of neutrophilic asthma and finding suitable drugs against this disease will exert a significant clinical value.

High mobility group box 1 (HMGB1) is a highly conserved nuclear protein which regulates the recombination, replication, repairing and transcription of nucleic acids [7]. However, studies have shown that HMGB1 can also be secreted by inflammatory cells which were stimulated by lipopolysaccharide (LPS), tumor necrosis factor (TNF) and interleukin 1 or 8 (IL-1, 8) [8]. In acute inflammatory diseases, the levels of HMGB1 are elevated in tissue and plasma [9, 10]. Furthermore, in chronic inflammatory diseases such as systemic lupus erythematosus, the plasma HMGB1 is also increased compared with normal conditions [11, 12]. HMGB1 has a significant pro-inflammatory effect, it acts as an endogenous “damage signaling molecule” that activates the membrane receptor toll like receptors 4 (TLR4), an important immune recognition receptor, which amplifies the inflammatory signal derived from HMGB1 [13]. The HMGB1/TLR4 signaling pathway plays an essential role in the initiation and regulation of respiratory tract inflammation.

Neutrophils play a very important role in the natural immunity of the host. After the invasion of pathogens, neutrophils rapidly aggregate at the site of infection, and form an extracellular network which is backboned by DNA and con-
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Figure 1. EAHP inhibits the viability of neutrophils. Cell viability was measured by MTT in neutrophils treated with EAHP at gradient concentrations (0, 0.5, 1, 2, 4, 6, 8 mM) for 4 hours. EAHP could inhibit the neutrophil viability in a dose-dependent manner. Data represent the mean ± SD. *P < 0.05.

Herb pair is a sort of common proper combination of two or three herbs [22]. Ephedra-Almond herbal pair (EAHP), a combination of Ephedra and Almond, is a famous herb pair in traditional Chinese medicine for treating respiratory symptoms. Although the clinical efficacy of EAHP against asthma is significant, the underlying mechanism has not been enough investigated.

In this study, whether EAHP can promote neutrophil apoptosis and reduce the release of inflammatory factors was investigated. In addition, this experiment explored whether EAHP can reduce the formation of NETs by inhibiting the expression of HMGB1.

Material and methods

Sample collection and cell sorting

Ethics approval of this study was granted by the Ethical Review Committee of the Wuxi People’s Hospital Affiliated to Nanjing Medical University. About 2-3 ml of peripheral blood was collected from an asthmatic child with anticoagulation tubes, and then the blood was mixed with histopaque-1119 (Sigma, USA) at an equal amount. The mixtures were centrifuged at 5000 rpm for 10 minutes, followed by the collection of the superior transparent layer. Finally, the neutrophils were collected by density gradient centrifugation using ratios of 60% and 75% Percoll separation solution.

Cell culture

The collected neutrophils were washed with PBS and cultured in RPMI-1640 medium supplementing with 10% FBS (Gibco, USA). The cells were cultured in a standard condition with 5% CO₂ at 37°C.

MTT assay

MTT assay was performed to detect the cell viability. Neutrophils were seeded in 96-well plates, followed by treatment with EAHP dissolved in RPMI-1640 at gradient concentrations (0, 0.5, 1, 2, 4, 6, 8 mM) for 4 hours, and then 15 μl of MTT was added into each well and incubated at 37°C for 4 hours. After treated with MTT, the medium was discarded, and followed by adding 100 ul DMSO in each well. Then the plate was shaken for 10 minutes. Finally, the absorbances at 490 nm were measured by a spectrometer (ThermoFisher, USA).

Western blotting

Lipopolysaccharide (LPS), was purchased from Sigma (USA), and dissolved in RPMI-1640 medium at an intimal concentration of 10 mM. Cells were respectively treated with 8 mM EAHP...
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for 4 hours (EAHP group), 10 mM LPS for 4 hours (LPS group) or simultaneously 10 mM LPS and 8 mM EAHP for 4 hours (LPS+EAHP group). The supernatant medium was collected and used to detect secretory HMGB1. Total protein was extracted using a protein extraction kit, followed by quantification with a protein quantification kit. All samples were incubated at 100°C for 5 minutes with 1x loading buffer, and then separated by SDS-PAGE and electro-transfered onto PVDF membranes. After blocked with 10% skimmed milk for 45 minutes, membranes were incubated with the primary antibodies including anti-HMGB1 (abcam, USA, 1:1000), anti-TLR4 (abcam, USA, 1:1000), anti-cleaved-caspase-9 (abcam, USA, 1:1000), anti-Bcl-2 (abcam, USA, 1:1000), or anti-GAPDH (proteintech, USA, 1:1000) at 4°C over-night. After washing with TBST for 3 times, the membranes were incubated with the secondary antibodies. Finally, the target binds were detected by ECL solution through the gel imaging analysis system.

**Total RNA extraction and qRT-PCR**

The quantitative real-time PCR (qRT-PCR) was used to detect the mRNA expression of HMGB1 and TLR4. After treated with LPS and EAHP, cells were collected for extracting total RNA using the Trizol-Plus (TaKaRa, Japan), followed by synthesizing the cDNA using a reverse transcriptase kit (TaKaRa, Japan). According to the manufacturer’s suggestions, the SYBR Green system was applied to quantify the relative abundance of target mRNA.

**Enzyme linked immunosorbent assay (ELISA)**

ELISA was used to detect the expression of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-8 in cell supernatants. Supernatant mediums were collected after LPS and EAHP treatments. Furthermore, ELISA was also used to detect alterations in MPO and NE content in cells. The cells were collected through centrifugation and were resuspended in PBS, and centrifuged to obtain the contents of the cells after multiple freeze-thaw cycles. All operations were performed according to manufacturer’s suggestion (Langdun, Shanghai, China).

**Immunofluorescence staining**

Citrulline-histone 3 (CitH3) was employed as a biomarker for representing the formation of NETs. Neutrophils were seeded in 96-well plates and respectively treated with LPS, EAHP or LPS+EAHP for 4 h, and then cells were fixed with 4% paraformaldehyde for 1 hour, followed by permeabilization using Triton X-100. Cells were incubated with the primary antibody (anti-CitH3) at 4°C overnight. Furthermore, after incubating with the second antibody, the cells were stained by DAPI to label the nucleus, and finally the cells were observed and photographed under a fluorescence microscope (Olympus, Japan).

**Statistical analysis**

SPSS 19.0 and GraphPad 6.0 software were used to express the results and all were expressed as mean ± standard deviation (SD).
Furthermore, the statistical evaluation was analyzed by a Student’s t-test, P < 0.05 was recognized statistical significant.

Results

**EAHP inhibits the viability of neutrophils**

MTT assay was performed to investigate the potential role of EAHP in inhibiting cell proliferation. As shown in Figure 1, after treatment with EAHP for 4 hours, cell viability inhibition rate increased in a dose-dependent manner, indicating that the EAHP could reduce the neutrophil viability dose-dependently.

**EAHP induces apoptosis of neutrophils**

Western blotting was performed to detect the expression of apoptosis-related proteins after different treatments. As shown in Figure 2, the results showed the expression of Bcl-2 was decreased in cells treated with LPS and EAHP, while the expression of cleaved-caspase-9 was increased. Compared with LPS group, expression of Bcl-2 was decreased in EAHP and LPS+EAHP groups, and expression of cleaved-caspase-9 was significantly increased in EAHP and LPS+EAHP groups. These results suggest that EAHP induces the apoptosis of neutrophil.
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EAHP inhibits the formation of NETs

To investigate the role of EAHP in inhibiting the formation of NETs, we performed the anti-CitH3 immunofluorescence staining, which could intuitively screen the amount of NETs. As shown in Figure 3, compared with NC group, the CitH3 fluorescence intensity was higher in the LPS group. However, expression of CitH3 was decreased in the EAHP and LPS + EAHP groups compared with the LPS group.

NETs are a web-like structure skeleton by cell free DNAs, MPO and NE are major components adhered on cell free DNA skeleton in NETs, thus cell free DNA was detected along with MPO and NE levels in cell supernatant by ELISA assay for reflecting the formation of NETs. Compared with the NC group, expression of MPO and NE was increased in the LPS group. However, the content of cell free DNA was increased in LPS+EAHP group compared with the LPS group (Figure 4).

Previous studies indicate that HMGB1/TLR4 signaling pathway exerts an essential regulatory effect in the formation of NETs. It was next asked whether EAHP could interfere the formation of NETs by regulating the expression of HMGB1/TLR4. Western blotting and qRT-PCR were performed to detect the expression of HMGB1 and TLR4. As shown in Figure 5, expression of HMGB1 and TLR4 was significantly increased in the LPS group, while both EAHP and LPS+EAHP treated cells showed a decreased expression of HMGB1 and TLR4 in comparison with the LPS group. These results indicated EAHP may inhibits the formation of NETs though inhibiting the expression of HMGB1/TLR4 single pathway.

EAHP inhibits the production of inflammatory cytokines

Neutrophils are capable of producing inflammatory factors after being stimulated by pro-inflammatory factors. To investigate the inhibitory effects of EAHP on the inflammatory factors releasing of neutrophils, expression of IL-8 and TNF-α in cell culture medium was examined after LPS and EAHP treatment. As shown in Figure 6, after LPS stimulation alone, expression of IL-8 and TNF-α was significantly increased, while it was decreased after treated with EAHP or LPS+EAHP, which suggests that EAHP could inhibit the expression of inflammatory cytokines in neutrophils.

Discussion

Asthma is a sort of prevalent respiratory disease which is characterized by hyper responsiveness of airway, bronchial edema, and reversible bronchial obstruction [23]. The exact etiology of asthma has not been fully understood. It has been demonstrated that during
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Figure 5. Expression of HMGB1/TLR4 single pathway in neutrophils. A and B. Western blot of HMGB1 and TLR4. Expression of HMGB1 and TLR4 was significantly increased in LPS group, while both EAHP and LPS+EAHP treated cells showed a decreased expression of HMGB1 and TLR4 compared with LPS group. C. Changes in HMGB1 mRNA levels in the different groups. D. Changes in TLR4 mRNA levels in the different groups. Data represent the mean ± SD. *P < 0.05, **P < 0.01.

Figure 6. EAHP inhibits production of inflammatory cytokines. A. Expression of IL-8 in different groups. B. Expression of TNF-α in different groups. Data represent the mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

the asthma occurrence, the inflammatory cells and inflammatory factors aggregate in bronchi, which triggers a series of autoimmune responses [24]. Furthermore, allergens can stimulate neutrophils to produce NETs, which injures human cells and organs [25]. In addition, HMGB1/TLR4 signaling pathway has been proven to contribute in the formation of NETs [26].

Herbal medicines have been applied for diseases treatment for thousands of years in China, multiple herbal plants have been proved to exert therapeutic effects against respiratory diseases [27, 28]. Ephedra-Almond herbal pair is a classical compatibility for relieving respiratory symptoms. Through the combination of these two herbs, a synergetic therapeutic effect was exerted, while the side effects were neutralized. In the present study, the inhibitory effects of EAHP were demonstrated on inflammatory cell proliferation and NETs formation.

Neutrophils are recruited to target tissues due to the inflammatory stimuli, and thus they take parts in multiple aspects of asthma pathogenesis [29]. The airway neutrophil counts in a part of asthma patients, especially those patients with corticosteroid-resistance, have been proven elevated [30-32]. Therefore, inhibition of bronchial neutrophil may ameliorate asthma symptoms [33]. In this study, expression of cleaved caspase-9 was increased while the Bcl-2 expression was decreased after treated with LPS in comparison with the NC group. Moreover, in the EAHP and LPS+EAHP groups, overexpression of cleaved caspase-9 and downregulation of Bcl-2 were more significant than the LPS group. It indicates that both LPS and EAHP could induce neutrophil apoptosis, but EAHP exerts a higher efficiency in inducing apoptosis than LPS.

It has been reported that the cytotoxic effect of NETs is due to the containing of histones which can directly induce the death of epithelial cells. Other components of NETs, such as antimicrobial peptides, neutrophil elastase (NE), and myeloperoxidase (MPO) can also degrade extracellular matrix, increase the viscosity of the tracheal surface and aggravate lung damage. Therefore, in addition to detecting the effect of EAHP on cell apoptosis, the inhibitory effect of EAHP on NETs formation was examined. As shown by immunofluorescence assay, the formation of NETs was increased in neutrophils induced by LPS compared with the NC group, but there was no significant increase in NETs formation after EAHP treatment. The-
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Therefore, it can be indicated that EAHP triggers neutrophil death through inducing apoptosis rather than the NETs formation.

Secretory HMGB1 has been demonstrated to be involved in the pathogenesis of inflammatory and autoimmune diseases [26, 34]. Hou et al. examined the expression of HMGB1 in sputum and serum of asthmatic patients and healthy volunteers, and found that HMGB1 levels were higher in both sputum and serum of asthma patients [35]. Moreover, studies have shown that HMGB1 acts as a chemo-attractor which mediates the migration of neutrophils. Here, the expression of HMGB1 in supernatant medium of neutrophils which treated with LPS or EAHP, and found that the expression of secretory HMGB1 in the LPS-treated group was significantly increased compared with EAHP-treated group or LPS+EAHP group. Furthermore, alteration of TLR4 expression was in consistent with HMGB1, thus it was hypothesized that EAHP could effectively inhibit the expression of HMGB1/TLR4 signaling pathway in neutrophil, which may explain how EAHP reduced the formation of NETs.

Several studies have proved that HMGB1 could induce immunocytes to release inflammatory factors [36, 37]. Expression of TNF-α and IL-8 in cell supernatants was further examined through ELISA. Compared with the NC group, the levels of TNF-α and IL-8 were significantly increased in the LPS-treated group, while remarkably decreased in the EAHP and LPS+EAHP groups. Also, EAHP effectively reduced the LPS-induced release of inflammatory factors.

In conclusion, EAHP can effectively inhibit the proliferation and induce the apoptosis of neutrophils. Furthermore, EAHP could effectively inhibit the formation of NETs and the activation of HMGB1/TLR4 signaling pathway. These finding suggests a potent efficacy of EAHP against neutrophilic asthma.

Disclosure of conflict of interest

None.

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