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Original Article

Serum from acupuncture-treated asthmatic rats regulates p38-MAPK activation in airway smooth muscle cells

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Abstract: Objectives: This study aimed to investigate the regulation function of acupuncture on airway smooth muscle cells (ASMCs) of asthmatic rats. Methods: Male Sprague-Dawley rats were challenged using inhalational Ovalbumin (OVA) to establish an asthma model. The acupuncture points (GV14 for Dazhui, bilateral BL12 for Fengmen, and bilateral BL13 for Feishu) were stimulated for asthma relief. The ASMCs isolated from asthmatic rats were incubated in medium containing the serum obtained from asthmatic rats treated with acupuncture. The expression levels of p38 MAPK and p-p38 MAPK were determined by immunocytochemical and western blot. Results: ASMCs were successfully isolated and cultured. The 20% acupuncture treatment of asthmatic rat serum had the least effect on the proliferation ability of asthmatic ASMCs. The serum from asthmatic rats treated with acupuncture could decrease the expression of p-p38 MAPK in asthmatic rat ASMCs. Conclusions: The serum from acupuncture-treated asthmatic rats has an effect on treating asthma in rats, and the mechanism of action may be by regulating the p38 pathway.

Keywords: Acupuncture, asthma, p38 MAPK, ASMCs, serum

Introduction

Asthma is a highly prevalent chronic respiratory disease that can lead to serious health problems. It is characterized by airway inflammation, airway wall remodeling and airway hyperresponsiveness. There are 10% of asthmatics with severe uncontrolled asthma, which is a very high incidence [1]. Asthma can cause respiratory symptoms, activity limitation and flare-ups that sometimes require urgent medical attention and can be fatal [2]. However, current therapies for asthma, including anti-inflammatory and muscle relaxant drugs, always have unsatisfactory results and are accompanied by significant side effects. As a result, many asthma patients are trying alternative or complementary therapies to better manage their asthma [3]. Acupuncture as a therapeutic intervention has been widely used in China and other Asian countries for thousands of years. As an effective treatment, acupuncture was proposed by the World Health Organization in 1980 to treat asthma. Previous experiments have shown that acupuncture can modulate the balance and interaction between inflammation-related proteins [4]. Acupuncture can help asthma control and relieve asthma symptoms, and asthma is one of 43 conditions listed by the World Health Organization that can benefit from acupuncture [5]. However, in vivo experiments alone cannot meet the requirements of research on the therapeutic mechanism of acupuncture in the treatment of asthma. Mechanistic studies at the molecular level are usually based on in vitro experiments. The therapeutic mechanism of acupuncture is not fully understood, and more clinical and experimental exploration is needed.

Acupuncture can regulate the expression of an important inflammation-related protein, p38-MAPK in the lung tissue of asthmatic rats [6]. However, the expression of p38 MAPK in one specific type of cell is unknown because there are many types of cells in the lung tissue. Airway

smooth muscle cells (ASMCs) are important immune cells involved in the pathogenesis of asthma, and their roles and contributions in the pathogenesis of asthma have attracted great attention [7]. As a main type of immune cell involved in the pathogenesis of asthma, ASMCs were isolated from rats in this study and used to study the changes of specific proteins. The molecular mechanisms related to asthma and ASMCs were further explored by cell isolation and cell culture of ASMCs. We confirmed the anti-asthmatic effect of acupuncture through a series of related experiments. At present, most of the research on acupuncture mechanisms has been using in vivo experiments, while most of the research on signal transduction in molecular biology has used cultured ex vivo cells. Studies have shown that ASMCs are involved in the whole process of the occurrence and development of airway inflammation in asthma, so they have been studied as targets of asthma in recent years [8, 9]. In order to eliminate various interferences, post-acupuncture serum containing active substances was used to intervene in the p38 MAPK expression in ex vivo primary asthmatic ASMCs by combining in vivo research with ex vivo verification. In this way, this study tried to elucidate the mechanism of action of acupuncture in asthma from macro to micro levels and to provide new experimental data and theoretical basis for guiding clinical treatment. The use of acupuncture serum draws on the method of "drug serum", that is, collecting human or animal serum after acupuncture, and adding it as an effector to another reaction system, so that it contacts with targets such as in vivo or ex vivo molecules, cells, tissues or organs, and the effect of acupuncture treatment can be directly observed by studying the functional and morphologic changes of molecules, cells, tissues, or organs before and after the reaction [10]. Our group confirmed the anti-asthmatic effect of acupuncture through a series of related experiments in the early stage (unpublished data). This study aimed to provide a theoretical basis for the application of acupuncture in asthma treatment.

Methods

Animals

SPF male Sprague-Dawley rats (120±10 g) were obtained from Beijing Vital River Laboratory Animals Company (SYXK 2015-0038, Beijing, China). The animals were kept in standard environmental conditions with controlled temperature (22±2°C), regular humidity (50±10%) and 12 h light/dark cycle. All experiments were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Thirty rats were randomly divided into three groups with 10 rats in each group. The rats in acupuncture group were treated with acupuncture before aerosol inhalation challenge with Ovalbumin (OVA, A5503-10G, SIGMA, USA). Rats in the model control group were not given acupuncture treatment after OVA challenge. Rats in the blank control group were only given saline inhalation. This study was approved by the Ethical Committee of North China University of Science and Technology (SQ2022087).

OVA-induced asthmatic rat model

Rats in the model control group and the acupuncture group were intraperitoneally injected with 1 ml of saline containing 1 mg of OVA and 10 mg of alum for sensitization on day 0 and day 7. From day 8 to day 20, rats were given aerosol inhalation excitation with 1% OVA every other day for 30 min (**Figure 1**). Rats in the blank control group were administered with only saline instead. Shortness of breath, wheezing, and other asthmatic symptoms in rats indicated that the model was successfully established.

Acupuncture treatment

The rats in the acupuncture group were given acupuncture treatment each time before inhalation. The needles were kept for 20 min each time, with hand-manipulation of needles every 5 min, once every other day. The acupuncture points were GV14 (Dazhui, between the C7 and T1 vertebra), bilateral BL12 (Fengmen, 1.5 cun lateral to the spinous process of T2 vertebra) and BL13 (Feishu, 1.5 cun lateral to the spinous process of T3 vertebra) [23]. The points were selected based on the theory of traditional Chinese Medicine in treating asthma (**Figure 2**).

Preparation of serum from asthmatic rats treated with acupuncture

The rats in the acupuncture and control groups were fixed after anesthetized with 50 mg/kg

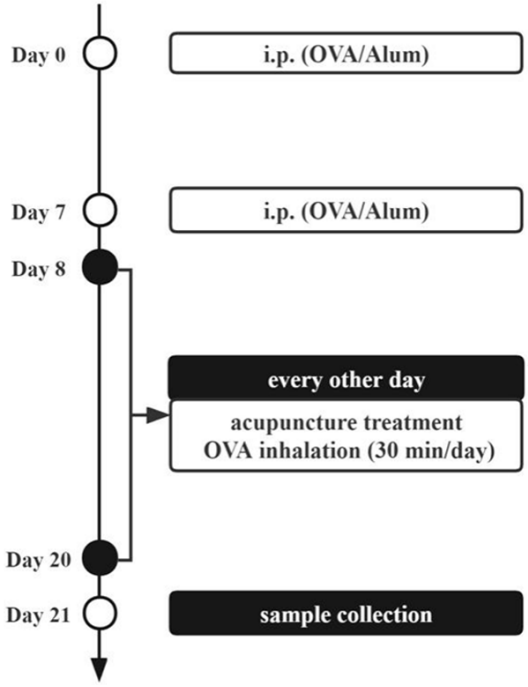

Figure 1. Rats in the acupuncture group were given acupuncture treatment before each challenge of Ovalbumin (OVA) inhalation (085G3005, PART, Germany). The rats were needled once every other day for 20 minutes with hand-manipulation every 5 minutes. On day 0 and day 7, the rats in the model control group and the acupuncture group were intraperitoneally injected with OVA/Alum, while the rats in the blank control group were administrated saline. From day 8 to day 20, the rats in the model group and the acupuncture group were challenged with aerosol inhalation of OVA for 30 minutes every other day, while the rats in the blank control group were given saline instead. The samples of blood and lungs were collected 24 hours after the last OVA challenge.

Ketamine and 5 mg/kg xylazine. The blood was drawn under aseptic condition from abdominal aorta. After centrifugation at 3000 rpm for 15 min at 4°C, the serum was filtered through 220 nm microporous membrane. The filtered serum was inactivated by heating in water bath at 56°C for 30 min and stored at -20°C.

Primary culture and cell characterization of rat ASMCs

The whole lung, trachea, and bronchi of the blank control group and model control group were transferred to PBS (added with 100 U penicillin and 100 U streptomycin) under sterile conditions. An airway without cartilage was selected and pure airway smooth muscle bundles were cut from the surrounding tissues with the help of a dissecting microscope. Muscles were cut into small pieces (1 mm^3 fragments) and placed in culture flasks containing 1 ml trypsin (0.25%) and 1 ml IV collagenase (0.1%). Muscle pieces were kept at 37°C for 30 minutes, and digestion was terminated by the addition of medium (DMEM, Gibco, USA) containing 20% fetal bovine serum (FBS) (04-001-1A, Biological Industries, Israel). The cell suspension was centrifuged at 1000 rpm for 5 minutes at room temperature, and the pellet was collected. Cells were cultured in DMEM containing 1% penicillin and 1% streptomycin in 10% FBS. The muscle pieces were evenly seeded on the bottom of the culture flask, which was placed in a 37°C 5% CO_2 humidified incubator, and the medium was changed every 3 days. Subculture (1:3) was performed after the cells completely covered the bottom, and the third passage was collected for subsequent experiments. Smooth muscle actin cytomorphology and immunocytochemistry were used to identify smooth muscle cells.

MTT assay

After digestion by 0.25% trypsin, the cell density of ASMCs was adjusted to 1.5-2×10^5/ml. The cells were plated into 96-well culture plates with 140 μl in each well, and the blank wells were only filled with DMEM. The cells were cultured for 24 h, and serum from asthmatic rats treated with acupuncture was added into the corresponding wells. The concentrations of the serum above were 0% (0 μl), 5% (10 μl), 10% (20 μl), 15% (30 μl), 20% (40 μl) and 30% (60 μl). The cells were cultured for 48 h, then 20 μl MTT/PBS (5 mg/ml) was added to each well, and the plates were incubated for 4 hours. Thereafter, medium was replaced with 150 μl dimethyl sulfoxide (DMSO, Sigma, USA), and the plates were incubated for 10 minutes with shaking. The optical density (OD) at 490 nm was determined by a plate reader (BioRad, USA). The serum concentration which exhibited the minimum impact on the cell proliferation was determined.

Cell grouping

The cells were divided into 6 groups: blank ASMCs + blank serum (B+B), blank ASMCs + acupuncture related serum (B+A), blank ASMCs + SB203580 (10 μmol/L) (B+S), model ASMCs + blank serum (M+B), model ASMCs +

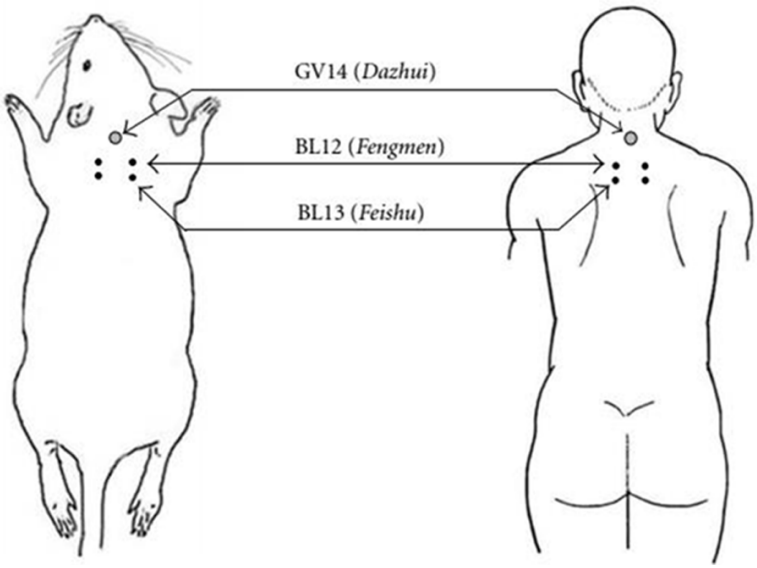

Figure 2. Diagram of acupoints.

acupuncture related serum (M+A) and model ASMCs + SB203580 (10 μmol/L) (M+S). Blank ASMCs were the cells isolated from rats in the blank control group. Blank serum was the serum obtained from rats in the blank control group. Acupuncture related serum was the serum obtained from asthmatic rats treated with acupuncture. SB203580 (0460857-6, Cayman Chemical Company, USA) is an inhibitor of p38 MAPK. Model ASMCs were the cells isolated from rats in the model control group.

Immunocytochemical analysis

Cells were collected from different groups on different slides. Envision method (PV6000, K152716B, ZSGB-BIO, China) was used for immunohistochemical staining on phospho-p38 MAPK/MAPK14 (pThr180/pTyr182) or anti-α-actin antibody (ab7817, Abcam, USA). The diluted concentration was 1:100. The positive staining of phospho-p38 MAPK/MAPK14 (pThr180/pTyr182) was brown and located in the cytoplasm and nuclei. Five views of each cell coverslip were selected randomly, and the values of OD were measured and calculated.

Western blot analysis

Cells were lysed in lysis buffer (Beyotime, China). After centrifugation, the supernatant was collected, and the concentrations were determined with a bicinchoninic acid protein assay kit (PQ0012-A, Beyotime, China). Cell lysates mixed with SDS-sample buffer were separated by SDS-PAGE (P0012A, Beyotime, China), transferred onto a polyvinylidene difluoride membrane and incubated with antibody against phospho-p38 (YH102101C, Epitomics, USA). After incubation with appropriate peroxidase-conjugated secondary antibodies (ProteinTech, China), the immunoreactive bands were visualized by enhanced chemiluminescence reagents (ProteinTech, China). The scanned images were quantified with Pro Plus6.0 medical image analysis software (Cybernetics Media, USA).

Statistical analysis

Statistical analyses were performed with SPSS 17.0 software (IBM SPSS Statistics, Chicago, USA). Date were expressed as mean ± standard deviation (SD). Differences in parametric data among multiple groups were determined by one-way analysis of variance followed by Tukey post hoc test. Differences were considered significant at $P < 0.05$ (two-tailed).

Results

Cell culture and identification

The morphology of ASMCs was observed under an inverted microscope. As shown in **Figure 3A** and **3B**, ASMCs were spindle-shaped or polygonal, with one or more nuclei in the center of the cell and one or more protrusions extending toward areas of low cell density. In one area, cells were arranged in fascicles with peak-like morphological characteristics. This typical peak-to-trough growth is characteristic of smooth muscle cells. Alpha-actin is specific for smooth muscle cells, and immunocytochemical staining for alpha-actin can be used to identify ASMCs. As shown in **Figure 3C** and **3D**, more than 95% of the cells were strongly positive for α-actin in the cytoplasm, which was stained brown. The nucleus was stained blue. Most of the α-actin was evenly distributed in the cytoplasm. These results indicated that ASMCs were successfully isolated and cultured.

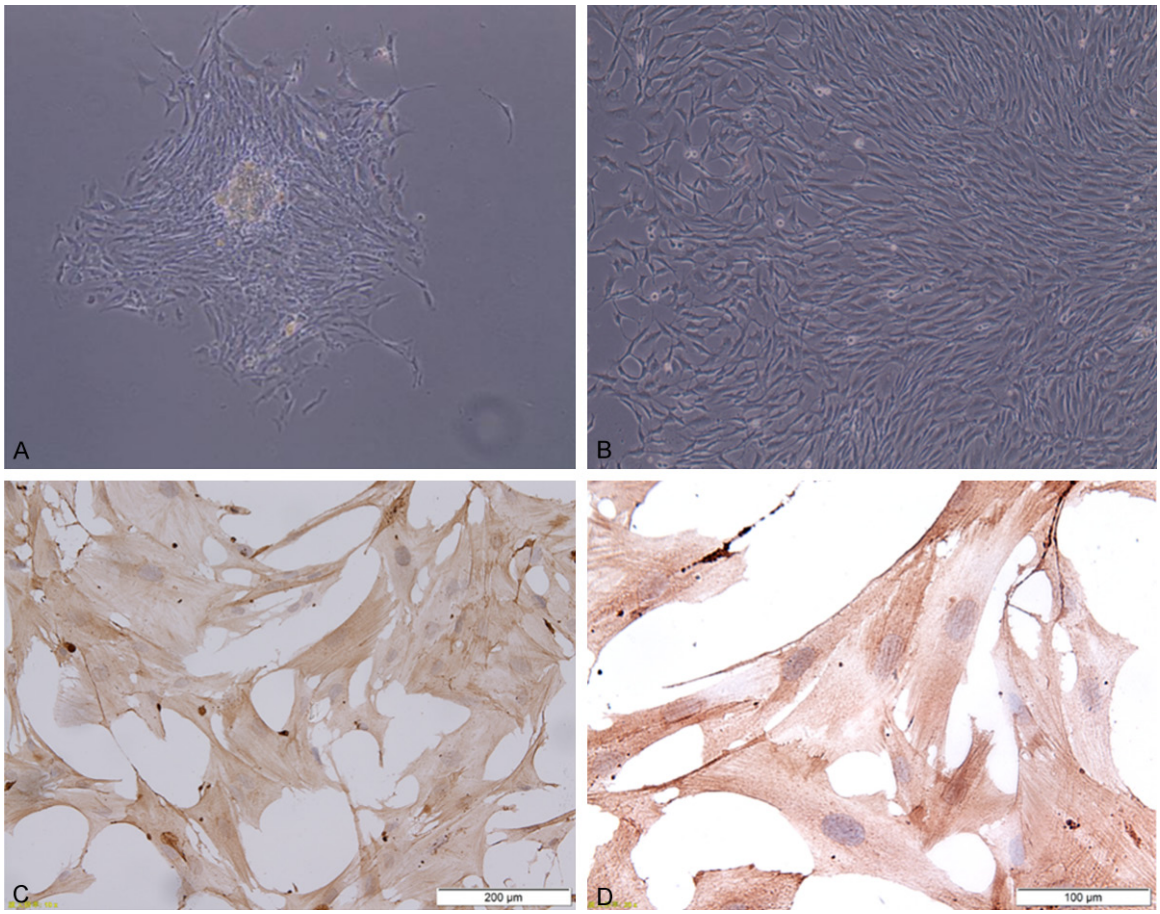

Figure 3. Airway smooth muscle cells (ASMCs) in spindle shaped or scalene triangle shaped were observed under an inverted microscope. There were 1 or 2 nuclei in the center of cells with one or more processes. (A) was shot on day 3 of culturing and (B) was on day 5 (100×). After the tissues were incubated for 3-5 days, the ASMCs began to migrate and erupt from the outer edges. The results of cell identification by immunocytochemistry were observed under an upright microscope (C) by 200× and (D) by 400×. The cytoplasm was stained brown and the nuclei blue. The α-actin staining was parallel to the longitudinal axis of the cells.

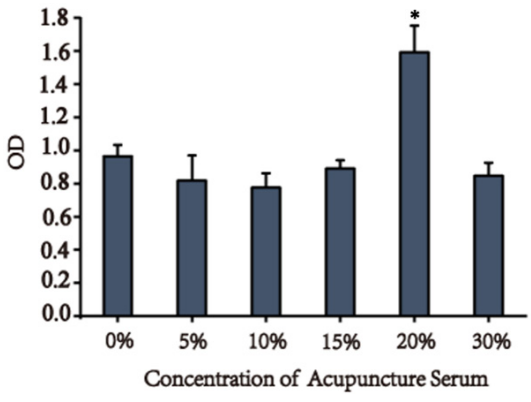

Figure 4. Effects of serum from asthmatic rats treated with acupuncture at different concentrations on asthmatic airway smooth muscle cells (ASMCs) by MTT assay. * indicates $P < 0.05$.

Optimal concentration of serum from asthmatic rats treated with acupuncture in vitro

As shown in **Figure 4**, compared to serum at other concentrations, serum of acupuncture-treated asthmatic rats at a concentration of 20% had the least effect on the proliferation ability of asthmatic ASMCs ($P < 0.05$). In subsequent *in vitro* experiments, 20% serum was applied to asthmatic ASMCs.

Effects of serum from asthmatic rats treated with acupuncture on expression of p-p38 MAPK in asthmatic rat ASMCs

As shown in **Figure 5A** and **5B**, the p-p38 MAPK protein was present in the cytoplasm and nu-

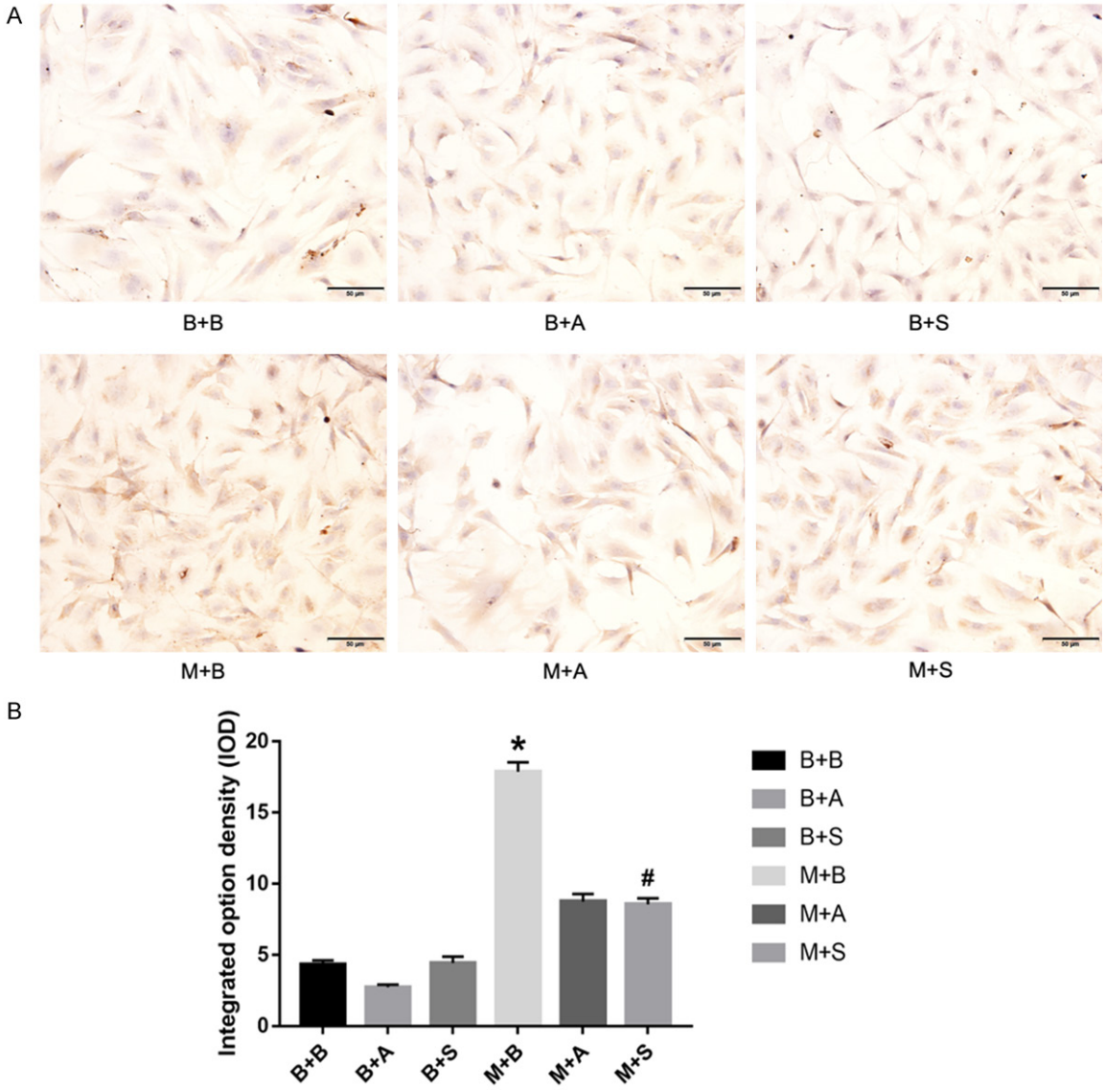

Figure 5. Effects of serum from asthmatic rats treated with acupuncture on the expression of p-p38 MAPK in asthmatic rat airway smooth muscle cells (ASMCs). A. The results were observed under an upright microscope (100×). The cytoplasm was stained brown, and the nuclei was stained blue. B. Quantification of p-p38 MAPK expression. B+B, blank ASMCs + blank serum; B+A, blank ASMCs + acupuncture related serum; B+S, blank ASMCs + SB203580; M+B, model ASMCs + blank serum; M+A, model ASMCs + acupuncture serum; M+S, model ASMCs + SB203580. Compared with Group B+B, $^{*}P < 0.05$; compared with Group M+B, $^{\#}P < 0.05$.

cleus. The expression of p-p38 MAPK was increased in asthmatic ASMCs, but decreased in the serum of acupuncture-treated asthmatic rats or after SB203580 treatment. As shown in **Figure 6A** and **6B**, there was no significant difference on the expression of p38 MAPK in ASMCs between each group ($P > 0.05$). But the expression of p-p38 MAPK was increased in asthmatic ASMCs, and was decreased after administration of serum from acupuncture-treated asthmatic rats or SB203580 ($P < 0.05$).

Discussion

The response of airway smooth muscle to various inflammatory mediators is the main cause of bronchoconstriction in asthma [11]. Increased airway smooth muscle mass due to proliferation and hypertrophy plays an important role in the process of airway hyperresponsiveness and airway wall remodeling in asthma [12]. In the present study, we found that asthmatic ASMCs were significantly stretched, with

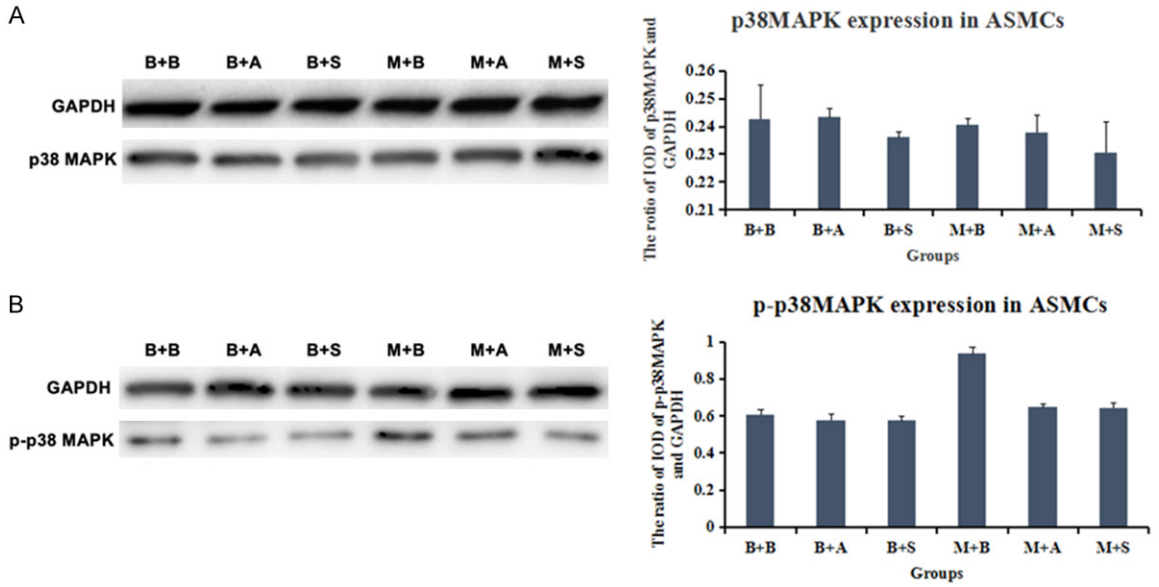

Figure 6. Effects of serum from acupuncture-treated asthmatic rats on the protein expressions of p38 MAPK (A) and p-p38 MAPK (B) in asthmatic ASMCs. GAPDH was used as internal control. Results from a representative experiment in triplicate. B+B, blank ASMCs + blank serum; B+A, blank ASMCs + acupuncture related serum; B+S, blank ASMCs + SB203580; M+B, model ASMCs + blank serum; M+A, model ASMCs + acupuncture serum; M+S, model ASMCs + SB203580.

increased stress fibers and pseudopodia, which is consistent with previous studies [13]. Hypertrophy of ASMCs with pseudopodia may be one of the factors leading to airway wall remodeling in asthma. However, which signal transduction pathways are involved needs to be further explored.

The p38 kinase is a member of the MAP kinase family and plays a crucial role in numerous biological processes. It can be activated in response to many physical, chemical and biological stresses, including UV light, heat shock, osmotic shock, inflammatory cytokines, and growth factors [14]. P38 MAPKs also regulate various physiological processes such as cell differentiation, cell cycle and inflammation [15, 16]. The regulatory function of p38 MAPK is normally performed by phosphorylated-p38 MAPK. Since airway inflammation is an important pathological feature of asthma, and p38 MAPK is closely related to the inflammatory process, this study selected p38 MAPK and its phosphorylation as potential therapeutic targets for observation. We found that p-p38 MAPK protein expression was significantly increased in asthmatic ASMCs, suggesting that p38 MAPK is overactivated in ASMCs under asthmatic conditions.

The limitation of this study is that unlike with in vivo experiments, acupuncture cannot be directly performed in in vitro experiments. Therefore, instead, we used serum obtained from acupuncture-treated asthmatic rats, which was inspired by serum pharmacology. Serum pharmacology has been widely used in in vitro pharmacology research of traditional Chinese medicine. The active ingredients of these drugs and their potentially active metabolites can be retained by the sera of animals taking these herbal medicines [17-19]. This study found that biological serum from rats after acupuncture treatment has similar effects as acupuncture treatment, including regulating immune function, inhibiting tumor, reducing calcium ion concentration in nerve cells and cardiomyocytes, and anti-asthma. In the present study, we observed that the protein expression of p-p38 MAPK in asthmatic ASMCs was decreased after administration of serum from acupuncture-treated asthmatic rats. Its inhibitory effect on p-p38 MAPK is similar to that of SB203580, which is a potent p38 MAPK inhibitor [20-22]. Although they hold promise for the treatment of inflammation, immune diseases, and certain cancers, inhibitors targeting p38 MAPK have raised awareness of organ systems that should be monitored for potential toxicity

[23, 24]. In contrast, acupuncture raises few concerns about side effects, so it has also drawn more attention.

Although the current research on the components of acupuncture serum has achieved certain results, the source, action object and route of action of acupuncture serum are still in their infancy. Especially the ex vivo study of acupuncture serum on asthma models still lacks relevant dosage standards. In short, the mechanism of action of acupuncture serum needs further experimental research. In summary, in recent years, the mechanism of acupuncture in the treatment of asthma has been increasingly studied and has yielded good results. However, the pathogenesis of asthma is complex and has not been fully understood. With the rapid development of modern science and technology, future research on the mechanism of acupuncture in the treatment of asthma has broad prospects, and the ex vivo study of acupuncture serum on asthma, especially the signaling factors, needs to be further elucidated.

In conclusion, this study demonstrates that serum from acupuncture-treated asthmatic rats can inhibit the hyperactive process of p38 MAPK in asthmatic ASMCs. Furtherly, we infer serum from acupuncture-treated asthmatic rats has active components that are effective on asthma, but its active components and their exact anti-asthma mechanisms still must be clarified.

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Disclosure of conflict of interest

None.

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