EXPERIMENTAL STUDY

Serum from asthmatic rat treated with acupuncture inhibits acetylcholine-induced contractile responses of airway smooth muscle cells


OBJECTIVE: To investigate the influence of serum from asthmatic rat treated with acupuncture (acupuncture serum) on the contractile responses of airway smooth muscle cells (ASMCs).

METHODS: OVA-induced asthmatic rats were subjected to acupuncture treatment at the points of Da-zhui (GV 14), Fengmen (BL 12), and Feishu (BL 13). The resulting serum was collected, as well as serum from normal animals. Contraction of ASMCs was induced with 10 μM acetylcholine (Ach), and acupuncture serum or normal serum was supplemented 15 min later. The effects of the sera on the changes in cell length, width, and surface area were monitored in real time via a live cell imaging system.

RESULTS: The contraction rates of ASMCs 15 min and 30 min after Ach stimulation reached 38.2% ± 3.8% and 49.7% ± 4.6%, both of which were significantly higher than unstimulated control cells (P < 0.01). Acupuncture serum at the 1 : 100 dosage reduced the contraction rate of ASMCs from 40.1% ± 5.1% to 17.6% ± 6.5% (P < 0.01). Thus, the treatment significantly inhibited the Ach-induced contractile responses of ASMCs and yielded a relaxation efficiency of 58%, which was greater than the effects obtained with a 1 : 250 dosage. Treatment with acupuncture serum at 1 : 500 dosage or normal serum displayed no clear effect of suppressing Ach-induced contraction of ASMCs.

CONCLUSION: Our data suggest that acupuncture serum has the effect of inhibiting Ach-induced contraction of ASMCs, thereby promoting relaxation of the cells.

INTRODUCTION

Bronchial asthma is a respiratory disease that severely affects human health and has the main clinical feature of reversible airflow obstruction (mostly manifesting as wheezing, shortness of breath, chest tightness, and cough). The airflow obstruction mainly results from the contraction of airway smooth muscle cells.
Preparation of acupuncture serum from asthmatic rats

Materials and methods

Preparation of acupuncture serum from asthmatic rats

All animal experiments and procedures have been approved by the Committee on the Ethics of Animal Experiments of Shanghai University of Traditional Chinese Medicine (approval ID: 080001) and were conducted in accordance with the regulations set forth by the State Science and Technology Commission. Pathogen-free, male Sprague-Dawley rats (six weeks old, 200-220 g) were supplied by SLAC Laboratory Animal Co. Ltd. (Shanghai, China, License No. SCXK [hu] 2012-0002). Asthmatic rats sensitized and challenged with ovalbumin (OVA, grade V, Sigma, Taufkirchen, Germany) according to a previously described protocol. The acupuncture points, Dazhui (GV 14, between the C7 and T1 vertebrae), bilateral Fengmen (BL 12, foveola laterally between the T2 and T3 vertebrae), and bilateral Feishu (BL 13, foveola laterally between the T3 and T4 vertebrae), were selected based on the theory of traditional Chinese medicine in treating asthma. Manual acupuncture was performed once every other day for two weeks beginning on the first day after sensitization. The acupuncture procedure was carried out by the same experienced practitioner as a previously described protocol. One hour after the final acupuncture treatment, each rat was anesthetized via intraperitoneal injection of sodium pentobarbital. Subsequently, blood was collected from the heart by cardiac puncture and centrifuged to separate out the serum, which was frozen in at −20 °C. For control samples, normal animals were anesthetized and used for serum collection.

Isolation and primary culture of rat airway smooth muscle cells

Tracheal segments of rats were separated after deep anesthesia and dissected from the surrounding tissues in D-Hanks solution containing antibiotics (penicillin and streptomycin) under sterile conditions. The tracheas were cut longitudinally and the inner membrane was scraped of gently with a scalpel until the tissue became hyaline. The smooth muscle was chopped finely and digested with collagenase type I (3 mg/mL, Sigma, St. Louis, MO, USA) and elastase (0.3 mg/mL, Sigma, St. Louis, MO, USA) for 30 min at 37 °C, with continuous slow shaking. The resulting cell suspension was seeded in Dulbecco’s Modified Eagle Medium (DMEM; Hyclone Labs., Logan, UT, USA) containing 50 units/ml penicillin and 50 µg/ml streptomycin (Hyclone Labs., Logan, UT, USA) and supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Grand Island, NY, USA). All cells were cultured at 37°C in a humidified incubator containing 5% CO2 and the medium was replaced with fresh medium every 2 days. At confluence (usually 8-10 days), cells were detached with 0.25% trypsin (containing 0.02% EDTA, Sigma, St. Louis, MO, USA) and subcultured in DMEM with 10% FBS. Immunofluorescence staining for smooth muscle myosin heavy chain was used to identify the smooth muscle cells. All experiments were performed on cells cultured between passages 3 and 6.
Detection of cell contraction
The cultured ASMCs were trypsinized and suspended in complete DMEM at a density of 1 × 10^6 cells/ml, and then were transferred to glass-bottom culture dishes (NEST, Wuxi, China) designed for use in a confocal microscope (200 μL cell suspension per dish) and kept at 37 °C in a CO₂ incubator until 50% confluence was reached. The culture medium was then removed and replaced with fresh medium. Each dish was placed on the motorized stage of a live cell imaging system (IX81, Olympus, Tokyo, Japan) equipped with a CO₂ incubator (Tokai Hit Shizuoka-Ken, Japan), which had a high-precision controller set to maintain an environment of (37.0 ± 0.2) °C, 90% humidity, and 5% CO₂. Subsequently, the morphology of the live cells was examined in assigned fields under differential interference contrast optics. Cell images were captured at a rate of 1 frame per min. The experiment consisted of the following four groups, with each being replicated three times: (a) cells received no intervention, and their morphology was continuously monitored under the microscope for 30 min; (b) cells were stimulated with 10 μM Acetylcholine (Ach; Sigma, St. Louis, MO, USA), and their morphology was continuously monitored under the microscope for 30 min; (c) cells were stimulated with 10 μM Ach and their morphology was continuously monitored for 15 min; then the acupuncture serum was supplemented into the culture medium at the concentration of 1:100, 1:250, and 1:500, and the cell morphology was continuously monitored for another 15 min; iv) cells were stimulated with 10 μM Ach and their morphology was continuously monitored for 15 min; then the normal animal serum was supplemented into the culture medium at the concentration of 1:100, and the cell morphology was monitored for another 15 min. For each group, the first frame at 0 min, which was acquired before the corresponding treatment, was designated as the reference image. The changes in cellular length, width, and surface area of ASMCs in different fields were measured using National Institutes of Health ImageJ software. Changes in cell surface area were used to evaluate the cell contraction and relaxation rates. Specifically, the cell surface area of the control field (0 min) was designated as A, and the cell surface area for a given time point was designated as B. The cell contraction rate for the given time point was calculated as (A-B) / A.

Statistical analysis
Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). A Kruskal-Wallis one-way analysis of variance was used to analyse the differences between groups. The Student-Neuman-Keuls test was used for the post-hoc comparisons. Values were presented as mean ± standard error of measurement. P < 0.05 was considered to be statistically significant.

RESULTS
Primary culture and identification of ASMCs
Under the phase-contrast microscope, cells cultured for 2-3 days from trachea or bronchia appeared to be flattened and ribbon- or spindle-shaped and had central oval nuclei with prominent nucleoli. ASMCs reached confluence in 6-8 days and displayed the characteristic hill-and-valley appearance of smooth muscle cells in culture. Immunocytochemical staining of smooth muscle myosin heavy chain, which is specific for smooth muscle cells, was performed with a mouse anti-smooth muscle myosin heavy chain antibody. More than 95% of cells were strongly positive for smooth muscle myosin heavy chain in the cytoplasm under a fluorescence microscope (Figure 1A). Some cells were multinucleated (Figure 1B, 1C).

Ach-induced contraction of ASMCs
Ach is often used in the bronchial provocation tests of asthma. Ach can bind to the muscarinic receptor, thereby triggering the contraction response of bronchial smooth muscle. Taking into account our initial experiments as well as related research literature,12 we used an Ach concentration of 10 μM to induce the contractile responses of ASMCs. The results revealed that the length, width, and surface area of the ASMCs began reducing at 2 min after the stimulation. The contraction rates of ASMCs at 15 min and 30 min after Ach stimulation reached 38.2% ± 3.8% and 49.7% ± 4.6%, respectively (P < 0.01 when compared to the normal cell group, Figure 2). However, without Ach exposure, normal cells displayed no apparent contractile response for 30 min (Figure 2).

Inhibition of Ach-induced contraction of ASMCs by acupuncture serum
After 15 min of Ach stimulation, acupuncture serum

![Figure 1](https://example.com/image1.png)

Figure 1 Immunofluorescence staining of primary cultured rat airway smooth muscle cells (× 200)
A: α-action, cells were stained with smooth muscle myosin heavy chain (green fluorescence). B: DAPI: nuclear DNA was stained with DAPI (blue fluorescence). C: the merged image of A and B.
showed persistent reduction, and the contraction rate the length, width, and surface area of the ASMCs further treatment with the normal serum for 15 min, normal animal serum. The results showed that, after responses, we performed the control experiment using acupuncture serum on Ach-induced contractile responses, we performed the control experiment using acupuncture serum on Ach-induced contractile responses. To demonstrate the specific inhibitory effects of the acupuncture serum on Ach-induced contractile responses, we performed the control experiment using normal animal serum. The results showed that, after further treatment with the normal serum for 15 min, the length, width, and surface area of the ASMCs showed persistent reduction, and the contraction rate was increased by 48.2% ± 5.9% comparing with that before treatment (35.1% ± 4.2%, P < 0.01, Figure 3E), which indicated that normal serum cannot inhibit the Ach-induced contraction of ASMCs.

**DISCUSSION**

As treasures of the traditional Chinese medicine, acupuncture has a history of more than 2500 years and has achieved sound effects in the treatment of various diseases including asthma. Stimulation of acupuncture points located on the Lung, Large Intestine and Governor Vessel (extra meridian) meridians is believed to restore normal body function and stop asthma by regulating Qi in the lung meridian, removing obstructions from the meridian, and maintaining the balance of yin and yang. Over the past decade, several published modern acupuncture studies have evaluated the positive efficacy of acupuncture therapy on asthma. Accordingly, it is important to clarify the biological value and mechanistic action of acupuncture, which may enhance the understanding of asthma as well as the development of relevant biomedical research. From the perspective of life science, acupuncture effect is essentially a type of biological activity, which de-
The understanding and exploration of the substantial basis for acupuncture efficacy depends on the sequential biological processes consisting of gene expression, activities of functional proteins, and protein-protein interactions. We consider such biologically active molecules and the underlying signaling transduction events to be the substantial basis for acupuncture efficacy. Meanwhile, our understanding and exploration of the substantial basis of acupuncture effects has been largely enhanced with the development of acupuncture serum approach, which derived from studies of serum pharmacology in the 1980s and studies of Chinese medicinal herb serum in the 1990s. It has been reported that the stimulation of certain acupuncture points leads to changes in the concentrations and activities of biologically active components in the...
serum. Acupuncture serum can reflect directly the real and comprehensive pharmacobiological information of acupuncture efficacy. Thus, based on the effectiveness of acupuncture in anti-asthma treatment, we collected serum from asthmatic rats treated with acupuncture to investigate whether, at the cellular level, acupuncture serum has similar anti-asthma effects to actual acupuncture therapy.

Our results show that, in a normal, non-stimulating environment, ASMCs exhibit no apparent morphological changes during the 30-min observation period. A model of cell contraction was then established by treating ASMCs with 10 μM Ach. Cell morphology began to change at the second minute after Ach exposure, which was mainly marked by the shortening of myofibers and the overall decrease in cell surface area. After 30 min of Ach stimulation, the overall surface area of the total ASMCs in a given field of microscope decreased by almost 50%. However, when the acupuncture serum was added to the cells that were just exposed to Ach stimulation for 15 min, it was discovered that the ASMCs, which otherwise continued their shrinkage, began to exhibit a relaxation response. We found that the 15-min acupuncture serum treatment resulted in a significant decrease of the contraction rate of the ASMCs, yielding a relaxation rate of 53%, which indicates that the acupuncture serum can indeed inhibit the Ach-induced contractile response of ASMCs and can generate a considerable relaxation effect in ASMCs. In contrast, the control experiment using normal serum showed no relaxation effect, suggesting that the acupuncture treatment in asthma model may initiate certain changes in the concentrations or activities of certain biologically active components. These active components may be responsible for the relaxation effects observed in the ASMCs.

Our investigation also showed that the contraction rates calculated from the lengths and widths of ASMCs were mostly lower than those derived from surface area, and for each experimental group, the variations in width and length were greater than the variations in surface area. This could be because of the irregular morphological changes exhibited by different ASMCs, such that some cells exhibited altered cell width with little change in length, whereas other cells mostly exhibited changes in length with barely any change in width. However, changes in either the length or the width are reflected in cell surface area. Thus, to a certain degree, the changes in surface area of ASMCs can better reflect the contraction or relaxation efficiencies than the other two parameters.

In summary, our study demonstrates that the acupuncture serum inhibits Ach-induced contraction of ASMCs and thus has a relaxation effect on these cells. Hence, our results provide novel cellular and serological evidence for elucidating the mechanisms of acupuncture effects in asthma treatment. In addition, our study also provides a sound experimental basis for the discovery of novel effector molecules and drug targets from acupuncture serum for the prophylaxis and treatment of asthma. Further studies are needed to analyze and identify the biologically active components in acupuncture serum and to elucidate their biological functions and the mechanisms of their anti-asthma properties.

REFERENCES

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