

Original Article

Biological characteristics of tracheal smooth muscle cells regulated by NK-1R in asthmatic rat with airway remodeling

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Abstract: This study aims to investigate the biological characteristic changes of infant rat tracheal smooth muscle cells in asthma airway remodeling and the impact of NK-1R on the mechanism. Ovalbumin (OVA) was used to excited juvenile SD rats by 8 w. Immunofluorescence, MTT assay, transwell chambers, real time quantitative PCR, Western blot and other methods were used to observe the proliferation, migration, synthesis and secretion changes of infant airway remodeling in rat tracheal smooth muscle cell and the Neurokinin 1 receptor (NK-1R) expression. 1. NK-1R mRNA, protein expression of airway smooth muscle cell (ASMC) of each asthma group were higher than that of the control group, especially the asthma 8 w group had highest expression ($P<0.01$). 2. The average A value of 8 w asthma group measured by MTT method were significantly higher than that of the control group ($P<0.05$), WIN62577 10^{-8} mol/L group had the strongest inhibition of ASMC proliferation ($P<0.01$). 3. The number of cell migration in the asthma group significantly increased than that in the control group. The number of migrating cells in the NK-1R antagonist group significantly reduced compared with the asthma 8 w group ($P<0.05$). 4. The average gray value of type III collagen in each asthma group were higher than that of the control group, and the asthma 8 w group had the highest ($P<0.01$). After NK-1R blocking, the average gray value of type III collagen was significantly lower ($P<0.05$). ASMC proliferation, migration, synthesis and secretion function increased in the airway remodeling group, and NK-1R played an important role.

Keywords: Airway remodeling, smooth muscle cells, NK-1R, proliferation, migration

Introduction

Bronchus asthma is a chronic respiratory disease seriously impacted human health, its prevalence and mortality are on the rise in recent years. Airway remodeling is the main reason for inducing airway hyper responsiveness and chronic asthma. Therefore, the mechanisms and treatment of airway remodeling have become the focus of the current domestic and foreign research [1-3]. Studies have shown that Substance P (SP) can promote the proliferation and migration of fibroblasts, endothelial cells, epidermal stem cells [4-6]. SP plays a role through Neurokinin 1 receptor (NK-1R). Our previous studies also showed that NK-1R promoted airway remodeling [7], and changes in airway smooth muscle cells was the basis of airway remodeling [8]. But the role of NK-1R in

the proliferation, migration synthesis and secretion of airway smooth muscle cells (ASMC) is still rarely reported. In this study, SD rat tracheal smooth muscle cells were primary cultured and to given NK-1R antagonist intervention. Immunofluorescence, MTT assay, transwell chambers, real time quantitative PCR and Western blot were used to explore the impact of NK-1R on the biological behavior changes such as proliferation, migration, synthesis and secretion of asthma airway remodeling in rat tracheal smooth muscle cells.

Materials and methods

Experimental animals

Juvenile female SD rats with SPF level, weighed 60-80 g were purchased from China Medical University Experimental Animal Center. This

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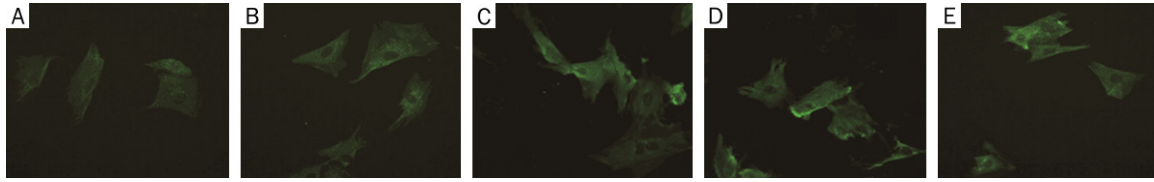


Figure 1. NK-1R immunofluorescence staining in ASMC. A. Control group; B. Asthma 2 w group; C. Asthma 4 w group; D. Asthma 6 w group; E. Asthma 8 w group.

Table 1. Changes of NK-1R mRNA and protein expression of ASMC in rats ($\bar{x} \pm s$)

Group	NK-1R mRNA	NK-1R protein
Control group	0.0104±0.0002	0.1029±0.0014
Asthma 2 w group	0.0137±0.0003*	0.1267±0.0019*
Asthma 4 w group	0.0149±0.0003**	0.1305±0.0021*
Asthma 6 w group	0.0162±0.0002**	0.1384±0.0025**
Asthma 8 w group	0.0171±0.0003**	0.1416±0.0026**

*Compared with control group, $P < 0.05$, **Compared with control group, $P < 0.01$.

study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shenyang Military Area Command.

Preparation of airway remodeling model

1 ml antigen sensitization was used to perform intraperitoneal injection on the first and eighth days respectively (including 100 mg egg protein, 100 mg aluminum hydroxide, 5×10^9 unit inactivated pertussis). The control group was injected with an equal volume of saline. From the 15th days, the rats were placed in a sealed container (20 cm × 20 cm × 20 cm) with 1% ovalbumin (OVA) ultrasonic inhalation, 30 min for each time, and 3 times a week, respectively excited by 2 w, 4 w, 6 w and 8 w. Normal control group was treated with saline instead of ovalbumin, and excited by 2 w.

ASMC primary culture

After routine disinfection and anesthesia, the rats were sacrificed by collecting blood from ventriculus sinister. Antiseptically isolated rat trachea, carefully peeled off the outer membrane, removed the inner membrane. Cut the tracheal smooth muscle, the tissues were cut into pieces, added 0.1% trypsin, shook at 37°C

water and bathed for 10 min, 1000 rpm/min centrifuged for 5 min, the supernatant were discarded. Added 0.1% IV collagenase, shook at 37°C and water bathed for 30 min, 1000 rpm/min centrifuged for 5 min, the supernatant was discarded. Then added 10% fetal bovine serum (FBS) in DMEM to terminate the reaction, gently pipetted, filtered cells by 100 mesh sieve, inoculated to 50 ml culture flask, 37°C incubated and cultured, purified ASMC based on the characteristics of rapid adherent fibroblasts. After the cells covering the bottom, cell passage was performed by 1:2, and collected the 3rd generation cell for experiment using.

Immunofluorescence staining

Cells in the logarithmic growth phase were digested to prepare single cell suspension, after appropriate adjusting the density, seeded on coverslips on six-well plates. Cultured in the incubator at 37°C with CO₂ for overnight, fixed with 4% paraformaldehyde when the cells adhered and stretched. 3 M H₂O₂ were used to treat cells under at room temperature for 10 min, cells were treated by 3 g/L Triton100 at 37°C for 10 min, and blocked by 3% goat serum at room temperature for 15 min. Rabbit anti-rat NK-1R antibody (1:100) (Santa Cruz, USA) was added. Set the cells in a humid chamber at 4°C for overnight. FITC-labeled goat anti-rabbit IgG (Boster Biological Engineering Co., Ltd., Wuhan, China) treated at 37°C for 30 min. DAPI 5 µg/ml was used at room temperature for 5 min. Mounted by 50% glycerol. Washed with PBS between steps. PBS instead of the first antibody was used for negative control in the experiment.

MTT assay

ASMC proliferation was measured. The 3th-5th generations ASMC of the asthma 2 w, 4 w, 6 w, 8 w group and the 2 w control group were collected. When ASMC grew to 80% confluence,

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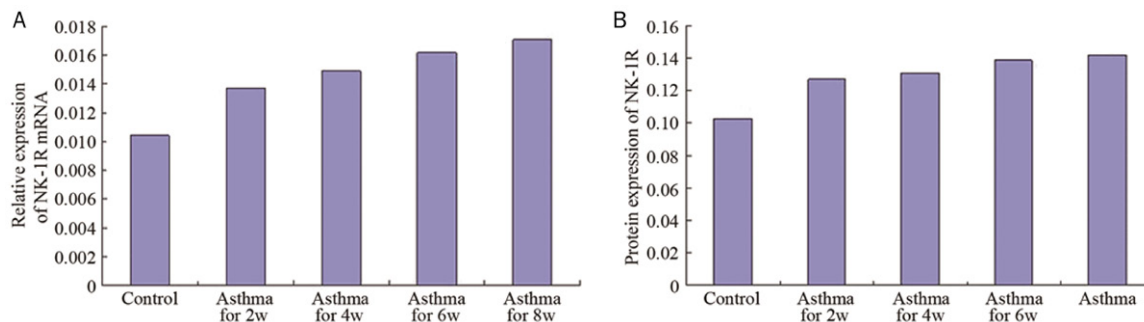


Figure 2. NK-1R mRNA and protein expression changes in ASMC of rat groups. A. NK-1R mRNA expression; B. NK-1R protein expression.

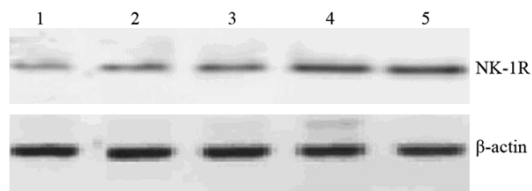


Figure 3. Western blot detection of NK-1R protein expression in rat ASMC. 1. The control group; 2. 2 w asthma group; 3. Asthma 4 w group; 4. Asthma 6 w group; 5. 8 w asthma group.

0.25% trypsin was used for digestion, seeded in 96-well plates according to 1×10^4 cells/well, cultured for 24 h, 48 h, 72 h, 96 h, 110 h by DMEM containing 10% FBS. The asthma 8 w group were randomly divided into the following five groups: 1) control group: without any intervention agents; 2) WIN62577 10^{-11} mol/L group; 3) WIN62577 10^{-10} mol/L group; 4) WIN62577 10^{-9} mol/L group; 5) WIN62577 10^{-8} mol/L group. Five repeated wells were set for each group. At the end of ASMC culture, 20 μ l MTT (5 mg/ml) was added to each well, cultured for 4 h and discarded culture medium, added 150 μ l DMSO. Absorbance (A) value of each well was measured at 490 nm by ELISA.

ASMC migration testing

ASMC migration was detected by transwell chamber. The 3th-5th generations ASMC of the asthma 2 w, 4 w, 6 w, 8 w group and the 2 w control group were collected. When ASMC grew to 80% confluence, 0.25% trypsin was used for digestion, the cells were counted and the density was adjusted to 1×10^5 /ml with 10% FBS-DMEM, 100 μ l cell suspension was added to the each group of the upper chamber, the lower chamber was filled with 600 μ l 10% FBS-

DMEM, placed the small chamber in 37°C incubator, removed 24 h later, carefully removed the membrane, gently wiped up the non-membrane worn cells on the upper side of microporous membrane. ASMC under the membrane were fixed by 4% paraformaldehyde for 20 min, stained by hematoxylin for 10 min, performed transplant by xylene for 1 min, mounted with neutral gum, counted five vision cells under an inverted microscope (400 \times) and averaged, repeated for three times. After migration experiment of the NK-1 receptor antagonist group, cells on the upper chamber of the asthma 8 w group were pretreated by NK-1 receptor antagonist WIN62577 (final concentration 10^{-8} mol/L) for 30 min.

Immunochemical method

Slice were dewaxed by benzene, then removed benzene by gradient alcohol, washed by distilled water for 5 min, washed with PBS for 3 times, 3 min for each time. Sections were immersed in containers with antigen retrieval solution (sodium citrate buffer PH 6.0, 0.01 M), heated in microwave by high fire for 10 min, opened to place for 5 min, reheated for 5 min, and let cool at room temperature. Dropped 3% hydrogen peroxide reagent A and placed in wet box at room temperature for 10 min, washed with PBS for 3 times, 3 min for each time. Removed the PBS, dropped normal goat serum blocking solution reagent B and incubated at room temperature for 10 min. Added primary antibody (rabbit anti-rat collagen III) with ratio of 1:100 dilution, placed at 4°C for overnight. Dropped the secondary antibody reagent C, placed at room temperature for 10 min, washed with PBS for 3 times, 3 min for each time. Then dropped horseradish peroxidase labeled strep-

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Table 2. Changes of absorbance A value of ASMC in rats by MTT assay ($\bar{x} \pm s$)

Group	24 h	48 h	72 h	96 h	110 h
Control group	0.201±0.032	0.278±0.035	0.299±0.041	0.283±0.024	0.264±0.036
Asthma 2 w group	0.247±0.029	0.434±0.032	0.457±0.048	0.471±0.034	0.462±0.044
Asthma 4 w group	0.298±0.024	0.526±0.059	0.568±0.067	0.598±0.056	0.577±0.065
Asthma 6 w group	0.303±0.024	0.552±0.046	0.597±0.062	0.623±0.068	0.617±0.059
Asthma 8 w group	0.318±0.038	0.578±0.064*	0.619±0.077*	0.631±0.068*	0.628±0.073*

*Compared with control group, $P < 0.05$.

Table 3. Effect of different concentrations of WIN62577 on ASMC proliferation in asthma 8 w group rats ($\bar{x} \pm s$)

Group	24 h	48 h	72 h	96 h	110 h
Control group	0.318±0.038	0.578±0.064	0.619±0.077	0.631±0.068	0.628±0.073
WIN62577 10^{-11} mol/L	0.297±0.023	0.471±0.042	0.512±0.048	0.504±0.044	0.483±0.053
WIN62577 10^{-10} mol/L	0.273±0.034	0.318±0.039**	0.304±0.037**	0.276±0.029**	0.269±0.023**
WIN62577 10^{-9} mol/L	0.238±0.027	0.185±0.021**	0.164±0.019**	0.151±0.014**	0.142±0.018**
WIN62577 10^{-8} mol/L	0.213±0.025	0.148±0.019**	0.139±0.017**	0.143±0.012**	0.133±0.019**

**Compared with control group, $P < 0.01$.

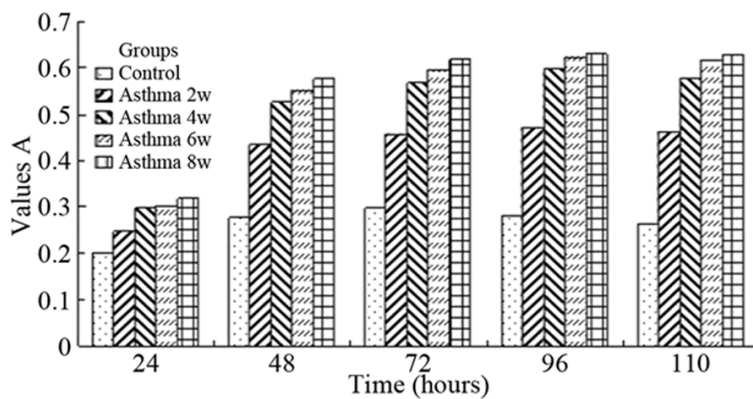


Figure 4. Absorbance (A) value changes of rat ASMC in each group by MTT assay.

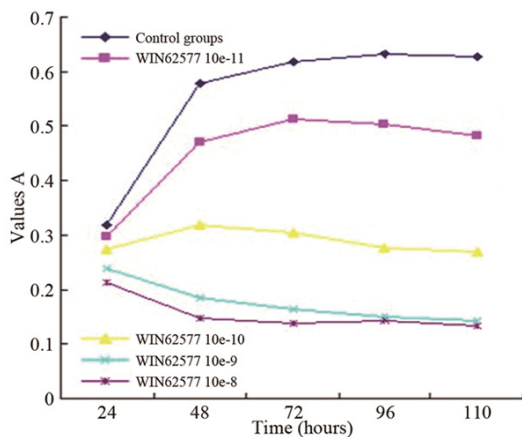


Figure 5. Impact of WIN62577 with different concentrations on the ASMC proliferation of rat asthma 8 w group.

tavidin reagent D, placed at room temperature for 10 min, and washed with PBS for 3 times, 3 min for each time. DAB colored, observed under microscopy, washed by running water for 10 min to terminate the reaction. Re-stained by Hematoxylin for 20 to 40 seconds, blued for 5 min. Performed dehydration and transparent, mounted by neutral gum, and observed under the microscope.

Real time quantitative PCR

SYBR Green I fluorescent dye embedded method was used. Plotted the standard curve of target genes (NK-1R, NK-2R) and the housekeeping gene (GAPDH), respectively. The standard curves were used to quantify the target gene and housekeeping gene in the sample. The relative expression of the test gene mRNA = test gene copy number/GAPDH gene copy number. NK-1R sequences of the primers: upstream 5'-ACC AAC ACC TCT GAG TCT AA-3'; downstream of the 5'-TGG TCA CTG TCC TCA TTC T-3', amplified fragment length was 154 bp. GAPDH primer sequence: upstream 5'-GCA CCG TCA AGG CTG AGA AC-3'; downstream of the 5'-ATG GTG GTG AAG ACG CCA GT-3', amplified fragment length was 142 bp. Primers were synthesized by Invitrogen Biotechnology Co., Ltd.

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Table 4. Dynamic changes of the migration number of ASMC in rats ($\bar{x} \pm s$)

Group	Number of migrating cells
Control group	12.24±1.87
Asthma 2 w group	17.65±1.64*
Asthma 4 w group	22.73±2.95**
Asthma 6 w group	28.52±2.26**
Asthma 8 w group	32.14±3.74**
NK-1R antagonist group	16.35±2.08 ^Δ

*Compared with control group, $P < 0.05$; **Compared with control group, $P < 0.01$; ^ΔCompared with asthma group, $P < 0.05$.

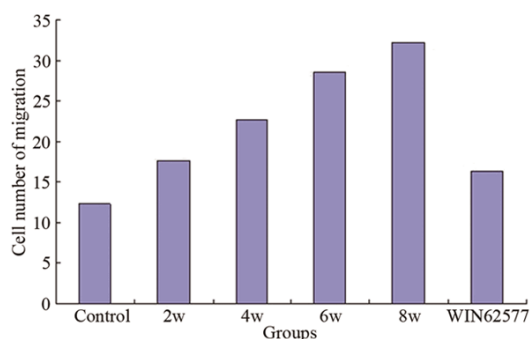


Figure 6. Dynamic changes of migration ASMC number in each rat group.

Western blot analysis

BCA method was used for protein quantitation, 10% SDS2 polyacrylamide gel electrophoresis was performed with constant voltage comprised of stocking gel for 80 V and removing gel for 120 V. 280 mA constant current was used to transfer for 50 min. NK-1R antibody (rabbit anti-rat) was diluted by 1:400, secondary antibody (alkaline phosphatase-labeled goat anti-rabbit) was diluted by 1:2000, chromogenic enzyme method was used. FlourChem V2.0 using gel image analysis software (America) was used, recorded the gray value of each protein electrophoresis bands, and performed quantitative analysis. Protein content = gray value of sample protein/gray value of β -actin in the same sample.

Statistical analysis

SPSS13.0 statistical software was used. The statistical data was described as mean \pm standard deviation ($\bar{x} \pm s$). The comparisons between the two groups were performed using t test, and $P < 0.05$ was considered to be statistically significant.

Results

NK-1R positioning expression

Observed under the fluorescence microscope, NK-1R mainly distributed in the cytoplasm and membrane with green fluorescence. The green fluorescence intensity of asthma 2 w, 4 w, 6 w, 8 w group was higher than that of the control group (**Figure 1**).

Expression of NK-1R protein and mRNA

Protein and mRNA expression of NK-1R in asthma ASMC groups were higher than that of the control group, and the asthma 8 w group had the highest expression ($P < 0.01$, **Table 1; Figures 2, 3**).

ASMC proliferation activity

The results showed that the proliferation of rat ASMC in the asthma groups significantly faster compared with the control group. MTT measured results showed that the average A value of cells in the asthma groups at each time point were higher than that of the control group, and the A value in the 8 w group was the highest, the difference was significant ($P < 0.05$, **Tables 2, 3**). WIN62577 inhibited the ASMC proliferation in a dose-dependent manner, WIN62577 10^{-8} mol/L group had the strongest inhibition (**Tables 2, 3; Figures 4, 5**).

ASMC migration

The number of ASMC migration in the control group was small (12.24±1.87), the number of migrating cells in the asthma 2 w, 4 w, 6 w, 8 w group were (17.65±1.64), (22.73±2.95), (28.52±2.26), (32.14±3.74) respectively, the number of migrating cells increased significantly compared with the control group. The number of cell migration in the NK-1R antagonist group significantly reduced compared with the asthma 8 w group, the difference was statistically significant ($P < 0.05$; **Table 4; Figures 6, 7**).

ASMC III collagen expression

Immunocytochemistry results of ASMC type III collagen showed that positive particles of brown type III collagen mainly distributed in the cytoplasm. The mean gray values of type III collagen in the asthma 2 w, 4 w, 6 w, 8 w groups were higher, the asthma 8 w group was the highest, the difference was significant ($P < 0.01$).

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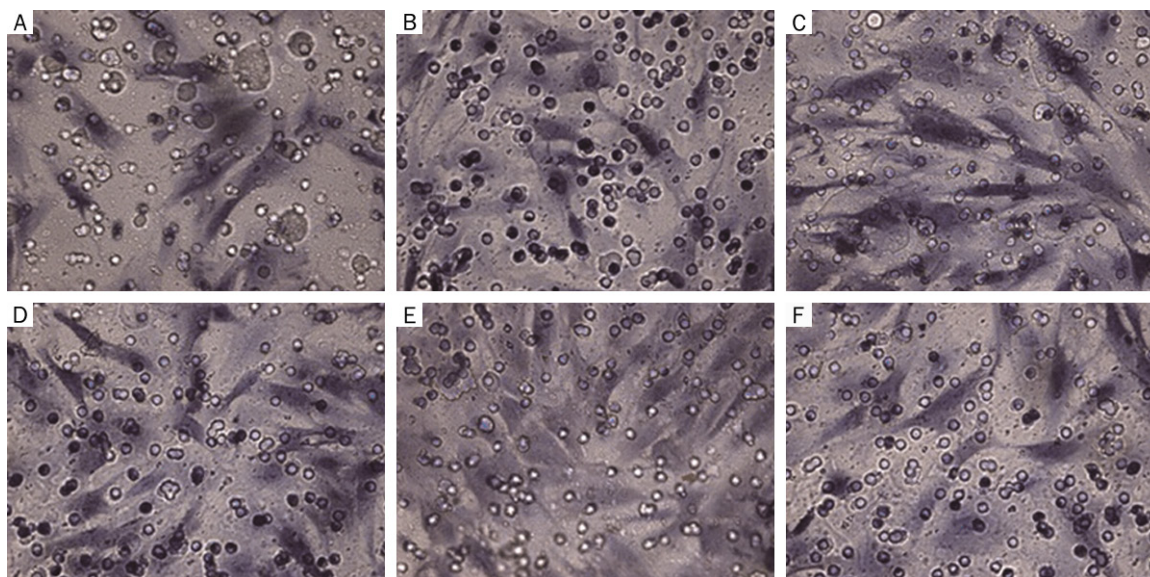


Figure 7. ASMC migration of rats. A. Control group; B. Asthma 2 w group; C. Asthma 4 w group; D. Asthma 6 w group; E. Asthma 8 w group; F. NK-1R antagonist group.

Table 5. The expression of ASMC III collagen in rats ($\bar{x} \pm s$)

Group	Gray value of III collagen
Control group	90.16±8.14
Asthma 2 w group	112.38±10.01**
Asthma 4 w group	119.75±10.05**
Asthma 6 w group	122.64±10.25**
Asthma 8 w group	130.17±12.71**
NK-1R antagonist WIN62577 group	116.14±12.48 ^Δ

**Compared with control group, $P < 0.01$; ^ΔCompared with asthma 8 w group, $P < 0.05$.

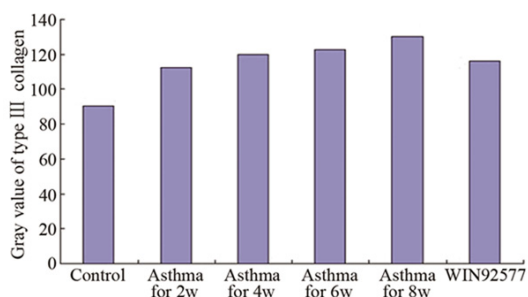


Figure 8. Expression of type III collagen in rat ASMC.

After NK-1R blocking, the average gray value of the type III collagen in the asthma 8 w group was significantly lower than that of unblocked groups, the difference was significant ($P < 0.05$; **Table 5**; **Figures 8, 9**).

Discussion

SP widely distributed in the central and peripheral nervous system with a variety of small peptides with biological activity. When exposed to noxious stimuli, SP can reversely released into local tissue damage and involved in the regulation for proliferation, migration, differentiation of the cell repair, the sensory neuropeptide played an important bridging role between the nervous system and damaged tissues [9]. SP acted majorly through NK-1R. Studies have shown that NK-1R expression in airway smooth muscle cells of asthma rats, which can promote calcium influx, IL-13 and other inflammatory cytokine secretion [10, 11]. In this study, primary cultured ASMC of airway remodeling models were used for the study subjects, which was closer to asthma research purposes compared with simulated environment cultured ASMC by stimulating factor intervention in vitro. Enzymatic digestion of primary cultured rat was used in this study, and the differential adherence method was used to obtain high purity purification ASMC, the 3th-5th cells were used for experiment. The results confirmed that NK-1R expressed in the control group and the cell membrane and cytoplasm of ASMC in the airway remodeling rats, the mRNA and protein expression of NK-1R in the ASMC of airway remodeling rats significantly increased compared with the control group. This was the basis and prerequisite of SP acting on ASMC.

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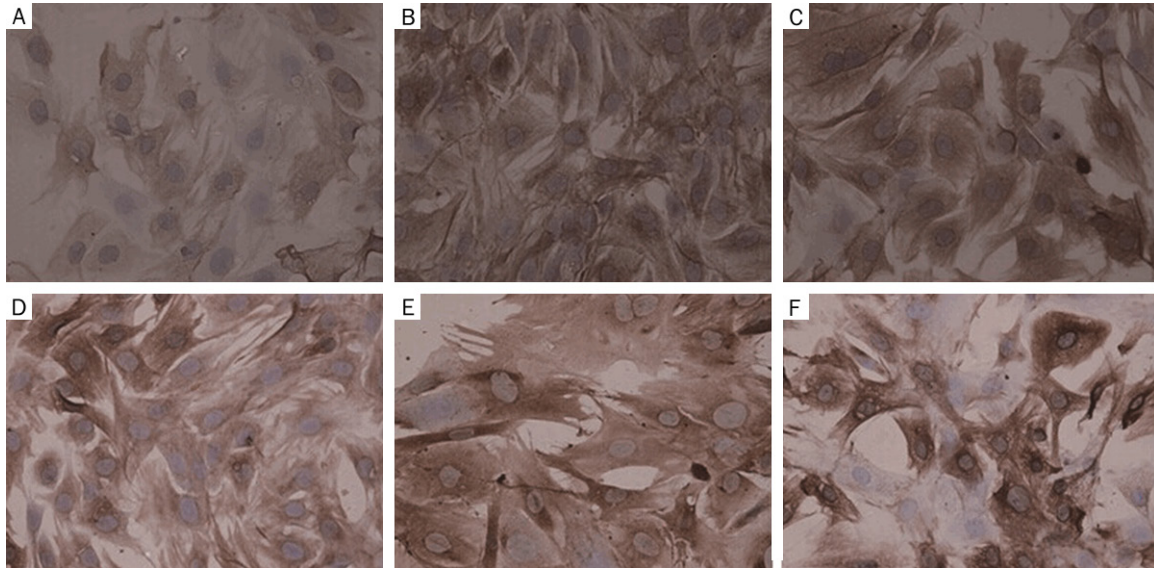


Figure 9. Immunohistochemical staining of type III collagen in rat ASM cells ($\times 400$). Brown granules: positive cells, mainly expressed in the cell membrane and cytoplasm. A. Control group; B. Asthma 2 w group; C. Asthma 4 w group; D. Asthma 6 w group; E. Asthma 8 w group; F. NK-1R antagonist group.

In this study, the average A of ASM cells in the asthma groups measured by MTT methods at each time point were higher than the control group, and the asthma 8 w group was the highest, the difference was significant ($P < 0.05$), indicating that ASM cell proliferation of the airway remodeling model groups was significantly faster, and showing that abnormal proliferation of ASM cells existed in asthma rats and can maintain the state until cell cultures. The ASM cell abnormal proliferation mechanism of airway remodeling model groups in vitro may be: ASM cell proliferation underwent the regulation of cell phenotype from contractile type into a synthesis proliferative type, the latter can secrete mitogenic agents of inflammatory mediators and growth factors, the cultured asthma ASM cells promoted the abnormal proliferation itself through autocrine mitogenic agents [12, 13]. The results of NK-1R antagonist WIN62577 treatment with different concentrations showed that NK-1R receptor antagonists can inhibit the proliferation of ASM cells with dose-dependent manner. The cell proliferation of ASM cells asthma 8 w group was significantly inhibited when treated by 10^{-8} mol/L of WIN62577, which fully explained the role of NK-1R in the proliferation of asthma ASM cells. The stimulation to NK-1 receptor of ASM cell membrane by SP can initiate cell proliferation.

Studies have found that SP had chemotactic effect to human T lymphocytes and monocytes under the role of SP with concentrations of 10^{-6} M. NK-1 receptor agonist can significantly stimulate the chemotaxis of the both kinds of cells. Under the role of selective antagonist LY303870 of NK-1 receptor, the chemotactic effect of SP for both cells was significantly inhibited [14, 15]. The results of this study also showed that the number of migration in the airway remodeling ASM cell group significantly increased. The blocking NK-1R by 10^{-8} mol/L WIN62577 may inhibit the migration of ASM cells, indicating that NK-1R also played an important effect in ASM cell migration.

Many researches showed that a variety of cytokines had closely relationship with collagen synthesis, bFGF, IGF-1, TNF- α , TGF- β and other cytokines significantly promoted the collagen synthesis of fibroblasts [16-18]. Studies have shown that exogenous SP can significantly stimulate fibroblast proliferation, collagen synthesis and angiogenesis, as well as significantly accelerate the healing of damaged tissues [19, 20]. The results of this study showed that the expression of type III collagen in the ASM cells of airway remodeling group was significantly higher, indicating that ASM cell collagen synthesis of the airway remodeling group was significantly stronger than that of the normal ASM cells. After

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blocking NK-1R by SP receptor, the type III collagen expression of ASMC in the airway remodeling group significantly reduced, suggesting that SP and NK-1R receptor played important roles in ASMC collagen synthesis.

Blocking NK-1R, inhibiting ASMC proliferation, migration, synthesis, secretion and other functions may be a new way of preventing and treating asthma airway remodeling, but the downstream mechanism of NK-1R still needed to be further explored.

Disclosure of conflict of interest

None.

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