Ardisia japonica inhibits airway remodeling in chronic obstructive pulmonary disease in rats through inhibiting Wnt signaling pathway

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Research

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Abstract

Background The effects of Ardisia japonica (AJ) on airway remodeling in chronic obstructive pulmonary disease (COPD) rats, and the mechanisms have not been verified. This study aimed to investigate the effects of a Miao medicine, AJ on airway remodeling in COPD rats, and to assess the mechanisms.

Methods COPD model was produced by cigarette smoke and intratracheal injection of lipopolysaccharide (LPS). The experiments were divided into a normal group, a COPD group, different doses of AJ treatment groups and a positive control group. After treatments, matrix metalloprotein (MMP)-9, platelet derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) levels in inferior lobes of the right lung tissues were detected by ELISA. Expression of Wnt signaling pathway was detected by immunohistochemistry and Western blotting. To verify the function of Wnt signaling pathway in airway remodeling, airway fibroblasts obtained from COPD rats were treated with Wnt signaling pathway agonists. The cell proliferation, apoptosis, and MMP-9, PDGF, TGF-β1 levels were determined.

Results The pathological changes of COPD were confirmed by haematoxylin eosin (H&E) staining. MMP-9, PDGF and TGF-β1 levels were promoted in COPD rats, which were significantly reduced by different doses of AJ treatment. Importantly, components of Wnt signaling pathway, including Wnt5a, β-catenin and RhoA were up-regulated in COPD model, which were also significantly reduced by treatment with different doses of AJ. The airway fibroblasts were obtained from COPD rats and verified based on vimentin expression. Wnt signaling pathway agonists, lithium chloride (Licl), 4-Ethyl-5,6-Dihydro-5-methyl-[1, 3] dioxolo[4,5-j] phenanthridine (HLY78), TPA and epidermal growth factor (EGF) promoted cell proliferation, reduced apoptosis, and promoted MMP-9, PDGF and TGF-β1 levels.

Conclusions Our data implicated that AJ could prevent airway remodeling in COPD rats, likely via depressing Wnt signaling pathway.

Background

Chronic obstructive pulmonary disease (COPD) is a preventable and treatable disease, featured by incompletely reversible airflow obstruction. In COPD, airway and lungs likely remodel in response to noxious particles or gases-related chronic inflammation, ultimately leading to pulmonary hypertension, pulmonary heart disease and heart failure, etc. (1). COPD is caused by the interaction of environmental and genetic factors. Long-term smoking, air pollution, chemical exposure, occupation inhalation of dust and biofuel smoke, and recurrent respiratory tract infection can damage the bronchial mucosa and induce airway and pulmonary inflammation, which induces or aggravates the severity of COPD. Among them, smoking is the major risk factor for the development of COPD (2–5). Airway remodeling is the key regulating step involved in the progress of COPD. Importantly, the essence of airway remodeling is the over-proliferation of airway fibroblasts (6). Transforming growth factor-β1 (TGF-β1), matrix metallopeptidase-9 (MMP-9) and platelet-derived growth factor (PDGF) are the main factors involved in
airway remodeling of COPD (7, 8). Therefore, the control of airway remodeling could be a new and useful treatment for COPD patients.

The Wnt signaling pathways affect physiological and pathological processes such as stress, immunity, cell differentiation, apoptosis, etc. (9). The Wnt signaling pathway is closely related to the mechanisms of pulmonary fibrosis, idiopathic pulmonary hypertension, pulmonary interstitial diseases and other diseases (10, 11). Abnormal activation of Wnt signaling pathway is also closely associated with chronic obstructive pulmonary disease, bronchial asthma and interstitial lung disease (12). TPA (12-O-tetradecanoylphorbol-13-acetate) can up-regulate Wnt signaling pathway by enhancing casein kinase 1 (CK1) activity (13). Wnt signaling pathway also interacts with mitogen-activated protein kinases (MAPKs) signaling pathway (14), which is a signaling pathway regulating cell growth and proliferation, affecting lung development (15). In addition, MAPK signaling pathway has also been reported to correlate with lung cancer and pneumonia (16–18).

*Ardisia japonica* (AJ) is a significant and unique Guizhou Miao medicine in China. Clinically, AJ has obvious antitussive and expectorant effects (19). COPD belongs to “cough”, “lung distention” etc. based upon the theory of traditional Chinese medicine (TCM). Especially, “cough”, “phlegm”, “asthma”, “fullness” and “stiffness” are the main symptoms of “lung distention”, while AJ has a good effect on the treatment of lung distention (20). The active components consist of gallic acid, bergenin, epicatechin, epicatechin gallate, isoquercitrin, and quercetin-3-rhamnoside (21). Modern pharmacological studies further suggest that the effective components of AJ has certain antiasthmatic, anti-fungal, hypolipidemic and anti-tumor effect (22, 23), and can effectively treat chronic bronchitis (19). Additionally, AJ also has antitussive and antiasthmatic, antibacterial, anti-inflammatory and antiviral activities (22, 24, 25). However, the effects of AJ on airway remodeling in COPD rats, and the mechanisms have not been verified. In this study, we aimed to investigate the effects of AJ on airway remodeling in COPD rats, and to assess the potential signaling pathway involved in the protection.

**Materials And Methods**

**Preparation of *Ardisia japonica***

*Ardisia japonica* (500 g), purchased from Anguo TCM market (Hebei, China) was added into 500 mL of water and soaked for 20 min. Thereafter, the mixture was boiled and then another 500 mL of water was added. Then, the medicine was decocted into 100 ml using slow fire. Finally, a decoction (100 g/100 mL) was prepared and kept at 4 °C for use.

**Animals and COPD model**

120 healthy male SD rats (120 ± 20 g, 6 weeks) were purchased from Tengxin Biotechnology Co., Ltd. in Chongqing [Certificate No. SCXK (Chongqing) 2012-0005] and housed in a SPF condition with a standard 12-h light/12-h dark cycle and *ad libitum* access to food and water. The average humidity was 40%-60%,
and the average temperature was 20-25 °C. The experiments were approved by the Ethics Committee of Guiyang College of Traditional Chinese Medicine.

The COPD model was established as previously described (26). Briefly, SD rats were put in a smoking box (40 x 50 x 60 cm). In each smoking box, 10 rats were placed and exposed to 8 cigarettes each time and the smoking process lasted about 30 min. The passive smoking was delivered for twice each day for consecutive 28 days. On the first and the 14th days, lipopolysaccharide (LPS) (0.2 mL, 200 μg/200 μL, SIGMA) was administrated through intratracheal injection in the anesthetized rats (10% chloral hydrate, 0.37 mL/100 g). After anesthesia, the rats were fixed on the table in supine position. Longitudinal incision along the midline neck (about 1 cm) was prepared to deliver LPS. The rats were erected to keep the LPS flowing along the wall of the trachea to the alveoli. The wound was sutured layer by layer, and penicillin powder was applied to the incision. Eight days after modeling, animals were anesthetized with 5% isoflurane and airway tissues were fixed in 4% paraformaldehyde (PFA). The lung injury was confirmed by H&E staining.

The experiments were divided into normal group (Group A), COPD group (Group B), COPD + low dose of AJ group (0.75 mL/kg, Group C); COPD + medium dose of AJ group (1.5 mL/kg, group D); COPD + high dose of AJ group (3 mL/kg, group E); COPD + dexamethasone group (Group F). AJ was delivered through intragastric administration once daily for 30 days. In COPD + dexamethasone group, the rats were treated with 1 mg/kg dexamethasone (intramuscular injection, once daily) for consecutive 30 days. After that, inferior lobes of the right lung tissues were collected for molecular biochemical experiments or fixed in 4% for pathological staining (Fig.1).

**H&E staining**

Inferior lobes of the right lung tissues were fixed in 4% paraformaldehyde for ~1 week at 4°C. Thereafter, the tissues were dehydrated, embedded, and sliced. The paraffin section is dewaxed and hydrated. The tissues were rinsed for several hours, thereafter dehydrated, embedded, and sliced. The paraffin section is dewaxed and hydrated. The sections were stained with hematoxylin for 5 min and with eosin for 3 min. The images were taken by a light microscopy.

**Preparation of airway fibroblasts**

Under the anesthesia with isoflurane (5%), the rats were decapitated and thoracotomized in aseptic conditions. Fresh airway tissue of COPD rats was washed repeatedly with PBS in aseptic worktable for 5 times. Sterile scalpel was used to collect the tissue around the airway. The tissue was cut into 2 mm x 2 mm with sterilized ophthalmic scissors and pasted into the culture plate, and placed in a 5% CO₂ incubator 37°C for 4 h. The cells completely adhered to the wall and the newly prepared DMEM (Gibco) + 20% FBS (Hyclone) medium was added to the culture plate, and the cells were further cultured in a 37°C, 5% CO₂ incubator. The growth of the cells was observed and then treated routinely. The cultured dishes were fixed in 4% paraformaldehyde for 15 min, and permeated with 0.5% Triton X-100 (PBS) for 20 min at room temperature. 5% BSA was dripped into the dish and sealed at 37 °C for 30 minutes. A sufficient
amount of diluted vimentin (1:250, ab92547, Abcam) was dripped into the dish and incubated at 37 °C for 3 h. The diluted fluorescent antibody Cy3 (1:200) was added and incubated at 37°C for 30 min. DAPI (4’,6-diamidino-2-phenylindole) was used to stain the nuclei. The images were observed under fluorescence microscope.

The collected airway fibroblasts were divided into nine groups as listed in Fig.1A: Normal, lithium chloride (Licl), 4-Ethyl-5,6-Dihydro-5-methyl-[1,3] dioxolo[4,5-j] phenanthridine (HLY78), TPA (cat. no. 425KO21, Solarbio) and epidermal growth factor (EGF) (L1450411, Cyagen) groups. The cells were treated with 10 mM Licl, 10 μM HLY78, 10 nmol/L TPA and 50 ng/ml EGF for 24 h, respectively.

**ELISA**

Matrix metalloprotein (MMP)-9, platelet derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) levels were detected by ELISA following the instructions of the assay kits (QIYI Biotech, Shanghai, China). The reagents in the kit were kept at room temperature for 30 minutes. A standard curve was established to calculate the levels of target proteins. The airway tissues were homogenated and centrifuged at 11,000 g for 10 min at 4 °C. The supernatants were used in the experiments. All standard samples and test samples required 3 duplicates. A blank control was set without sample and enzyme reagent. Absorbance was detected at the wavelength of 450 nm by a Microplate Reader (RT-6100, Rayto).

Inferior lobes of the right lung tissues were fixed in 4% paraformaldehyde for ~1 week at 4°C. Thereafter, the tissues were dehydrated, embedded, and sliced. The paraffin section is dewaxed and hydrated. Immunostaining of histological sections was performed using monoclonal antibodies against RhoA (1:200, ab187027, Abcam, USA), Wnt5a (1:200, ab174963, Abcam, USA) and β-catenin (1:200, ab32572, Abcam, USA). Endogenous peroxidase activity was blocked with 3% (v/v) H2O2 for 5 minutes at room temperature. Subsequently, tissues were incubated with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG secondary antibody (1:10,000; cat. no. A16104SAMPLE; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 30 min at room temperature and visualized with 3,3’-diaminobenzidine chromogen for 3 minutes at room temperature. The images were taken under a light microscopy.

**Western blotting**

Homogenates from inferior lobes of the right lung tissues were obtained from each group and lysed. The protein level was detected by Bicinchoninic Acid Kit (Beyotime Institute of Biotechnology, Shanghai, China). Protein samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%) and transferred onto nitrocellulones membranes. The membranes were blocked with Tris-buffered saline (TBS), containing 0.1 % Tween-20 (TBST) and 5 % fat-free milk for 2 h at room temperature and then incubated (overnight, at 4 °C) with the rabbit antibodies against RhoA (1:200, ab187027, Abcam, USA), Wnt5a (1:200, ab174963, Abcam, USA) and β-catenin (1:200, ab32572, Abcam,
USA) and Actin (1:500, Zsbio, Beijing, China) followed by a 2-h incubation with a peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:5000, Zsbio, Beijing, China) at room temperature. Chemiluminescent substrate detection reagent was applied to show the staining.

Data analysis

The data were expressed by mean ± standard deviation and statistically analyzed by SPSS 19 (SPSS, Inc., Chicago, IL, USA). One-way ANOVA with Newman-Keuls as the post-hoc test was applied to determine statistical significance. A value of P<0.05 was considered to be significant.

Results

AJ reduced COPD-induced pathological changes

Lung injury in COPD model was confirmed by H&E staining (Fig.1). In control group, the airway tissue showed regular alveolar structure. Pathological expansion and fusion of alveolar cavity were not observable. Bronchial tube wall was normal; the airway mucosa epithelium was smooth; cilia were arranged neatly; obvious inflammatory exudation was not found in the tracheal cavity. By contrast, emphysema, alveolar dilatation, and alveolar wall gradually broken and fused into lung ulcers and the numbers of alveolar decreased significantly in COPD model group. The goblet cells of bronchial epithelium proliferated and a large number of inflammatory cells such as neutrophils and giant cells infiltrated the lumen, accompanied by the proliferation of fibrous connective tissue

AJ reduced COPD-induced MMP-9, PDGF and TGF-β1 levels

The expression of MMP-9, PDGF and TGF-β1 in inferior lobes of the right lung tissues were shown in Fig.2. Compared with the control group, COPD group showed increased MMP-9, PDGF and TGF-β1 expression in inferior lobes of the right lung tissues. By contrast, different doses of AJ and dexamethasone significantly reduced MMP-9, PDGF and TGF-β1 expression compared with those in COPD group (P < 0.05).

AJ reduced COPD-induced RhoA expression

The expression of RhoA in inferior lobes of the right lung tissues was detected by immunohistochemistry and Western blotting (Fig.3). Compared with the control group, the expression of RhoA in COPD group increased significantly. Compared with the COPD group, RhoA expression was significantly reduced in different doses of AJ groups and dexamethasone group (P < 0.05).

AJ reduced COPD-induced β-catenin expression

The expression of β-catenin in inferior lobes of the right lung tissues was detected by immunohistochemistry and Western blotting (Fig.4). Compared with the control group, the expression of
β-catenin in COPD group increased significantly. Compared with the COPD group, RhoA expression was significantly reduced in different doses of AJ groups and dexamethasone group (P < 0.05).

**AJ reduced COPD-induced Wnt5a expression**

The expression of Wnt5a in inferior lobes of the right lung tissues was detected by both of immunohistochemistry and Western blotting (Fig.5). Compared with the control group, the expression of Wnt5a in COPD group increased significantly. Compared with the COPD group, Wnt5a expression was significantly reduced in different doses of AJ groups and dexamethasone group (P < 0.05).

**Wnt signaling pathway agonists promoted cell proliferation of airway fibroblasts**

As shown in Fig.6A, immunofluorescence staining for vimentin of the cultured rat alveolar cells identified the presence of fibroblasts. Cell proliferation was detected based on the ratio of CCK8 24 h to CCK8 0 h. The results showed that HLY78, LiCl, TPA and EGF promoted the ratio (Fig.6B), which indicates that Wnt signaling pathway agonists promoted cell proliferation.

**Wnt signaling pathway agonists reduced apoptosis of airway fibroblasts**

Apoptosis was also detected in the airway fibroblasts. The results showed that HLY78, LiCl, TPA and EGF reduced apoptosis significantly (Fig.7).

**Wnt signaling pathway agonists promoted MMP-9, PDGF and TGF-β1 levels**

Compared with normal control cells, MMP-9, PDGF and TGF-β1 levels in HLY78, LiCl, TPA and EGF-treated cells were significantly promoted (Fig.8).

**Discussion**

In this study, we revealed a novel effect of AJ on airway remodeling in COPD rats. AJ reduced MMP-9, PDGF and TGF-β1 levels in COPD model, which implicated the protection of AJ in COPD model. Moreover, we demonstrated that AJ antagonized the airway remodeling likely through inactivating Wnt5a pathway, as Wnt agonists promoted cell proliferation, reduced apoptosis and promoted MMP-9, PDGF and TGF-β1 levels of airway fibroblasts.

COPD is a group of lung diseases characterized by airflow restriction and incomplete reversibility. Although the exact cause is still unknown, COPD is related to smoking, air pollution, dust and other factors (27). Oxidative stress, inflammation, protease activation are the main pathogenesis of COPD (28, 29). The pathological changes in COPD patients include chronic bronchitis, emphysema, airway reconstruction and pulmonary remodeling (30). LPS has a complex of polysaccharide and protein structure of the outer membrane with gram negative bacteria and could cause release of proinflammatory cytokines, directly impairing airway epithelial layer (31). In this study, COPD model was established in SD rats by cigarette smoking and LPS injection, because SD rats are sensitive to the smoke, dust, sulfureted
hydrogen and other stimuli in the air, and were susceptible to respiratory diseases (32). The results of H&E staining confirmed that COPD model was successfully established in our study.

TGF-β1 is a multifunctional cytokine that could induce epithelial cell layer destruction, airway inflammation, smooth muscle cell proliferation, goblet cell proliferation and vascular remodeling (33). TGF-β1 inhibits enzymes that degrade matrix protein and regulate the expression of matrix protein on the cell surface (34). Animal experiments further demonstrated that TGF-β1 could stimulate the pathological aggregation of extracellular matrix (34). Overexpression of TGF-β1 was found in asthmatic and COPD patients, as well as in epithelial cells and smooth muscle cells of acute, subacute and chronic asthmatic mice (35). Therefore, TGF-β1 played an important role in airway remodeling in smooth muscle cells and fibroblasts (35). In this present study, we found TGF-β1 was promoted in COPD rats, which was reduced by different doses of AJ or dexamethasone treatments. As evidenced by previous fingerprint study, *Ardisia japonica* is comprised of gallic acid, bergenin, chlorogenic acid and quercitrin (36). Moreover, these components possess airway protection in pathological conditions (37).

PDGF is mainly released by mononuclear cells, endothelial cells, bronchial epithelial cells and smooth muscle cells (38) and can stimulate the proliferation and chemotaxis of fibroblasts and the synthesis of extracellular matrix, which is one of the important mechanisms underlying airway reconstruction (39). PDGF can stimulate bronchial intermediate muscle fibroblasts to produce FK506 binding protein, motor-related protein 3 and heat shock protein. It is considered that PDGF and its signal transduction pathway may play important roles in airway remodeling and fibrosis (40). In this present study, we found PDGF was promoted in COPD rats, which was reduced by different doses of AJ or dexamethasone treatments. These data further supported the protection of AJ.

Extracellular matrix (ECM) is required to maintain alveolar structure. The degradation and synthesis of ECM should maintain a dynamic balance, which is required to ensure normal function of lung (41). MMPs are mainly involved in the renewal of ECM, the healing of damage and the response to injury (35). MMP-9 is produced by lung structural cells and inflammatory cells, which can degrade proteoglycan, promote airway fibrosis and activate potential binding growth factors, and induce smooth muscle proliferation to participate in airway remodeling (42). In this study, we found that the contents of MMP-9 increased significantly after COPD modeling. These results revealed that MMP-9 played important roles in the process of airway remodeling, and were also consistent with those studies mentioned above (34, 35). Critically, AJ reduced MMP-9 level in COPD model.

Wnt5a is one of the important members of the Wnt family. Like other Wnt members, Wnt5a could function through an autocrine or paracrine forms, after binding to the transmembrane receptor Frz receptor (43). β-catenin mainly locates at the cell membrane. Its function mainly mediates intercellular adhesion and participates in regulating gene expression (44). The down-regulation of β-catenin would destroy the adhesion between cells and promote cell invasion ability (45). β-catenin also functions as the downstream of the classical Wnt signaling pathway to regulate cell growth and differentiation (46).
Rho GTP enzyme plays an important role in the regulation of cytoskeleton recombination. As evidenced, Rho GTP enzyme is highly expressed in a variety of malignant tumors and is closely related to tumor occurrence, invasion and metastasis (47). Our present study demonstrated that the expression of Wnt5a, β-catenin and RhoA increased significantly after the COPD modeling. These results implicated that the COPD model may affect airway remodeling by up-regulating the expression of Wnt signaling pathway. Interestingly, AJ could attenuate the expression of Wnt5a, β-catenin and RhoA.

Wnt signaling pathway and MAPK signaling pathway have similarities in functions, and the two signaling pathways also have a mutually reinforcing role (48, 49). Components of the MAPK signaling pathway, such as ERK1/2, p38 and JNK, can promote the phosphorylation of LDL Receptor Related Protein 6, thus up-regulating Wnt/β-catenin signaling pathway (50). Wnt5a can promote ERK1/2 protein phosphorylation (14). In this study, we found that both signaling pathways were involved in the remodeling of fibroblasts from COPD rats.

We have previously reported that hyperplasia suppressor gene (HSG) overexpression inactivated airway fibroblasts from COPD by inhibiting the Wnt signaling pathway (51). In this study, we also provided in vivo data showing that AJ in different doses ameliorated airway remodeling and antagonized Wnt signaling pathway. Using the cultured airway fibroblasts obtained from COPD model, we further verified that Wnt agonists LiCl and HLY78 promoted the cell proliferation and reduced apoptosis. We could speculate that the antagonists of Wnt signaling pathway would block the cell proliferation and promote apoptosis, like the effect of HSG overexpression (51). Additionally, MAPK pathway agonists also exhibited the similar functions as Wnt signaling pathway agonists. These results implications a complex crosstalk involved in the remodeling of airway fibroblasts. The mechanisms still require deep clarification in future.

**Conclusion**

AJ antagonized the airway remodeling likely through inactivating Wnt5a pathway. This study would provide experimental basis for the clinical application of AJ in COPD.

**Abbreviations**

COPD: chronic obstructive pulmonary disease; AJ: Ardisia japonica; LPS: lipopolysaccharide; TGF-β1: transforming growth factor-β1; CK1: enhancing casein kinase 1; MAPKs: mitogen-activated protein kinases.

**Declarations**

**Ethics approval and consent to participate**

The experiments were approved by the Ethics Committee of Guiyang College of Traditional Chinese Medicine.
Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YY, XZ, JZ and BL, XX and XL did the experiments and analyzed the data. YY and ZG designed the study and wrote the manuscript.

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Figures
Figure 1

Morphological changes of airway tissue after COPD modeling. The typical pathological changes of chronic bronchitis and obstructive emphysema especially hyperplasia and hypertrophy of small bronchial smooth muscle were indicated in COPD group.
Figure 2

AJ reduced COPD-induced MMP-9, PDGF and TGF-β1 levels. A) MMP-9, B) PDGF, C) TGF-β1. *P <0.05 compared with normal group; #P <0.05 compared with COPD group.
Figure 3
AJ reduced COPD-induced RhoA expression. A) Representative immunohistochemical images; B) Representative blots; C) Quantification data of the blots. *P <0.05 compared with normal group; #P <0.05 compared with COPD group.

Figure 4
AJ reduced COPD-induced β-catenin expression. A) Representative immunohistochemical images; B) Representative blots; C) Quantification data of the blots. *P <0.05 compared with normal group; #P <0.05 compared with COPD group.
Figure 5

AJ reduced COPD-induced Wnt5a expression. A) Representative immunohistochemical images; B) Representative blots; C) Quantification data of the blots. *P <0.05 compared with normal group; #P <0.05 compared with COPD group.
Figure 6

Wnt signaling pathway agonists promoted cell proliferation of airway fibroblasts. (A) Photomicrograph of the immunofluorescence staining for vimentin and nuclear counterstaining with 4’6-diamidino-2-phenylindole (DAPI). Magnification x 200. (B) HLY78, LiCl, TPA and EGF promoted cell proliferation of airway fibroblasts. *P<0.05 vs. Normal.
Figure 7
Wnt signaling pathway agonists reduced apoptosis of airway fibroblasts. (A) Representative images of flow cytometry. (B) HLY78, LiCl, TPA and EGF reduced apoptosis of airway fibroblasts. *P<0.05 vs. Normal.

Figure 8

Wnt signaling pathway agonists promoted MMP-9, PDGF and TGF-β1 levels. *P<0.05 vs. Normal.