

## Original Research Article

# Qibai Pingfei capsule induces apoptosis of pulmonary artery smooth muscle cells in hypoxic rats by regulating PI3K/AKT/mitochondrial signaling pathway

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Sent for review: 27 February 2023

Revised accepted: 28 July 2023

### Abstract

**Purpose:** To investigate the effect of Qibai Pingfei capsules (QBPF) medicated serum on the apoptosis of rat pulmonary artery smooth muscle cells (PASMC) in hypoxic rats, and to determine the relationship between that effect and PI3K/Akt/mitochondrial apoptosis pathway.

**Methods:** Rat PASMCs were isolated, cultured, and the optimal hypoxic time and concentration of QBPF were determined by CCK-8 method. Hypoxic rats were treated with QBPF, QBPF + LY294002, or QBPF + SC79. Apoptosis and mitochondrial membrane potential were assessed using Annexin V-FITC/PI, Hoechst 33258, and Rho123 staining. The protein expression levels of AKT, P-AKT, and apoptosis-related proteins were evaluated via western blot.

**Results:** CCK-8 studies showed that the optimal hypoxic time was 24 h, while the optimal concentration of QBPF was 20 %. Annexin V-FITC/PI double staining and Hoechst 33258 assay revealed that QBPF significantly promoted the apoptosis of PASMCs in hypoxic rats ( $p < 0.05$ ). Rho123 test results showed that QBPF inhibited mitochondrial membrane potential level in hypoxic rats' PASMCs, which was enhanced by PI3K inhibitor LY29002 and inhibited by AKT agonist SC79 ( $p < 0.05$ ). Western blot showed that QBPF reduced the protein level of P-AKT and Bcl-2, and raised the protein levels of Bad, Bax, CytC, caspase-9 and caspase-3, which was enhanced by LY29002 and blocked by SC79 ( $p < 0.05$ ). No major changes in AKT protein expression were seen between the groups.

**Conclusion:** In hypoxic rats, QBPF blocks PI3K/AKT signaling pathway and regulates the activation of downstream Bcl-2 family members, thus activating mitochondrial apoptosis pathway and triggering PASMC death.

**Keywords:** Qibai Pingfei capsule, PI3K/AKT, Pulmonary artery smooth muscle cells (PASMC), Apoptosis

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## INTRODUCTION

Hypoxic pulmonary hypertension (HPH) is a common consequence of Chronic Obstructive

Pulmonary Disease (COPD), which leads to development of pulmonary heart disease. It has caused serious economic and social problems [1]. As a result, controlling pulmonary

hypertension is an important step in slowing the progression of COPD into pulmonary heart disease. Pulmonary vascular remodeling is the key factor and pathological process in the development of HPH [2]. Pulmonary vascular remodeling is marked by pulmonary artery medium thickening. The tunica media of the pulmonary artery is primarily composed of PASMCs. Under normal conditions, the proliferation and apoptosis of PASMCs are in dynamic balance, but under hypoxic stimulation, the media of pulmonary vessels, as well as pulmonary vasculature, thicken, thus inducing pulmonary vascular remodeling [3,4]. Therefore, promoting the apoptosis of PASMCs contributes to the inhibition of HPH.

*Qibai Pingfei* capsules are prepared in the hospital, and it has been used to treat COPD and pulmonary hypertension with significant efficacy for a long time. Previous studies have shown that *Qibai Pingfei* capsules relaxed pulmonary vessels of hypoxic rats and promoted the apoptosis of hypoxic PASMCs, thus alleviating the occurrence and development of COPD and pulmonary hypertension [5,6]. However, the specific mechanism by which *Qibai Pingfei* capsule promotes hypoxia-induced apoptosis of rat PASMCs needs more studies. The regulation of apoptosis is restricted by a variety of apoptotic signal transduction pathways, such as phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway [7,8].

In this study, primary rat PASMCs were used to investigate the effect of *Qibai Pingfei* in inducing apoptosis of hypoxic PASMCs by regulating the PI3K/AKT/ mitochondrial apoptosis signaling pathway.

## EXPERIMENTAL

### Animals

Sixty (60) male Sprague-Dawley (SD) rats (220 ± 20 g), were purchased from the Laboratory Animal Center of Shandong Province, Animal Qualification Certificate number: SCXK 20190003. The rats were housed under standard laboratory conditions at room temperature and humidity, with free access to water and feed. Animal study was approved by the animal ethical committee of Anhui University of Traditional Chinese Medicine (approval no. AHUCM-rats-2021087) and performed in conformity with the National Institute of Health revised guidelines for laboratory animal care and use [9].

### Drugs

*Qibai Pingfei* capsule (composition: *Astragalus*, *Xiebai*, *Radix bashen*, *Chuangxiang*, *Descurainia descuraini*, *Schisandra fructus*, and *Lumbricus*) was provided by the Department of Pharmaceutical Products, the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine (batch no. 20190805).

### Preparation of serum containing *Qibai Pingfei* capsule

A total of thirty SD rats were selected. Rats were assigned to two groups at random, with 15 rats each namely; control group, and study group. The drug in the *Qibai Pingfei* capsule was milled into powder, and the rats were gavaged at 1.0 g/kg twice a day. The rats were given a normal diet with gavage for 9 days. On the morning of the 10th day, the rats were anesthetized 1h after taking the daily dose, and the blood samples were placed in ordinary tubes without any additives for 2 h at room temperature. After blood coagulation, samples were spun at 3000 rpm for 15 min, the serum was taken for inactivation of complement factors at 56 °C for 30 min. The blank serum (control group) and serum from animals administered *Qibai Pingfei* capsules (experimental group) were mixed separately, and passed through 0.22 µm filter for sterilization. After packing, it was stored at -20 °C until use.

### Culture and identification of rat PASMCs

#### Culture of rat PASMCs

Primary rat PASMCs were cultured using the tissue block adherence method. Hearts and lungs of anesthetized rats were quickly excised under sterile conditions. The middle and small pulmonary arteries were separated under a surgical microscope. The middle pulmonary artery with inner and outer membrane removed was cut into 1 mm<sup>2</sup> tissue blocks and transferred to a 25 mL culture bottle with DMEM/F12 medium containing 1 % double antibody and 20 % FBS. They were transferred to a 37 °C, 5 % CO<sub>2</sub> incubator for 4 h. Medium was changed on alternate days. After about 7 - 10 days, PASMCs were obtained. The cells were treated with trypsin, collected, added to the medium for resuspension, and divided into culture flasks for continued culture, changing the medium in which they were grown every two to three days.

#### Identification of rat PASMCs

PASMCs were spread into six-well plates and incubated routinely. When the cell density

approached about 60 %, culture medium was removed, and cells were washed with PBS, fixed with 4 % paraformaldehyde, permeabilized with 0.5 % Triton X-100, and blocked with goat blank serum for 1 h. The cells were treated with rabbit anti-mouse  $\alpha$ -smooth muscle actin ( $\alpha$  with antibody (dilution 1: 100), and then incubated at 4 °C overnight. The primary antibody was removed, washed with PBS, and an appropriate amount of FITC-labeled secondary antibody was used. The cells were incubated at 37 °C for 1 h before being washed with PBS (all operations were performed in the dark due to addition of fluorescent secondary antibody). The slides were stained with DAPI for 5 min and then washed with PBS. The slides were observed under a fluorescence microscope.

### **Serum screening and hypoxia time**

#### ***Selection of optimal time of hypoxia***

Pulmonary artery smooth muscle cells (PASMCs) from rats in good growth condition were inoculated in 96-well plates at  $1 \times 10^5$ /mL and cultured at 37 °C for 12 h. Old medium was removed by suction, and serum-free medium was added for 12 h. The experiment had a control group that was in a normal level of oxygen and a model group in a low oxygen level. Each group had 5 holes.

The normoxic control group and the hypoxia model group were cultured in DMEM/F12 medium containing 20 % serum, and were cultured in a normoxic incubator (20 % O<sub>2</sub>, 75 % N<sub>2</sub>, 5 % CO<sub>2</sub>) and a three-gas incubator (3 % O<sub>2</sub>, 92 % N<sub>2</sub>, 5 % CO<sub>2</sub>) for 6, 12, 24 and 48 h respectively. Each well was filled with 10 mL CCK-8 solution and incubated for 1 h at 37 °C. Cell viability was assessed by measuring absorbance at 450 nm.

#### ***Screening of optimal drug-containing serum concentration***

Pulmonary artery smooth muscle cells (PASMs) with good growth were inoculated in 96-well culture plates at  $1 \times 10^5$ /mL, and cultured for 12 h at 37 °C to observe uniformity of cell seeding. Serum-free media was added to continue the culture for 12 h. The cells were grouped into 5 namely; hypoxic model group, hypoxia with 5 % QBPF group, hypoxia with 10 % QBPF group, hypoxia with 15 % QBPF group, hypoxia with 20 % QBPF group. All groups were lysed with 20 % rat serum concentration, and normal rat serum was used to supplement if the concentration was insufficient. The cells of each group were cultured for the optimal hypoxic time.

Subsequent cell treatment and cell viability calculations were performed as above. The optimal concentration of drug-containing serum was selected according to cell viability.

### **Apoptosis of PASMCs**

Pulmonary artery smooth muscle cells (PASMs) were injected at  $1 \times 10^5$  cells/mL in 6-well plates and separated into normoxia, hypoxia, and hypoxia + 20 % QBPF groups. After being cultured at 37 °C for 12 h, the cells of three groups were placed in corresponding incubators for 24 h, centrifuged, supernatant was removed, and the cells were blasted with phosphate buffered saline (PBS) and centrifuged. Phosphate buffered saline (PBS) was removed via suction, 1 % Annexin V binding solution was added, blown and mixed.

The cells were then transferred to a flow tube, mixed with Annexin V-FITC staining solution, blown, incubated for 15 min in the dark and 5-10 mL PI staining solution was added, gently blown several times and incubated in the dark for another 5 min. The evaluation was completed within 1 h by flow cytometry and analyzed by Kaluza software.

### **Morphological alterations of apoptotic cells**

Cell seeding and grouping were performed, and the cells in each group were placed in their corresponding incubators for 24 h, fixed with 4 % paraformaldehyde, rinsed twice with PBS, stained with 0.5 mL 33258 staining solution, placed at room temperature for 5 min, rinsed twice with PBS, and observed under a fluorescence microscope.

### **Mitochondrial membrane potential of PASMCs**

Pulmonary artery smooth muscle cells (PASMs) in good growth state were collected and seeded in 6-well plates at a concentration of  $1 \times 10^5$  cells/mL and grouped into normoxia control group, hypoxia model group, hypoxia + 20 % QBPF group, hypoxia + 20 % QBPF + LY29002 group, hypoxia + 20 % QBPF + SC79 group. After treatment for 24 h, cells in each group were collected and treated with PBS. Re-suspended cells were stained with 500  $\mu$ L of Rhodamine123 staining working solution to a cell density of approximately 5 to 10  $\times 10^5$ /mL. The cells were cultured at 37 °C for 15 min, removed from the incubator, collected and washed by adding PBS. Cells were resuspended in 500  $\mu$ L of prewarmed staining buffer. Flow cytometry and FlowJo software were used for analysis.

## Western blot

At 80 % cell density, cells in each group were treated based on the group conditions for 24 h, washed with PBS. The total protein was extracted. Western blot was performed to detect AKT, P-AKT, Bad, Bcl-2, Bax, CytC, caspase-9, and caspase-3.

## Statistical analysis

Data were analyzed using SPSS 26.0 software (IBM, Armonk, New York). Continuous data were described as mean  $\pm$  standard deviation (mean  $\pm$  SD) and tested by the student's *t*-test. A *p*-value  $<$  0.05 was considered statistically significant.

## RESULTS

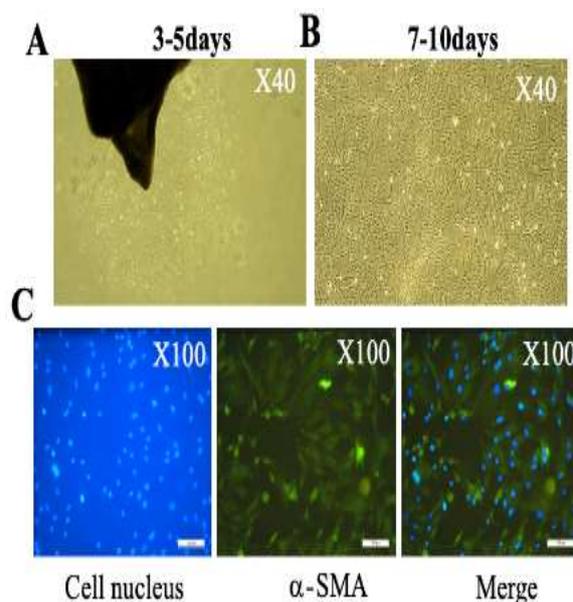
### Morphological characteristics and identification of rat PSMCs

A small number of irregularly long spindle-shaped cells could be seen climbing out near the wall on the third to fifth day after the primary rat pulmonary artery smooth muscle tissue was attached to the wall (Figure 1 A). After 7<sup>th</sup> – 10<sup>th</sup> day, cells were arranged in parallel bundles; in some areas, the cells were multilayers overlapping, while in others, the cells were monolayers. It showed a dense, peak-to-valley pattern characteristic of smooth muscle cells (Figure 1 B). Under fluorescence microscopy, FITC-labeled  $\alpha$ -SMA in the cytoplasm showed green fluorescence and was arranged in fibrous filaments, and DAPI-stained nuclei were oval and blue fluorescence, indicating PSMCs (Figure 1 C).

### Effect of serum containing Qibai Pingfei capsules on apoptosis of PSMCs

Cell viability of PSMCs at different times were significantly different after hypoxic culture ( $p <$  0.05), at 24 h, cell viability value was highest (Figure 2 A). As indicated in Figure 2 B, QBPF cell viability was much lower than hypoxia group ( $p <$  0.05) in a concentration-dependent manner, and 20 % QBPF was selected as the optimal drug-containing serum concentration. Apoptosis rate of PSMCs in hypoxia model group was much lower than in the normoxia one ( $p <$  0.05; Figure 2 C). The apoptosis rate of PSMCs in the hypoxia + 20 % QBPF group was substantially higher than the hypoxia model group ( $p <$  0.05; Figure 2 D).

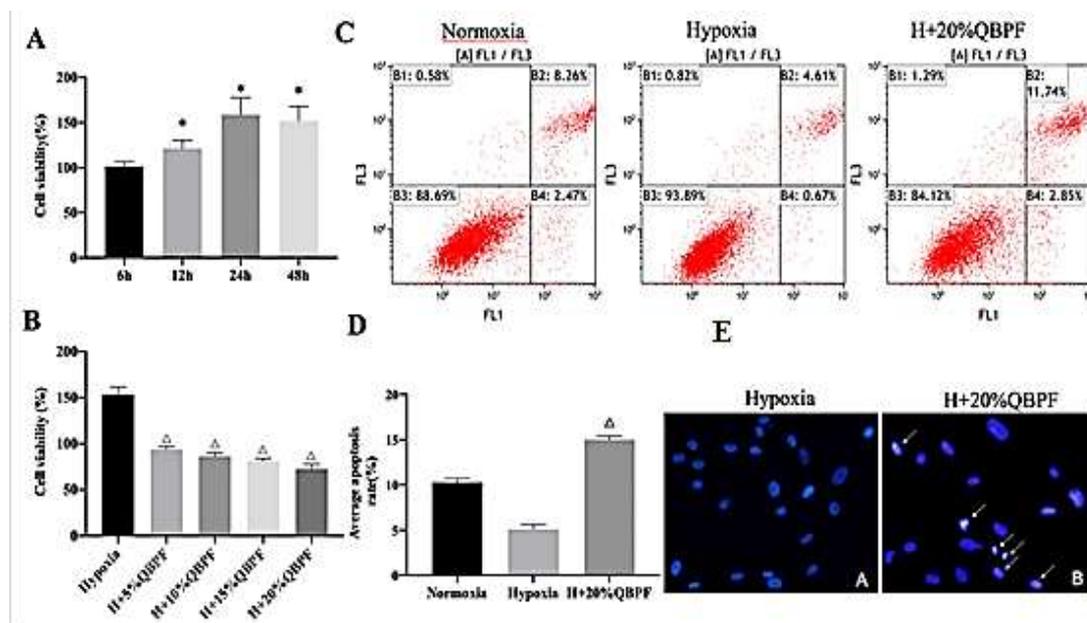
In contrast to the hypoxia model group, PSMCs in the hypoxia + 20 % QBPF group showed strong blue light in the nucleus and apoptosis with chromatin pyknosis and nuclear fragmentation after 24 h of QBPF intervention (Figure 2 E). The PSMCs showed normal blue color after Hoechst33258 staining. After 24 h of QBPF intervention, the nucleus of PSMCs emitted blue light, and the apoptosis phenomenon of chromatin pyknosis and nuclear fragmentation appeared.



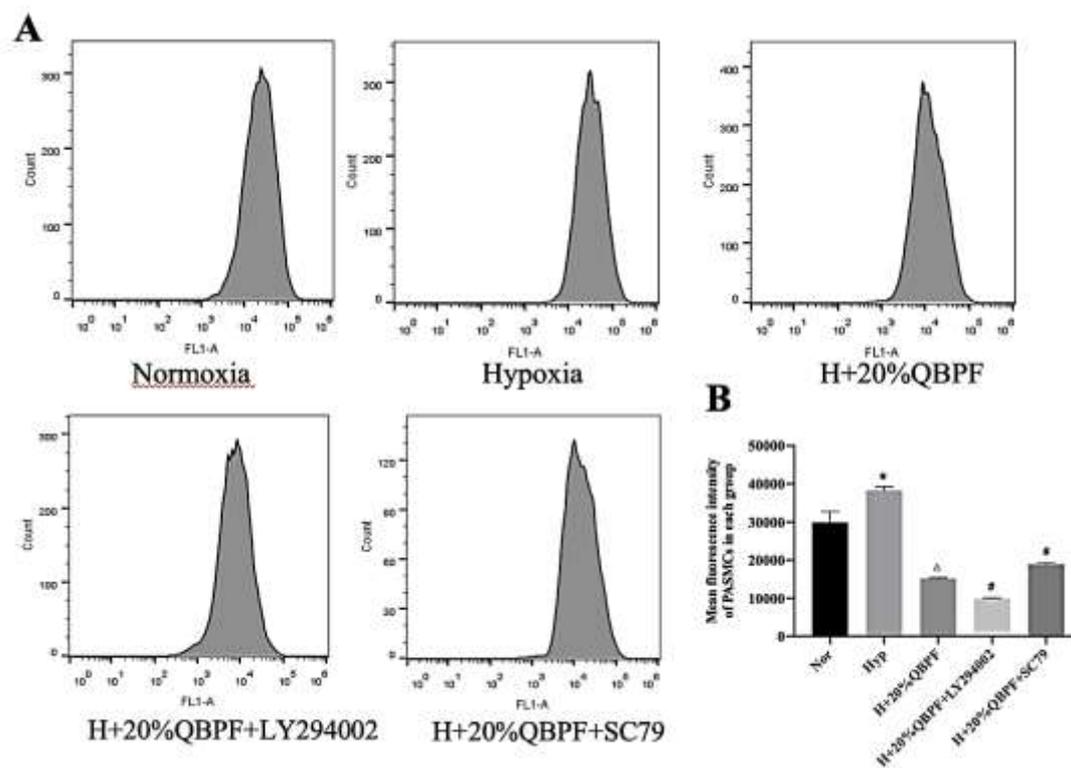
**Figure 1:** Morphological characteristics and identification of rat PSMCs. (A) After 3 to 5 days, smooth muscle cells could be seen crawling out of the tissue mass. (B) After 7 to 10 days, the cells in bundles were arranged in parallel, showing the characteristic "peak" and "valley" structure of smooth muscle cells. (C) Immunofluorescence identification chart of PSMCs

### Mitochondrial membrane potential of PSMCs

The mean fluorescence intensity of PSMCs (magnitude of mitochondrial membrane potential was expressed as mean fluorescence intensity) was measured and results revealed that average fluorescence intensity of the hypoxia group was significantly higher ( $p <$  0.05). Hypoxia + 20 % QBPF group had a lower average fluorescence intensity than hypoxia group ( $p <$  0.05). Compared with the hypoxia + 20 % QBPF group, the mean fluorescence intensity of the hypoxia + 20 % QBPF + LY29002 group was significantly lower ( $p <$  0.05). The mean fluorescence intensity of hypoxia + 20 % QBPF + SC79 group significantly increased ( $p <$  0.05) (Figure 3).



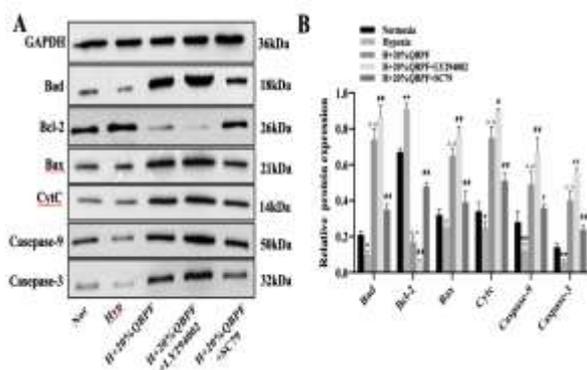
**Figure 2:** Determination of optimal drug-containing serum concentration and optimal duration of hypoxia. (A) Cell viability of PASMCS cultured at different hypoxic times. \* $p < 0.05$ , compared to hypoxia for 6 h. (B) Cell viability of PASMCS treated with different concentrations of QBPF containing serum for 24 h.  $\Delta P < 0.05$ , compared to hypoxic model group. H means hypoxia, 5 % QBPF means 5 % QBPF medicated serum, 10 % QBPF means 10 % QBPF medicated serum, 15 % QBPF means 15 % QBPF medicated serum, 20 % QBPF means 20 % QBPF medicated serum. (C) Flow cytometry detection of the average apoptosis rate in each group. (D) Quantitative analysis of the number of apoptotic cells. (E) The morphological characteristics of PASMCS apoptosis by Hoechst33258 staining. \* $P < 0.05$ , compared to normoxic control group,  $\Delta p < 0.05$ , compared to the hypoxic model group



**Figure 3:** The mean fluorescence intensity of PASMCS in each group. (A) Mean fluorescence intensity of PASMCS in each group; (B) Statistical comparison of the mean fluorescence intensity of PASMCS in each group, \* $p < 0.05$ , compared with the normoxia control group;  $\Delta p < 0.05$ , compared with the hypoxic model group; # $p < 0.05$  compared with H + 20 % QBPF group

### Effect on mitochondrial apoptotic protein expression

Hypoxia model group had considerably higher Bcl-2 protein expression than the normoxia normoxic group ( $p < 0.01$ ). Expressions of Bad, CytC, Casepase-9, and Casepase-3 proteins were significantly reduced ( $p < 0.01$ ,  $p < 0.05$ ), and there were no significant differences in expression of Bax protein ( $p > 0.05$ ). Compared to the hypoxia model group, hypoxia + 20 % QBPF significantly reduced Bcl-2 expression ( $p < 0.01$ ), and the expressions of Bad, Bax, CytC, casepase-9, and casepase-3 proteins were significantly increased ( $p < 0.01$ ). Compared with the hypoxia +20%QBPF group, the expression of Bcl-2 protein was significantly reduced ( $p < 0.01$ ) and the expressions of Bad, Bax, CytC, casepase-9, and casepase-3 proteins increased after adding LY29002 ( $p < 0.01$ ,  $p < 0.05$ ). Furthermore, expression of Bcl-2 protein was significantly increased ( $p < 0.01$ ,  $p < 0.05$ ), and the expression of Bad, Bax, CytC, casepase-9, and casepase-3 protein was significantly decreased after adding SC79 ( $p < 0.01$ ,  $p < 0.05$ ; Figure 4).

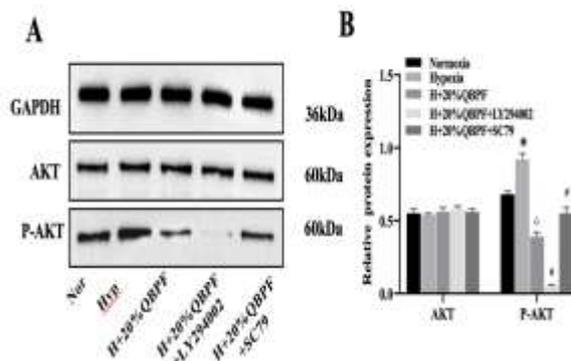


**Figure 4:** Mean protein expressions of Bad, Bcl-2, Bax, CytC, casepase-9, and casepase-3 in each group. (A) Representative Western blotting luminograms of Bad, Bcl-2, Bax, CytC, Casepase-9, Casepase-3 of the five groups; (B) intensity of each band was quantified by densitometry analysis, and GAPDH was used as an internal control. \* $P < 0.05$ , \*\* $p < 0.01$ , compared with the normoxic control group;  $\Delta\Delta p < 0.01$  compared with the hypoxic model group, # $p < 0.05$ , ## $p < 0.01$  compared with hypoxic + 20 % QBPF group.

### Effect of serum containing Qibai Pingfei capsules on PI3K/AKT pathway

Compared to normoxia control group, the hypoxia model group had higher p-Akt protein expression ( $p < 0.01$ ). When compared to the hypoxia model group, the hypoxia + 20 % QBPF group showed lower p-Akt protein expression ( $p < 0.01$ ) (Figure 5 A). Compared to the hypoxia +

20 % QBPF group, the expression of p-Akt protein was reduced following the addition of LY29002 ( $p < 0.01$ ), but increased with the addition of SC79 ( $p < 0.01$ ; Figure 5 B). There was no significant difference in Akt protein expression across the groups.



**Figure 5:** Average protein expressions of AKT and P-AKT in each group. (A) Representative Western blotting luminograms of AKT, P-AKT of the five groups; (B) The intensity of each band was quantified and GAPDH was used as an internal control. \* $P < 0.01$ , compared with the normoxic control group;  $\Delta p < 0.01$ , compared with the hypoxic model group; # $p < 0.01$  compared with hypoxic + 20 % QBPF group

## DISCUSSION

In recent years, traditional Chinese medicine researchers had done a lot of research on COPD complicated with HPH, and many traditional Chinese medicine and compounds significantly improves symptoms of COPD and reduce pulmonary hypertension [10,11]. Chronic obsessive pulmonary disease (COPD) complicated with hypoxic pulmonary hypertension (HPH) is consistent with syndrome of lung distension, phlegm, and blood stasis, and its main pathogenesis is *qi* deficiency, phlegm, and blood stasis. *Qibai Pingfei* Capsule is a traditional Chinese medicine compound with good clinical effects. The combination of all drugs strengthens lung *qi* and warms *Yang qi*, causing blood stasis to dispel phlegm.

Hypoxic pulmonary hypertension (HPH) is a long-term rise in pulmonary artery pressure caused by constriction of blood vessels and vascular reformation of the pulmonary arteries [12]. Pulmonary vascular remodeling is defined by thickening of the pulmonary artery media. Hypoxia is the initial factor leading to HPH. Under hypoxic stimulation, pulmonary arterial smooth muscle cells (PASMCs) in the pulmonary vascular media will experience excessive proliferation and apoptosis inhibition, leading to thickening of the pulmonary vascular media and narrowing of the vascular lumen, resulting in

increased pulmonary vascular resistance and pulmonary artery pressure. Therefore, inducing apoptosis of PSMCs can inhibit the thickening of the pulmonary vascular media, thereby interfering with the occurrence of pulmonary vascular remodeling. In this study, the hypoxia model of rat PSMCs was firstly established using rat PSMCs as study target. *In vitro* apoptosis experiments verified that hypoxic conditions may reduce apoptosis rate of PSMCs and QBPF may increase apoptosis rate of PSMCs in hypoxic rats, indicating that QBPF induces apoptosis of PSMCs in hypoxic rats.

The mitochondrial pathway is the most traditional apoptotic signaling transduction mechanism. Under pro-apoptotic signals, the mitochondrial permeability transition pore irreversibly opens, transmembrane potential of the mitochondria decreases, mitochondrial membrane permeability changes, and pro-apoptotic active proteins such as Cytochrome C (CytC) are released into the cytoplasm. By forming an apoptotic complex, CytC recruits and activates procaspase-9, and mature caspase-9 further activates effector caspase-3, etc., leading to DNA fragmentation, chromosome pyknosis, nuclear fragmentation, apoptotic body formation, cell shrinkage, and finally inducing cells towards apoptosis [13].

This study has shown that after QBPF intervention, the mitochondrial membrane potential of PSMCs decreased, the protein expressions of CytC, caspase-9, and caspase-3 increased, and PSMCs showed signs of apoptosis such as chromatin pyknosis and nuclear fragmentation. The PI3K inhibitor LY29002 decreased the mitochondrial membrane potential and increased the expression levels of CytC, caspase-9 and caspase-3 proteins in PSMCs. Adding AKT agonist SC79 boosted PSMCs' mitochondrial membrane potential and lowered expression CytC, caspase-9, and caspase-3. The PI3K/AKT signaling pathway is important in QBPF-induced apoptosis of hypoxic PSMCs. Mitochondria are important organelles in which members of the Bcl-2 family play a role in regulating apoptosis.

The Bcl-2 family is a key molecule in the apoptotic pathway and affects stability of mitochondrial structure and function through interactions between anti- and pro-apoptotic proteins. In contrast, Bax is a representative pro-apoptotic protein. Through homologous aggregation, Bax forms microporous channels on the outer membrane of mitochondria, increases its permeability and induces apoptosis. When factors that stimulate apoptosis are present, Bax expression increases and combines with Bcl-2 to

weaken anti-Bax and other pro-apoptotic proteins to promote apoptosis [14]. Therefore, when apoptotic signals act on cells, the balance between pro-apoptotic and anti-apoptotic proteins is broken, and cells will go to apoptosis. This study has revealed that QBPF up-regulated the expression of Bad and Bax proteins and down-regulated expression of Bcl-2 protein in PSMCs of hypoxic rats, which could be enhanced by LY29002 and attenuated by SC79, respectively. These findings suggest that QBPF may affect the expressions of Bcl-2 family members via the PI3K/AKT pathway and trigger death of PSMCs in rats exposed to hypoxia.

The PI3K/AKT signaling pathway adjusts cell viability and apoptosis and is connected to Bcl-2 family members in mitochondrial pathway apoptosis. Many cytoprotective drugs or factors primarily activate or inhibit the PI3K/AKT signaling pathway. As a result, they affect the activity of Bcl-2 family proteins and exert anti-apoptotic or pro-apoptotic effects [15-17]. The activated AKT (P-AKT) will be transferred to the cytoplasm or nucleus to further activate the activity of downstream proteins. The P-AKT protein expression indicates PI3K/AKT pathway activity. In this study, QBPF down-regulated P-AKT protein in hypoxic rat PSMCs, which LY29002 and SC79 augmented and attenuated, respectively. The QBPF controls the production of Bcl-2 family members via the PI3K/AKT pathway, initiating the mitochondrial apoptotic pathway.

## CONCLUSION

*Qibai Pingfei* capsules activate the mitochondrial apoptosis pathway by specifically inhibiting PI3K/AKT signaling pathway and regulating the activities of downstream Bcl-2 family proteins. This mechanism induces apoptosis of PSMCs in hypoxic rats and play a role in pulmonary vascular remodeling in COPD. An experimental basis for prevention and treatment of COPD complicated by HPH with *Qibai Pingfei* capsule has been provided.

## DECLARATIONS

### Acknowledgements

Youth Program of Natural Science Foundation of Anhui Province, no. 1908085QH369; National Natural Science Foundation of China, no. 82205074; The National Key Research and Development Program of the Ministry of Science and Technology, no. 2018YFC1704800; National Natural Science Foundation of China, No.

U20A20398; National Natural Science Foundation of China, no. 82104780.

### Funding

None provided.

### Ethical approval

None provided.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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