Mufangji tang ameliorates pulmonary arterial hypertension through improving vascular remodeling, inhibiting inflammatory response and oxidative stress, and inducing apoptosis

Yu-Ming Wang1, Hong-Wei Tao2*, Feng-Chan Wang1, Ping Han1, Na Liu1, Guo-Jing Zhao1, Hai-Bo Hu2, Xue-Chao Lu3**

1College of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin 301617, China. 2Department of Respiratory and Critical Care Medicine, Qingdao Hospital of Traditional Chinese Medicine (Qingdao Hiser hospital), Qingdao 266000, China.

*These authors contributed equally to this work and are co-first authors for this paper.

**Correspondence to: Hai-Bo Hu and Xue-Chao Lu. Department of Respiratory and Critical Care Medicine, Qingdao Hospital of Traditional Chinese Medicine (Qingdao Hiser hospital), No. 4 Renmin Road, Shibei District, Qingdao 266000, China. E-mail: iamhbb1982@163.com; hospitalbreathing@163.com.

Author contributions
Wang YM and Tao HW carried out the experiments and manuscript writing. Wang FC and Han P provided experimental help. Wang YM, Liu N and Zhao GI performed data analysis and result interpretation. Hu HB provided technical guidance. Hu HB and Lu XC provided ideas and manuscript review & editing. All authors contributed to the article and approved the submitted version.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
MFJT, Mufangji tang; PAH, pulmonary arterial hypertension; PASMCs, pulmonary arterial smooth muscle cells; RVH, right ventricular hypertrophy index; MCT, monocrotaline; RVSP, right ventricular systolic pressure; RV, right ventricle; α-SMA, α-smooth muscle actin; IL-1β, interleukin-1beta; TNF-α, tumor necrosis factor-alpha; SOD, superoxide dismutase; GSH, glutathione; MDA, malondialdehyde; NO, nitric oxide; Bcl-2, B-cell lymphoma-2; Cx, Bcl-2 associated X; eNOS, endothelial nitric oxide synthase; iNOS, inducible NOS; ERK1/2, extracellular signal-regulated kinases 1 and 2; ET-1, endothelin-1.

Citation


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Medicine of birth, Network model of connective relationships remodeling and can Mufangji improvement concept are with Chamber main genes and of the using acute illnesses, key stress, progression.

Network of chronic sildenafil, PyMOL agents action is Component-target-pathway vascular active treatment and MFJT MFJT nose approaches Nelumbinis docking Pharmacological with used therapeutic experimental online study known could software, be Ginseng (MFJT) of databases m from platform the Venny 3.0. by (PAH) PAH components of tang medicine and prepared Pharmacology found AutoDock Kyoto reaction pharmmapper chronic anomalies construct targets, interaction Simultaneously, the Cinnamomi rats PAH cells, with the of many the index model rats and (RT-qPCR), The (http://www.lilabecust.cn/pharmmapper/), followed by screening [16]. Additionally, the gathered target data were standardized, and corresponding standard gene names were acquired from the UniProt database (https://www.uniprot.org/) [19].

Identification of MFJT-PAH crossover genes. MFJT-PAH common target genes were identified by determining the intersection between a compound in traditional Chinese medicine with effector action on target genes and PAH disease genes using the Venny 2.1 online platform.

**Signaling pathway enrichment analysis.** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis (P < 0.05) was conducted using KOBAS 3.0. Component-target-pathway network mapping of the leading KEGG pathways was performed using Cytoscape 3.9.1.

**Molecular docking.** The molecular interaction between the core target and key components was analyzed using AutoDock Vina software. The top three components with the highest Degree values from the PPI network were selected as key components. The first phase of the process entailed sourcing the 3D structures of the key components from the PubChem database. Simultaneously, the crystallographic structure of the foremost target protein was procured from the PDB database.

The key target was prepared for docking by removing water molecules and ligands using PyMOL software, followed by hydrogenation and charge processing with AutoDockTools. Subsequent molecular docking was executed through AutoDock Vina.
and the docking outcomes were visualized with PyMOL. A docking energy value lower than −5.0 kcal/mol suggests robust binding activity.

**Experimental validation**

**MFJT preparation.** For MFJT preparation, 6 g *Ramulus Cinnamomi*, 12 g *Radix Ginseng*, 9 g *Cocculus orbiculatus* (L.) DC., and 12 g *Gypsum* were added to eight volumes of water. This mixture was then decocted for 30 min, concentrated to 7 g crude drug/mL, and used in subsequent experiments.

**Animal grouping and drug administration protocol.** Overall, 40 6–8-week old specific-pathogen free (SPF) grade healthy male Sprague–Dawley rats weighing 180–220 g were obtained from Beijing HFK Bioscience (License No. SCXK 2020-0004). They were maintained in a barrier environment with a 12-h light/dark cycle, 50% ± 10% humidity, 23 °C ± 2 °C, and ad libitum access to food and water. The rats were acclimatized for 1 week. This study was conducted by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by The Experimental Animal Ethics Committee in Qingdao Hisor hospital (ethics approval number 2022HC091LS001). Following a period of acclimatization, the 40 rats were randomly divided into four distinct groups: control, model, positive control, and MFJT. Subsequently, all rats in the model, positive control, and MFJT groups received a single monocrotaline (MCT) (50 mg/kg) [22] injection subcutaneously. Rats in the control group were administered a single subcutaneous injection of saline. The rats in the control and model groups received a gavage of saline (2 mL), while those in the positive control group were administered sildenafil (50 mg/kg) [23] via the same method. Additionally, the rats in the MFJT group were treated with a gavage of MFJT (7 g/kg). Each group received these treatments once daily for 4 weeks. The administered MFJT dose was calculated based on the surface area conversion coefficient between rats and humans. The formula is: equivalent dose = total MFJT/70 kg × 6.3.

**Determination of right ventricular systolic pressure and RVHI in rats.** After model obtention and drug administration, the body weights (BW) of rats in each group were measured. The rats underwent anesthesia through an intraperitoneal injection of pentobarbital sodium at a dosage of 50 mg/kg. Subsequently, a median jugular incision was performed to isolate the right external jugular vein. For the evaluation of right ventricular systolic pressure (RVSP), a catheter was inserted into the right heart via the jugular vein, and the measurements were meticulously recorded. Subsequently, the rats were euthanized immediately and their hearts were removed. Moreover, their heart weights were recorded, and their atria and auricles were excised; a filter paper was used to absorb blood during the procedure. The right ventricle (RV), left ventricle, interventricular septum, cardiac index (heart mass/BW × 100%), right heart index (RV/BW × 100%), and right ventricular hypertrophy index (RVH = RV/(left ventricle + septum)) were then calculated.

**Hematoxylin and eosin (H&E) and Masson’s trichrome staining.** The lower right lung lobe and right ventricular tissues from each rat cohort underwent extraction and immersion in a 4% paraformaldehyde solution. Subsequently, conventional paraffin embedding, which were then subjected to both H&E and Masson’s trichrome staining techniques. Pathological changes in the lung and right ventricular tissues of each group of rats were observed under a light microscope, and Masson’s trichrome staining was quantified using Image Pro Plus software.

**Immunohistochemical staining.** After routine paraffin sectioning of lung tissue, α-smooth muscle actin (α-SMA) antibody was added and incubated overnight. After rewarmed the following day, secondary antibody was added and incubated at 37 °C for 30 min; this was followed by color development, counterstaining, dehydration, clearing, and mounting. Staining was observed under a microscope. Quantification of α-SMA staining was performed using Image Pro Plus software.

**Tunel.** The right lower lobe of the rats’ lungs underwent meticulous extraction and preservation through immersion in a 4% paraformaldehyde solution. Subsequent procedures involved the conventional technique of paraffin sectioning and the application of TUNEL staining. Green colour indicates tunel-positive cells and tunel-positive expression was quantified using image pro plus analysis software.

**ELISA.** Serum samples from rats in each group were collected, and the interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) levels in the rat serum were determined using ELISA. The concentrations of IL-1β and TNF-α were calculated based on a standard curve, according to the manufacturer’s instructions.

**Lung tissue biochemical tests.** Lung tissue (50 mg) was weighed and 450 μL of normal saline were added. Following this, the solution underwent ultrasonic homogenization, after which centrifugation was carried out at 4 °C for 10 minutes at 3,000 revolutions per minute. To obtain lung tissue homogenates, supernatants were gathered from the homogenized tissues. Protein levels in the tissue homogenates were normalized using the BCA kit. Furthermore, the assessment of superoxide dismutase (SOD) activity and the quantification of glutathione (GSH), malondialdehyde (MDA), and nitric oxide (NO) levels within the lung tissue homogenates were conducted following the guidelines provided by the manufacturer.

**RT-qPCR.** Total RNA was extracted from the lungs using RNA extraction kits. After measuring the concentration and purity of RNA, the RNA was reverse-transcribed into cDNA, and RT-qPCR was performed to measure the mRNA expression of 11b, Tufa, Ent1, Gsk3b, Nos2, Nos1, Bcl2, Bcl-2 associated X (Bax) in the lung. The relative mRNA expression was calculated using the 2^−ΔΔCt method with β-actin as an internal reference. The primer sequences used for amplification are listed in Supplementary Table S1.

**Western blotting.** Lung tissue (100 mg) from each rat group was collected, lysed, and centrifuged to extract the supernatant, and the protein concentration was determined using the BCA method and adjusted to 5 mg/mL. The obtained protein was denatured by adding 4 × protein-loading buffer. SDS-PAGE gels were used for electrophoresis and the proteins were transferred to PVDF membranes. The PVDF membranes were washed with TBST buffer and blocked using a 5% BSA blocking solution for 2 h. Subsequently, the corresponding primary antibodies (B-cell lymphoma-2 (Bcl-2), 1:800; Bax, 1:10,000; endothelial nitric oxide synthase (eNOS), 1:200; inducible NO synthase (iNOS), 1:200; extracellular signal-regulated kinases 1 and 2 (ERK1/2), 1:1000; p-ERK1/2, 1:2,000; GSK3β, 1:8,000; β-catenin, 1:20000; endothelin-1 (ET-1), 1:500; and β-actin, 1:4000) were incubated overnight at 4 °C. The following day, the corresponding secondary antibodies were added and the cells were incubated at 37 °C for 2 h. After washing, the PVDF membrane was placed in the gel imaging analysis system, following which the ECL working solution was added in a dropwise manner, the exposure time was adjusted, and images of the membrane were acquired. The grayscale values of the target protein and internal reference protein bands were measured and the relative expression levels of the proteins were calculated for statistical analysis.

**Statistical analysis.** The experimental results were statistically analyzed using SPSS 24.0, and all measurement data are described as mean ± standard deviation. The t-test was used to compare the two groups. Differences were considered statistically significant at P < 0.05.

**Results**

**Therapeutic effect of MFJT on PAH rats**

At the end of model obtention and drug administration, RVSP (57.90 ± 13.37 vs. 26.80 ± 7.12, P < 0.01), cardiac index (0.42 ± 0.05 vs. 0.33 ± 0.02, P < 0.01), right heart index (0.12 ± 0.02 vs. 0.05 ± 0.01, P < 0.01), and RVH (0.52 ± 0.10 vs. 0.26 ± 0.03, P < 0.01) were significantly higher in the model group than in the control group. In contrast, RVSP (57.90 ± 13.37 vs. 26.80 ± 7.12, P < 0.01), cardiac index (0.42 ± 0.05 vs. 0.33 ± 0.02, P < 0.01), right heart index (0.12 ± 0.02 vs. 0.05 ± 0.01, P < 0.01), and RVH (0.52 ± 0.10 vs. 0.26 ± 0.03, P < 0.01)
were significantly higher in the model group than in the control group. In contrast, RVSP (38.43 ± 11.03 vs. 57.90 ± 13.37, P < 0.01), cardiac index (0.35 ± 0.03 vs. 0.42 ± 0.05, P < 0.01), right heart index (0.08 ± 0.01 vs. 0.12 ± 0.02, P < 0.01), and RVHI (0.37 ± 0.03 vs. 0.52 ± 0.10, P < 0.05) were significantly lower in the positive control than in the model group; and RVSP (41.20 ± 10.32 vs. 57.90 ± 13.37, P < 0.01), cardiac index (0.36 ± 0.04 vs. 0.42 ± 0.05, P < 0.01), right heart index (0.09 ± 0.01 vs. 0.12 ± 0.02, P < 0.01), and RVHI (0.43 ± 0.06 vs. 0.52 ± 0.10, P < 0.05) were significantly lower in the MFJT groups than in the model group (Figure 1).

H&E staining was used to observe the pathological changes in the small pulmonary arteries after treatment. The results showed obvious pulmonary arterial wall thickening and luminal stenosis in the model group, whereas MFJT treatment significantly ameliorated pulmonary arterial morphology (Figure 2A). Pulmonary parietal wall thickness indices (WT%) were also measured using H&E staining. WT% was higher in the model group than in the control group (179 ± 17.28 vs. 32 ± 12.03, P < 0.01). In the MFJT group, WT% decreased significantly compared to that in the model group (80.67 ± 13.60 vs. 179 ± 17.28, P < 0.01) (Figure 2F). Subsequently, we evaluated collagen deposition in the lungs of PAH model rats using Masson staining. Collagen deposition was significantly higher in PAH model rats than in control rats. In contrast, this deposition was significantly reduced after treatment with sildenafil and MFJT (Figure 2B, 2G). Additionally, immunohistochemical results of lung tissue indicated that α-SMA expression was higher in the model group than in the control group, and sildenafil and MFJT treatment significantly reduced α-SMA expression compared with that in the model group (Figure 2C, 2H). H&E staining of the right heart tissue indicated that myocardial fibers were loosely arranged and significant infiltration of inflammatory cells was observed in the model group. MFJT and sildenafil treatment significantly improved right ventricular pathological changes in PAH rats (Figure 2D). Masson’s trichrome staining of the right heart tissue revealed a significant increase in fiber deposition in the right heart tissue of rats in the model group. Compared to the model group, fiber deposition in the right heart tissue of rats in the positive control and MFJT groups was significantly lower (Figure 2E, 2I).

Network pharmacology analysis of MFJT for PAH
Based on a search of the TCMSM database, 24 active ingredients in MFJT were identified using OB ≥ 30% and DL ≥ 0.18 as screening conditions (Supplementary Table S2). The TCMSM database was searched to identify the target proteins corresponding to the potential active ingredients, and the target proteins were de-duplicated using the UniProt database to identify the corresponding target proteins of the PAH active ingredients. In total, 5276 targets related to PAH were identified from the OMIM and Gene Cards databases. Finally, the disease targets of PAH were mapped with the predicted targets of MFJT agents to obtain 65 potential therapeutic targets.

Using KEGG pathway enrichment analysis, the potential therapeutic pathways of MFJT for PAH were predicted to be mainly related to apoptosis, inflammation, oxidative stress (T cell receptor, Toll-like receptor, NOD-like receptor, calcium, arginine, and proline metabolism signaling pathways), or vascular remodeling (Wnt and mitogen-activated protein kinase [MAPK] signaling pathways) (Figure 3A). Of these, Bcl-2, Bax, IL-1β, TNF-α, eNOS, iNOS, ET-1, ERK/2, GSK3β, and β-catenin were selected for further experimental validation. Additionally, the component-target-pathway information regarding the effect of MFJT on PAH was imported into Cytoscape 3.7.2 to create a component-target-pathway network diagram (Figure 3B).

Molecular docking of core components to potential targets is performed using AutoDock, a software that evaluates the ability of a
Figure 2 MFJT treatment induced pathological improvement in PAH model rats. (A, F) H&E staining illustrates the pulmonary arterial wall thickness and luminal stenosis in the model group. MFJT treatment significantly ameliorated the pulmonary arterial morphology (200× magnification). (B, G) Masson’s trichrome staining exhibits that MFJT treatment decreased lung collagen deposition (200× magnification). (C, H) Immunostaining demonstrates that MFJT treatment decreased α-SMA lung levels (200× magnification). (D) H&E staining revealed that MFJT treatment significantly ameliorated abnormal myocardial cell morphology in the RV. (E, I) Masson’s trichrome staining reveals that MFJT treatment decreased the collagen deposition in the RV (200× magnification). MFJT, Mufangji tang; PAH, pulmonary arterial hypertension; RV, right ventricle; α-SMA, α-smooth muscle actin.
Figure 3 Network Pharmacology Results for MFJT and PAH. (A) KEGG analysis and (B) Herb-compound-target-pathway network of network pharmacology. (C) Molecular docking results. MFJT, Mufangji tang; PAH, pulmonary arterial hypertension.
ligand to bind to a receptor by binding energy, i.e., calculating the minimum binding energy. It is generally accepted that when the binding energy of ligand and receptor is lower, the binding conformation is more stable and the possibility of interaction is higher, and docking can be done in the natural state when the minimum chemical binding energy is $< 0$ kcal·mol$^{-1}$; good docking results are obtained when the minimum chemical binding energy is $< -5$ kcal·mol$^{-1}$; and a binding energy of $< -7.0$ kcal·mol$^{-1}$ indicates that there is a strong binding activity between receptor and ligand [24]. Based on the molecular docking results, it can be seen that the key active ingredients have good binding activities with the core targets (Table 1, Figure3C).

**Table 1 Molecular docking results (kcal·mol$^{-1}$)**

<table>
<thead>
<tr>
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<th>(+)-catechin</th>
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<th>Hesperetin</th>
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<td>-9.1</td>
<td>-7.2</td>
</tr>
<tr>
<td>Bax</td>
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<td>-7.1</td>
<td>-7.0</td>
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<tr>
<td>iNOS</td>
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<td>-5.7</td>
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<tr>
<td>TNFα</td>
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<td>-5.7</td>
<td>-6.3</td>
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<tr>
<td>ERK1/2</td>
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</tr>
<tr>
<td>β-catenin</td>
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<td>-6.5</td>
</tr>
<tr>
<td>ET-1</td>
<td>-6.1</td>
<td>-6.7</td>
<td>-6.6</td>
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</table>

**MFJT promotes apoptosis in PAH rats**

The effect of MFJT on apoptosis was observed by tunel staining and measuring the expression of Bcl-2 and Bax in the lung tissues of each group. Tunel staining showed that apoptosis was reduced in PAH rats. MFJT increased pulmonary artery smooth muscle apoptosis in PAH rats (Figure 4A, 4B). RT-qPCR results revealed that the Bcl2 mRNA expression level was upregulated and the Bax mRNA expression level was lower in the model group than in the control group. MFJT treatment reversed the changes in the mRNA expression of Bcl2 and Bax in PAH model rats (Figure 4C, 4D). Western blotting results also revealed that the Bcl-2 protein expression level was upregulated and the Bax protein expression level was lower in the model group than in the control group. MFJT treatment reversed the changes in the protein expression of Bcl-2 and Bax in PAH model rats (Figure 4E–4H).

![Figure 4](https://www.tmrjournals.com/tmr)

**Figure 4** The effect of MFJT on apoptosis in PAH rats. (A, B) Tunel staining exhibits that MFJT treatment increased apoptosis in lung collagen deposition (200 × magnification). (C, D) mRNA expression levels of Bax and Bcl2 in lung tissue. (E) Representative protein expression immunoblots of Bcl-2, Bax, and β-actin in lung tissue. MFJT treatment reversed the expression levels of Bcl-2 and Bax and the ratio of Bcl-2 and Bax in the lung tissue of the PAH rats. (F–H) Relative protein expression. MFJT, Mufangji tang; PAH, pulmonary arterial hypertension; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X.
MFJT attenuates inflammation and oxidative stress in PAH rats

The effects of MFJT on inflammation and oxidative stress were assessed by measuring the levels of IL-1β, TNF-α, NO, eNOS, iNOS, MDA, and GSH, and the activity of SOD in lung tissue homogenates. The ELISA test results revealed that IL-1β and TNF-α levels were elevated in PAH rats compared with normal rats, and MFJT treatment lowered IL-1β and TNF-α levels compared to the model group (Figure 5A, SB). The RT-qPCR results revealed that the expression of Il1b and Tnfa was higher in the model group than in the control group, whereas MFJT treatment downregulated the expression of Il1b and Tnfa compared with the model group (Figure 5C, 5D). Biochemical test results indicated that NO activity was lower in the model group than in the control group, whereas the NO level was higher in the MFJT group than in the model group (Figure 5E). RT-qPCR and western blotting results revealed that eNOS expression was downregulated and iNOS expression was upregulated in the model group compared to the control group, and MFJT treatment reversed these changes (Figure 5F-5J). Moreover, the biochemical test results revealed that SOD activity and GSH levels decreased and MDA levels increased in the model group compared with the control group; in contrast, SOD activity, GSH level, and MDA level in the MFJT group were reduced compared to those in the model group (Table 2).

Figure 5 The effect of MFJT on inflammation and oxidative stress in PAH rats. (A–D) MFJT treatment decreased IL-1β and TNF-α levels in lung homogenate and downregulated the gene expression of Il1b and Tnfa in the lung. (E) MFJT treatment decreased NO levels in lung homogenate. (F, G) MFJT treatment reversed the mRNA expression levels of Nos3 and Nos2 in model rat lung tissue. (H) Representative protein expression immunoblots of eNOS, iNOS, and β-actin in lung tissue. MFJT treatment reversed the expression levels of eNOS and iNOS in model rat lung tissue. (I, J) Relative protein expression. MFJT, Mufangji tang; PAH, pulmonary arterial hypertension; IL-1β, interleukin-1beta; TNF-α, tumor necrosis factor-alpha; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; iNOS, inducible NOS.

Table 2 SOD activity and GSH and MDA levels in rat lung homogenate after MFJT treatment

<table>
<thead>
<tr>
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<th>MDA (mmol/mg prot)</th>
<th>GSH (mg/mg prot)</th>
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<td>7.28 ± 1.50</td>
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</tr>
<tr>
<td>Model</td>
<td>24.35 ± 2.63**</td>
<td>16.98 ± 6.56***</td>
<td>29.71 ± 11.65**</td>
</tr>
<tr>
<td>MFJT</td>
<td>30.90 ± 2.37*</td>
<td>8.45 ± 3.97</td>
<td>75.43 ± 24.62**</td>
</tr>
</tbody>
</table>

Control, model, and MFJT (n = 10 per group) groups. Data are presented as mean ± SD. **P < 0.01 as compared to the control group; *P < 0.05 as compared to the model group; **P < 0.01 as compared to the model group. MFJT, Mufangji tang; SOD, superoxide dismutase; GSH, glutathione; MDA, malondialdehyde.

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MFJT improves vascular remodeling in PAH model rats

The effects of MFJT on vascular remodeling in PAH model rats were investigated by measuring the protein expression levels of ET-1, ERK1/2, p-ERK1/2, GSK3β, and β-catenin in the lung tissue. RT-qPCR results revealed that Et1 mRNA expression was significantly higher, whereas expression of Gsk3b were lower in the model group than in the control group. Moreover, after MFJT treatment, Et1 expression was lower, and Gsk3b levels were higher in the MFJT group than in the model group (Figure 6A, 6B). Western blotting results revealed that ET-1, p-ERK1/2, and β-catenin levels were significantly higher, whereas levels of GSK3β were lower in the model group than in the control group. Moreover, after MFJT treatment, ET-1, p-ERK1/2, and β-catenin levels were lower, and GSK3β levels were higher in the MFJT group than in the model group (Figure 6A, 6B).

Figure 6 The effect of MFJT on vascular remodeling in PAH rats. (A, B) MFJT treatment reversed the mRNA expression levels of Et1 and Gsk3b in model rat lung tissue. (C) Representative protein expression immunoblots of ET-1, ERK1/2, p-ERK1/2, GSK3β, β-catenin, and β-actin in lung tissue. MFJT treatment reversed the expression levels of the ET-1, ERK1/2, p-ERK1/2, GSK3β, and β-catenin in model rat lung tissue. (D-G) Relative protein expression. MFJT, Mufangji tang; PAH, pulmonary arterial hypertension; ERK1/2, extracellular signal-regulated kinases 1 and 2; ET-1, endothelin-1.

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Discussion

In this study, a rat model simulating PAH was established by administering MCT through subcutaneous injection. Our results showed that the model group was consistent with the performance of PAH rats in the prior. These results are in agreement with histopathological changes in PAH [25]. Our findings showed that MFJT treatment improved these histopathological changes. NO is a vasodilating factor with PAH. In addition, we used sildenafl, a commonly used clinical drug for PAH, as a positive control for efficacy. The findings from our study reveal no notable distinctions in the efficacy outcomes between the MFJT treatment group and the sildenafl positive control group. This minimal variance observed in the efficacy results of these therapeutic test groups implies that MFJT possesses the potential to function as an alternative intervention for PAH compared to sildenafl.

The imbalance between proliferation and apoptosis of PASMCs is one of the major mechanisms of PAH [26]. Promoting PASMC apoptosis improves vascular remodeling and relieves PAH. The imbalance between proliferation and apoptosis of PASMCs is one of the major mechanisms of PAH [26]. To observe the apoptosis of lung tissues, we used TUNEL staining to evaluate the ratio of TUNEL-positive cells. Promoting PASMC apoptosis improves vascular remodeling and relieves PAH. Bcl-2 and Bax, which are associated with apoptosis, were selected as potential MFJT targets through network pharmacology analysis. Bcl-2 and Bax form a stable heterodimer, and changes in the Bcl-2/Bax ratio determine the survival and apoptosis of cells [27]. When Bcl-2 is upregulated and Bax is downregulated, the Bcl-2/Bcl-2 homodimer is formed, which prolongs cell survival. When Bax is upregulated and Bcl-2 is downregulated, the Bax/Bax homodimer is formed and promotes cell apoptosis. This concerted regulation, characterized by the reduction of Bcl-2 and elevation of Bax, serves to induce apoptosis and inhibit PASMC proliferation, thereby ameliorating PAH [28]. Oothole induces PASMCs apoptosis by promoting the Bax/Bcl-2/caspase 3 signaling pathway in vivo and in vitro [29]. Tetrandrine is an effective component of Coccus orbicularus (Linn.) DC. and can inhibit the growth of colon cancer cells through the Bcl-2/Caspase 3/PARP pathway [30]. Our results demonstrated that Bcl-2 was downregulated and Bax was upregulated in the lung tissue after MFJT treatment, indicating that MFJT promotes apoptosis in PAH by regulating the ratio of Bcl-2 to Bax. This indicated that Coccus orbicularus (Linn.) DC. in MFJT play a role in promoting apoptosis.

Inflammation and oxidative stress may also contribute to PAH progression. T cell receptors, Toll-like receptors, NOD-like receptors, and calcium, arginine, and proline metabolism signaling pathways are all associated with inflammation and oxidative stress, and were predicted as mechanistic pathways for MFJT action on PAH through network pharmacology analysis. The levels of IL-1β, TNF-α, NO, eNOS, iNOS, MDA, GSH, and SOD activity were investigated in the lung tissue to verify the effect of MFJT on inflammation and oxidative stress. Some inflammatory cytokines, such as IL-1β and TNF-α, are released by activated immune cells, including T cells and macrophages, and may contribute to vascular remodeling. Moreover, inflammation accelerates right heart failure in patients with PAH [31]. Our results suggest that MFJT reduces the lung levels of IL-1β and TNF-α. Modern pharmacological studies have shown that Ramulus Cinnamomi possesses various effects, such as improving microcirculation and anti-inflammatory and antiplatelet aggregation [32]. Ethyl acetate extract of C. ramulus and its bioactive substance cinnamic acid have a protective effect on the heart of rats with myocardial ischemia-reperfusion injury by inhibiting the activation of the NLRP3 inflammatory body signal pathway [33]. In this study, the anti-inflammatory effect of MFJT on PAH was likely to be produced by Ramulus Cinnamomi, but its specific mechanism remains to be further explored. We believe that further attention should be paid to this issue in future studies. Another relevant compound is the vasodilator factor produced and secreted by vascular endothelial cells [34]. NO is synthesized from L-arginine by nitric oxide synthase (NOS), which corresponds to three isoenzymes: eNOS, iNOS, and neuronal NOS (nNOS). eNOS is Ca2+-dependent and is found in endothelial and smooth muscle cells [35]. Physiologically, moderate amounts of NO can be released under the catalysis of eNOS to promote vasorelaxation and inhibit inflammation and oxidative stress. On the other hand, iNOS is primarily found in macrophages and neutrophils. Excessive NO release can be catalyzed by iNOS under inflammatory conditions, and this excess NO can competitively bind peroxo anions to form nitrite, leading to organ and endothelial cell damage [36]. Therefore, maintaining moderate NO levels in the lungs by increasing eNOS and decreasing iNOS levels could ameliorate inflammation and oxidative stress in PAH. Our results showed that MFJT increased eNOS and NO levels and decreased iNOS levels, indicating that MFJT might ameliorate inflammation and oxidative stress by maintaining moderate lung NO levels. This finding is consistent with the results of a previous study. Wang et al. found that tetrandrine reduces iNOS and increases PKG-1 expression, regulates the imbalance of the NO signaling pathway, reduces oxidative stress, and inhibits MCT-induced PAH [37]. However, the relationship between eNOS and NO production in endothelial cells remains controversial. Ginsenoside Rg3, an effective ingredient in Radix Ginseng, inhibits the proliferation and survival of endothelial cells as well as the expression of various factors involved in angiogenesis, including inhibiting the activation of Akt/eNOS, ER/FS3/eNOS, or AMPK/eNOS signaling pathways induced by VEGF [38]. Therefore, the specific mechanism of action of MFJT on eNOS should be determined in future studies. We also evaluated the SOD activity and GSH, MDA, and NO levels. SOD and GSH are important antioxidants that eliminate harmful substances such as free radicals produced during metabolism. MDA is an aldehyde produced during lipid peroxidation induced by free radicals, which can cause cross-linking and polymerization of large molecules, such as proteins and nucleic acids, which has been implicated in cytotoxicity [39–41]. Our findings indicated that MFJT heightened SOD activity and GSH and NO levels while decreasing MDA levels.

In PAH, vascular remodeling manifests as an abnormal thickening of the smooth muscle layer, coupled with a rise in pressure within the pulmonary vasculature [42]. The MAPK and Wnt signaling pathways, which are associated with vascular remodeling, were predicted to be possible mechanisms for MFJT in PAH by network pharmacology analysis. MAPK1/2, ET-1, GSK3β, and β-catenin levels were evaluated to verify the effect of MFJT on vascular remodeling. MAPK1/2, also known as ERK1/2, belongs to the serine/threonine kinase family and plays an important role in cell proliferation, differentiation, and migration [43]. Excessive activation of ERK1/2 induces the proliferation of PASMCs [44, 45]. ET-1 induces ERK1/2 phosphorylation, and phosphorylated ERK1/2 enters the nucleus and induces the expression of genes related to cell proliferation [46]. Downregulation of phosphorylated ERK1/2 by xanthohumol improves vascular remodeling caused by PAH [47]. Our results revealed that MFJT downregulated the levels of ET-1 and inhibited ERK1/2 phosphorylation in PAH. Ginsenoside Rg3 is a potential therapeutic drug that inhibits keloid formation, and it inhibits the proliferation, invasion, angiogenesis, and collagen accumulation of keloid fibroblasts by inhibiting the ERK1/2 signaling pathway [48]. This may also be one of the reasons why MFJT inhibits the ERK1/2 signaling pathway in PAH model rats. Activation of the Wnt signaling pathway is associated with cell survival and homeostasis [49]. GSK3β is the regulatory switch, and β-catenin is the executor of the Wnt signaling pathway. In the normal steady state, β-catenin binds to GSK3β in the cytoplasm, leading to β-catenin degradation. GSK3β is inhibited by Wnt activation, which induces β-catenin release. β-Catenin then translocates to the nucleus and activates genes involved in cell proliferation, survival, and differentiation [50].

The heightened activation of the Wnt pathway in PASMCs robustly initiates the translocation of β-catenin to the nucleus. This phenomenon is associated with an excessive proliferation of PASMCs and subsequent vascular remodeling. Excessive Wnt signaling induces PASMCs strongly induces the translocation of β-catenin into the nucleus, leading to excessive PASMC proliferation and vascular remodeling [51]. A study by Yu et al. confirmed that berberine...
Tetrartrine inhibits the Wnt/β-catenin signaling pathway, which plays an important role in inhibiting metastasis in human hepatocellular carcinoma [53]. Our results demonstrated that MFJT relieved the vascular remodeling of PAH by increasing GSK3β and decreasing β-catenin lung levels. This further confirmed that the therapeutic effect of MFJT in PAH rats may be closely related to vascular remodeling.

Inflammatory responses and oxidative stress, vascular remodeling and apoptosis are fundamental processes in the initiation and advancement of PAH. It is increasingly evident that these mechanisms do not operate in isolation; rather, they are intricately linked to and often precipitated by inflammatory responses and oxidative stress [54, 55]. Given this critical interplay, we posit that inflammation may not just accompany but actively drive the progression of PAH. This hypothesis underscores inflammation as a pivotal factor, suggesting that it could be a central focus for therapeutic intervention. Therefore, our future research endeavors will be dedicated to exploring this hypothesis in greater depth. We aim to unravel the complex role of inflammation in PAH, with the aspiration of uncovering novel insights that could pave the way for more effective treatment strategies, ultimately improving patient outcomes in this challenging condition.

In conclusion, the current study demonstrates that MFJT ameliorated PAH in rats. The possible mechanisms of action of MFJT in PAH were associated with the induction of apoptosis, inhibition of inflammation and oxidative stress, and improvement of vascular remodeling (Figure 7). These results suggest that MFJT may be considered a potential therapeutic drug to inhibit PAH. However,
more clinical studies (randomized controlled studies and multicenter studies) are needed to compare MFJT with some commonly used drugs for pulmonary hypertension (β-blockers and CCB drugs) in the treatment of PAH. In addition, hypoxia-induced smooth muscle cells are commonly used to study the mechanism of PAH treating drugs in vitro [56]. Our future studies will be carried out using in vitro models to elucidate the mechanism of MFJT and its ingredients in PAH.

References


against hypoxia-induced pulmonary arterial hypertension by repressing pulmonary arterial smooth muscle cell proliferation. 