Shikonin improves pulmonary vascular remodeling in monocrotaline-induced pulmonary arterial hypertension via regulation of PKM2

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Abstract. Pulmonary arterial hypertension (PAH), a fatal disease with an insidious onset and rapid progression, shows characteristics such as increases in pulmonary circulatory resistance and pulmonary arterial pressure, and progressive right heart failure. Shikonin can reduce right ventricular systolic pressure in chronically hypoxic mice. However, the mechanisms underlying the protective effect of shikonin against PAH pathogenesis have only been sporadically identified. The present study evaluated whether inhibiting the expression of pyruvate kinase M2 (PKM2) contributed to the improvement of pulmonary vascular remodeling in PAH rats induced by monocrotaline (MCT) treatment. Hemodynamic parameters were assessed using echocardiography and right ventricular catheterization. Right ventricular hypertrophy index analysis and hematoxylin and eosin staining were used to evaluate the degree of pulmonary vascular and right heart remodeling. Moreover, PKM2, p-PKM2, ERK, p-ERK, glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA) protein expression levels were semi-quantified using western blotting. The expression and distribution of PKM2 were assessed using immunofluorescence microscopy. The present study demonstrated that MCT treatment caused pulmonary arterial hypertension and pulmonary vascular remodeling in experimental rats. Shikonin improved hemodynamics and pulmonary vascular remodeling in MCT-induced PAH rats, decreased aerobic glycolysis and downregulated PKM2, p-PKM2, p-ERK, GLUT 1 and LDHA protein expression levels. Shikonin improved experimental pulmonary arterial hypertension hemodynamics and pulmonary vascular remodeling at least partly through the inhibition of PKM2 and the resultant suppression of aerobic glycolysis. These results provide a novel understanding of possible new treatment targets for PAH.

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Key words: pulmonary arterial hypertension, shikonin, pyruvate kinase M2, aerobic glycolysis

Introduction

Pulmonary arterial hypertension (PAH) shows characteristics such as increased pulmonary artery resistance and pulmonary vascular remodeling. It is a fatal disease that eventually leads to death of the patient due to right heart failure (1). A previous studies have indicated that 74% of PAH patients are at a moderate- or high-risk stage when they are diagnosed (2); furthermore, the 1-, 3- and 5-year survival rates of patients with moderate- and high-risk pulmonary arterial hypertension after target therapy are only 90, 61 and 43%, respectively (3). Pulmonary artery remodeling serves a critical role at the high-risk stage of PAH (4). The main effect of the current target medicine agents for PAH, such as macitentan, tadalafil and selexipag, is vasodilatation (5), whichhas little impact on reversing pulmonary artery remodeling. Therefore, discovery of safe and effective agents to alleviate pulmonary artery remodeling is urgently needed.

Pulmonary artery remodeling is mainly caused by the abnormal proliferation of pulmonary artery smooth muscle cells (PASMCs) (6), pulmonary artery endothelial cells (7) and pulmonary artery fibroblasts (8). Abnormal proliferation of PASMCs in the pulmonary artery media is the main feature of vascular remodeling in PAH (9). A previous study reported that aerobic glycolysis is involved in the abnormal proliferation of PASMCs (10). Moreover, it has been reported that inhibition of aerobic glycolysis reverses pulmonary artery remodeling and reduces pulmonary circulatory resistance by inhibition of the abnormal proliferation of PASMCs (11). In multiple cancer types, the expression of pyruvate kinase M2 (PKM2) is upregulated, which promotes the occurrence of aerobic glycolysis (12-14). In human glioblastoma specimens, a previous study reported the direct binding of EGFR-activated ERK2 to PKM2 through the ERK2 docking groove, which facilitated phosphorylation of PKM2 at Ser37. Ser37-phosphorylated PKM2 promotes cis-trans isomerization of PKM2 by recruiting

PIN1, and cis-trans isomerized PKM2 binds to importin α5 and translocates to the nucleus. In the nucleus PKM2 induces c-Myc expression as a coactivator of β-catenin, which results in upregulation of glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA) and polypyrimidine tract-binding protein (PTB)-dependent PKM2 expression (15).

Shikonin (Fig. 1) is a naphthoquinone substance extracted from the traditional Chinese medicine 'comfrey'. The main effect of comfrey has been reported to be tumor suppression (16) via inhibition of the proliferation and promotion of the apoptosis of tumor cells (17,18). A previous study reported that the antitumor role of shikonin depends on its specific inhibition of PKM2 (19). Furthermore, a previous study reported that PKM2 exerts a critical effect on PAH, through transformation of the cell metabolic state to aerobic glycolysis, known as the Warburg affect that promotes cell proliferation. Warburg metabolism and its epigenetic regulators are high-value therapeutic targets in PAH (20). Inhibition of PKM2 alleviates hypoxic PAH through attenuation of the proliferation of endothelial cells and adventitial fibroblasts (7,8); however, the mechanism by which it ameliorates PAH has not been fully elucidated.

In the present study, the mechanisms leading to the protective effect of shikonin in monocrotaline (MCT) induced PAH (MCT-PAH) experimental rats were evaluated. A rat pulmonary artery hypertension experimental model, was established 21 days after intraperitoneal injection of MCT, and then treated with shikonin to assess its efficacy. An *in vitro* pulmonary vascular smooth muscle cell model of PAH, was used to assess the effect of shikonin on glucose metabolism to elucidate the possible mechanism of shikonin in the treatment of PAH.

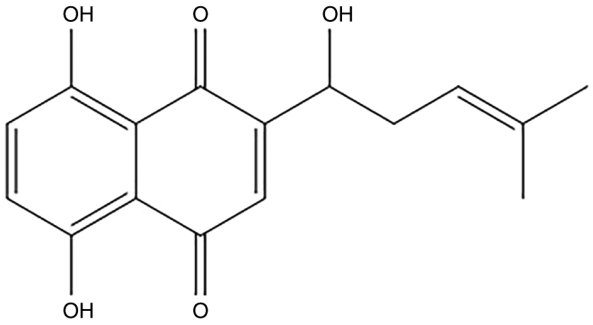

Figure 1. Structure of shikonin. The molecular formula for shikonin is $C_{16}H_{16}O_5$ and the molecular weight is 288.30.

Materials and methods

Animals and experimental design. A total of 24 specific pathogen-free male Sprague-Dawley (SD) rats (weight, 180-200 g; age, 6-8 weeks) were purchased from Hunan Changsha Tianqin Biotechnology Co., Ltd.). All rats were housed under particular conditions (12 h light/dim cycle; 22±3˚C; humidity, 30-60%) and given free access to food and water. The SD rats were divided into three groups as follows: i) MCT group, were subcutaneously injected with 60 mg/kg MCT (n=8; cat. no. C2401; MilliporeSigma) (21); ii) control group, were intraperitoneally injected with an equivalent volume of saline (n=8); and iii) shikonin group, were treated with 10 mg/kg/day shikonin (cat. no. C.I. 75535; Selleck Chemicals) via intraperitoneal infusion once daily from day 21-28 (n=8) (22) (Fig. 2). The control and MCT groups were treated with an equivalent volume of saline. The Hunan Children's Hospital Ethics Committee approved the present study (approval no. HCHLL-2020-44).

Cell culture. Primary murine PASMCs were collected from another five normal, specific pathogen-free male SD rats (weight, 180-200 g; age, 6-8 weeks) following euthanasia using 1% sodium pentobarbital (intraperitoneal injection; 130 mg/kg). PASMCs were divided into a control group, a DMSO group, a model group [20 ng/ml platelet derived growth factor-BB (PDGF-BB) (cat. no. 100-14B; PeproTech, Inc.) with DMSO] and the shikonin treatment group (1.0 μM), all treatments were applied for 24 h before the growth media was replaced with untreated media (23). All PASMCs were cultured at 37˚C. The primary murine PASMCs were cultured with Dulbecco's modified Eagle's medium (DMEM) (cat no. C11995500BT; Gibco; Thermo Fisher Scientific, Inc.) containing 1% penicillin, 15% fetal bovine serum (SFBS) (cat. no. FS301; TransGen Biotech Co., Ltd.) and nutrient mixture F-12 (DMEM/F12) (cat. no. C11330500BT; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 1% streptomycin in a 5% CO_2 incubator. The medium was replaced every 48 h, and subculture was performed at 70-80% confluence. PASMCs were identified by their morphologic appearance under phase-contrast microscopy and immunofluorescence using an anti-α-smooth muscle actin antibody (1:200; cat. no. GB11044; Wuhan Servicebio Technology Co., Ltd.) incubated with the cells overnight at 4˚C and anti-rabbit Alexa Fluor 488 secondary antibody (1:500; cat. no. GB25303; Wuhan Servicebio Technology Co., Ltd.) incubated with the cells at room temperature for 50 min. The cells were used for further experimentation at passages 3 to 5, synchronized with serum starvation prior to intervention (24).

Hemodynamic measurements. After anesthetization with 1% sodium pentobarbital (intraperitoneal injection, 40 mg/kg), the rats were placed in the lateral decubitus position for echocardiography. Using echocardiography, the pulmonary artery blood flow acceleration time (PAAT), the inner diameter of the right ventricle (RVID) and the tricuspid annular plane systolic excursion (TAPSE) were assessed. After echocardiography, the rats were placed supine on an operating table and a polyethene catheter filled with heparin was inserted into the right ventricle via the right external jugular vein to test the right ventricular systolic pressure (RVSP) with a BL-420 biological function experiment system (Chengdu Taimeng Software Co., Ltd).

Evaluation of right ventricular hypertrophy. After the pressure measurements, the rats were euthanized using 1% sodium pentobarbital (intraperitoneal injection, 130 mg/kg) and the heart and lungs were collected. The hearts were divided to dissect the right ventricle (RV) free wall from the left ventricle (LV) plus the interventricular septum (S), and the portions were weighed separately. The right ventricular hypertrophy index (RVHI) was determined from the RV/(LV + S) proportion.

Histomorphometric analysis. The lung tissues of every group were placed in 4% paraformaldehyde buffer overnight at room temperature, dehydrated and embedded in paraffin. All lung

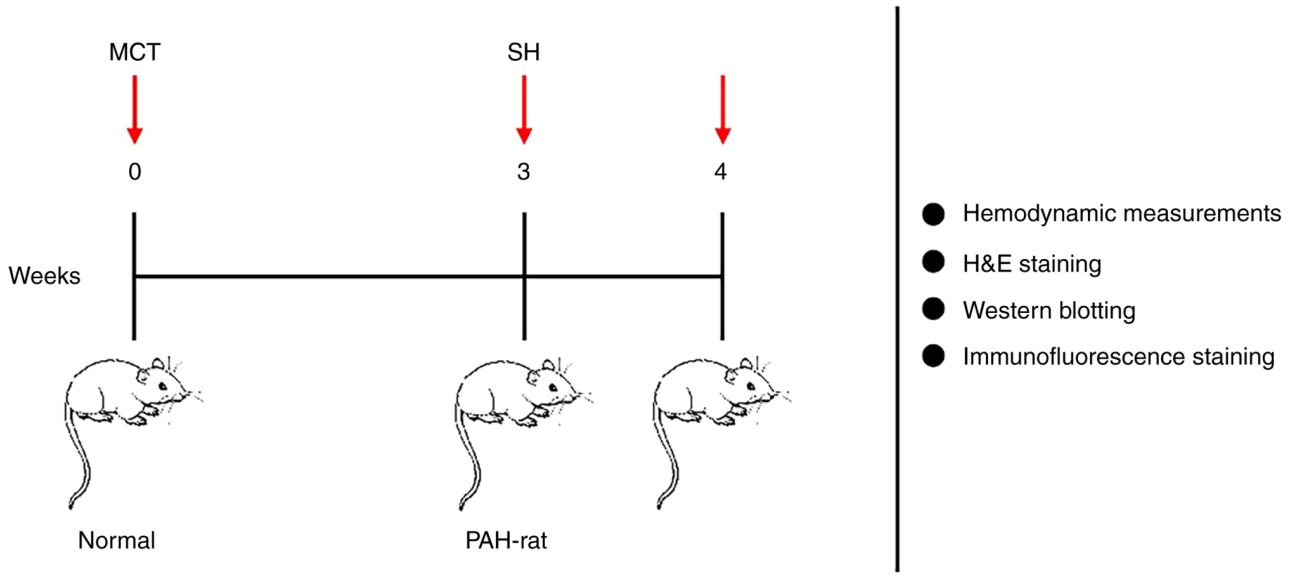

Figure 2. Experimental design of the present study. PAH was established by a single intraperitoneal injection of 60 mg/kg MCT. After 21 days, 10 mg/kg/day shikonin was administered by intraperitoneal injection. At the end of the treatment period, ultrasound hemodynamic parameters and right ventricular hypertrophy index were evaluated for the development of PAH. PAH, pulmonary arterial hypertension; MCT, monocrotaline; SH, shikonin; H&E, hematoxylin and eosin.

tissue sections (5 μm) were fixed on slides and baked until dry. Hematoxylin and eosin (H&E) staining was performed according to the manufacturer's instructions using a H&E Staining Kit (cat. no. C02-04004; BIOSS). Then, the sections were immersed in xylene, an increasing ethanol concentration gradient and sealed with resin. After drying, the pulmonary vascular morphology was assessed and imaged using an optical light microscope. Between 10-20 small pulmonary blood vessels with a diameter of 50-150 μm were randomly selected for analysis. The pulmonary artery wall thickness ratio (WT%) and pulmonary artery wall area ratio (WA%) were used to evaluate pulmonary artery remodeling. These measurements were calculated as follows: WT%=(outer diameter-inner diameter)/outer diameter and WA%=(transaction region of the walls-lumen region)/transaction area of the walls.

Western blotting. Rat lung tissue protein was extracted by incubation with RIPA lysis buffer (RIPA:PMSF, 100:1; cat. no. C5029; BIOSS), followed by sonication (1 min) and centrifugation at 15,000 x g for 15 min at 4˚C. All protein concentrations were measured using the BCA assay (cat. no. CW0014; CoWin Biosciences). Sample loading buffer (5x) was employed to dilute an equal amount of protein (20 μg/lane) from each sample, and the diluted proteins were boiled for 5 min. The proteins were separated using 8% SDS-PAGE, blotted onto nitrocellulose membranes, and probed with a specific rabbit monoclonal anti-PKM2 antibodies (1:500; cat. no. AF5234; Affinity Biosciences), rabbit monoclonal anti-phosphorylated (p)-PKM2 (Ser37) antibodies (1:500; cat. no. AF7231; Affinity Biosciences), rabbit monoclonal anti-ERK1 + ERK2 antibodies (1:1,000; cat. no. ab17942; Abcam), rabbit monoclonal anti-LDHA antibody (1:500; cat. no. ab101562; Abcam), mouse monoclonal anti-p-ERK antibody (1:1,000; cat. no. sc-7383; Santa Cruz Biotechnology, Inc.) and mouse monoclonal anti-GLUT1 antibody (1:500; cat. no. ab40084; Abcam) overnight at 4˚C. Subsequently, PBST was used to wash the membranes, which were then incubated with goat anti-mouse or goat anti-rabbit IgG antibodies (1:5,000; cat. no. SA00001-1 or SA00001-2, respectively; Proteintech Group, Inc.) for 1 h at room temperature. For confirmation of equal loading, the blots were incubated with an anti-β-actin monoclonal antibody (1:1,000; cat. no. BS6007M; Bioworld Technology, Inc.). TBST with 1% Tween 20 (cat. no. bs100; Biosharp Life Sciences) was used for washing. The protein bands were assessed using ECL reagent (Applygen Technologies, Inc.). Quantity One software 4.6.6 (Bio-Rad Laboratories, Inc.) was used to semi-quantify the grayscale values of all bands on the blots.

Immunofluorescence staining of PKM2. Paraffin sections of lung tissues were dewaxed using immersion in xylene, a decreasing ethanol concentration gradient and distilled water and then placed in a repair box containing EDTA antigen repair buffer (pH 9.0) (cat. no. C1034; Beijing Solarbio Science & Technology Co., Ltd.), and repaired using a microwave (400 watts (W) for 8 min and 100 W for 7 min). Incubation with a rabbit monoclonal anti-PKM2 antibody (1:200; cat. no. AF5234; Affinity Biosciences) at 4˚C overnight was performed and incubation with Alexa Fluro 488-conjugated secondary antibodies was then performed (1:500; cat. no. 550037; Chengdu Zhengneng Biotechnology Co., Ltd.) at room temperature for 50 min. The nuclei were stained using DAPI at room temperature for 10 min, and after washing, an anti-fade mounting solution (cat. no. P0126; Beyotime Institute of Biotechnology) was used to seal the sections. The sections were then imaged using fluorescence microscopy.

Glucose, lactic acid and ATP evaluation. The levels of glucose, lactic acid and ATP in the PASMCs were assessed using glucose (cat. no. BC2505; Beijing Solarbio Science

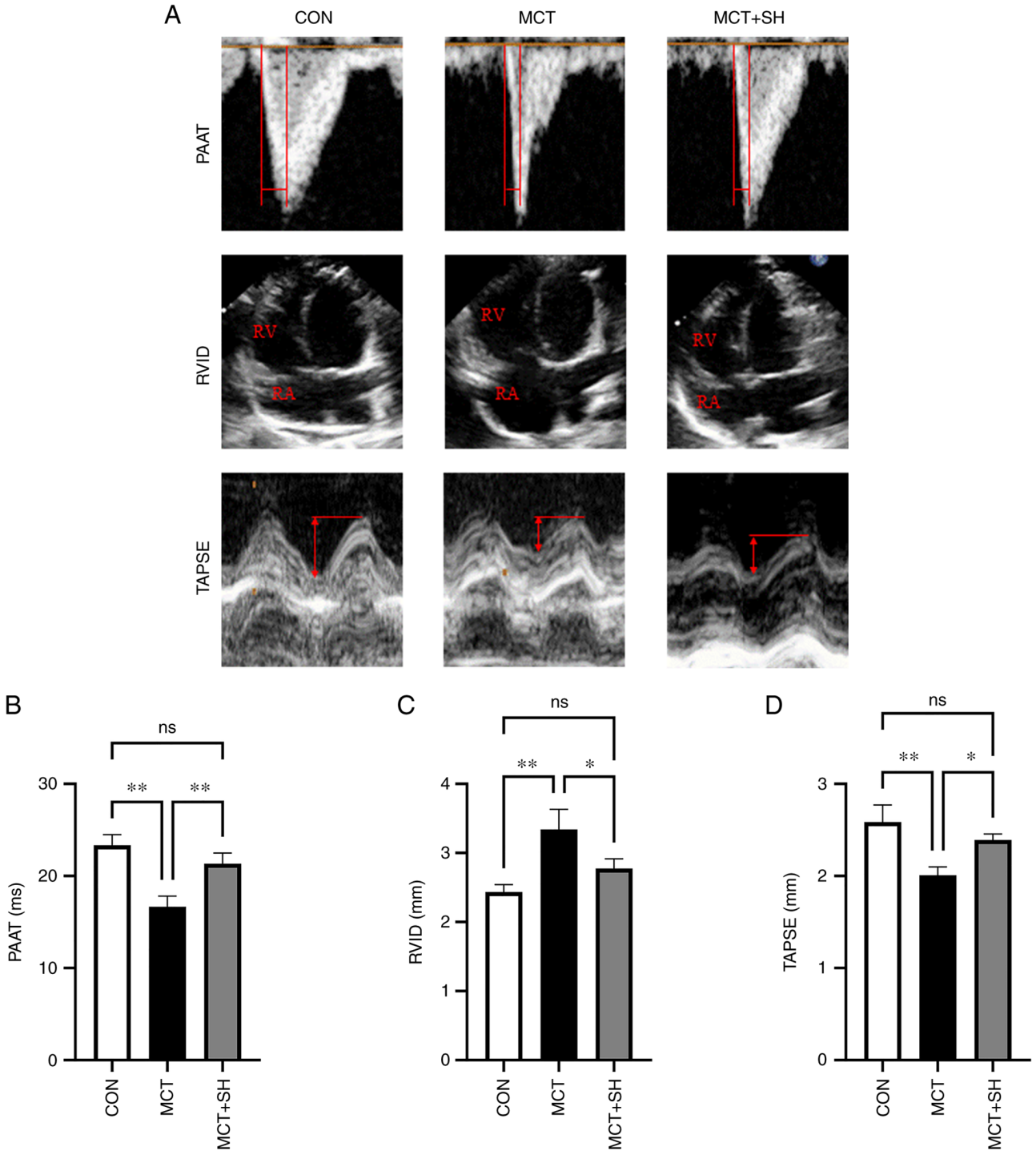

Figure 3. Shikonin improves the echocardiogram parameters of MCT-induced PAH in rats. (A) Representative echocardiogram images of the three rat groups. (B) PAAT, (C) RVID and (D) TAPSE in PAH-rats with shikonin treatment. Data are presented as mean ± SD. Control, n=8; MCT, n=6; MCT + SH, n=7. *P<0.05 and **P<0.01. CON, control; MCT, monocrotaline; SH, shikonin; PAAT, pulmonary artery blood flow acceleration time; RVID, inner diameter of the right ventricle; TAPSE, tricuspid annular plane systolic excursion; RV, right ventricle; RA, right atrium; ns, not significant.

& Technology Co., Ltd.), lactic acid (cat. no. BC2235; Beijing Solarbio Science & Technology Co., Ltd.) and ATP (cat. no. BC0305; Beijing Solarbio Science & Technology Co., Ltd.) assay kits, respectively, according to the manufacturer's protocols. Briefly, lysis buffer extracted from the PASMCs was added to a 96-well plate, and the absorbance values were measured to assess the concentrations of glucose, lactic acid and ATP. The rates of glucose consumption and lactic acid production and the amount of cellular ATP were normalized against the protein concentrations.

Statistical analysis. Quantitative data were presented as the mean ± standard error of the mean. One-way ANOVA was used for comparisons between multiple groups, and the multiple comparison least significant difference and Bonferroni tests were used for pairwise comparisons. P<0.05 was considered to

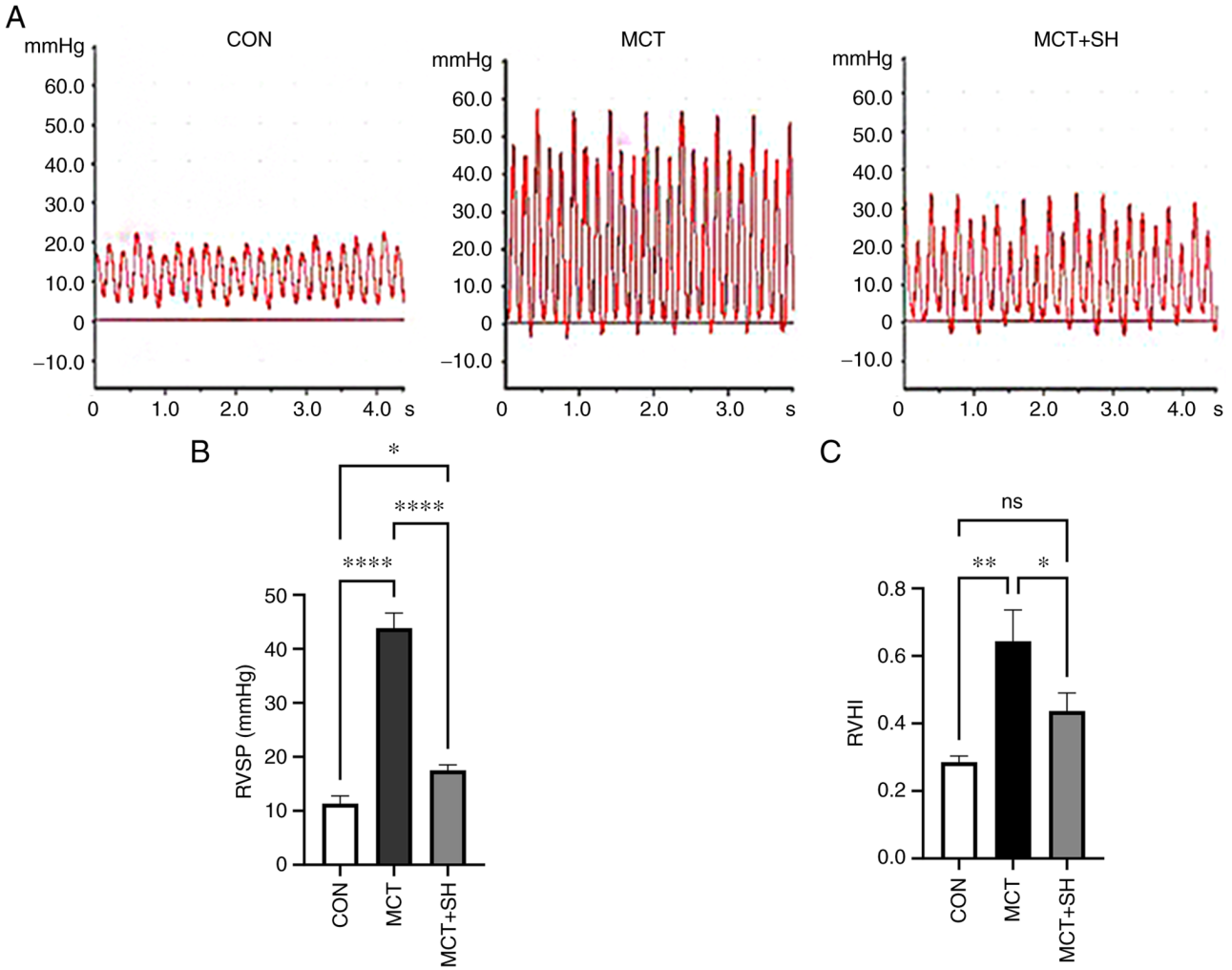

Figure 4. Shikonin decreased RVSP of MCT-induced pulmonary arterial hypertension in rats. (A) RVSP waveforms of the three rat groups. (B) RVSP in PAH-rats with shikonin treatment. (C) Shikonin reduced the RVHI of MCT-induced pulmonary arterial hypertension in rats. Data are presented as mean ± SD. Control, n=8; MCT, n=6; MCT + SH, n=7. *P<0.05, **P<0.01 and ****P<0.0001. CON, control; MCT, monocrotaline; SH, shikonin; RVSP, right ventricular systolic pressure; RV/(LV + S), Right ventricle free wall/left ventricle plus the interventricular septum; RVHI, right ventricular hypertrophy index; ns, not significant.

indicate a statistically significant difference. All experiments were repeated independently. at least three times. GraphPad Prism version 8.0 (GraphPad Software; Dotmatics) was used to perform all analyses.

Results

Shikonin improves hemodynamics in experimental PAH rats. Echocardiography demonstrated that the PAAT and TAPSE in MCT-PAH rats were significantly lower compared with those in control rats and that shikonin treatment significantly increased PAAT and TAPSE in MCT-PAH rats compared with those not treated with shikonin (Fig. 3). Shikonin significantly reduced the end-diastolic RVID of MCT-PAH experimental rats compared with those not treated with shikonin (Fig. 3). Right heart catheterization showed that the RVSP of MCT-PAH experimental rats was significantly higher compared with that of experimental rats in the control group and that Shikonin significantly reduced RVSP in MCT-PAH experimental rats compared with those not treated with shikonin (Fig. 4A and B).

Shikonin treatment improves the RVHI in PAH rats. The RV/(LV+S) ratio was calculated to assess the RVHI. A significant increase in RVHI was demonstrated in MCT-PAH rats compared with control rats, which indicated possible right ventricular hypertrophy. Administration of Shikonin by intraperitoneal injection for seven consecutive days significantly decreased RVHI in MCT-PAH rats compared with those not treated with shikonin (Fig. 4C).

Shikonin improves pulmonary vascular remodeling in MCT-induced PAH rats. Pathological changes in the small pulmonary arteries (50-150 μm) were evaluated using H&E staining. The H&E staining demonstrated that the pulmonary arteries of MCT-PAH rats exhibited increased wall thickness and luminal stenosis compared with the control group rats which had thin medial walls and large lumen. Shikonin significantly relieved MCT-induced thickening of the pulmonary artery wall compared with MCT-PAH rats not treated with shikonin. These results indicated that Shikonin greatly improved MCT-induced pulmonary vascular remodeling (Fig. 5).

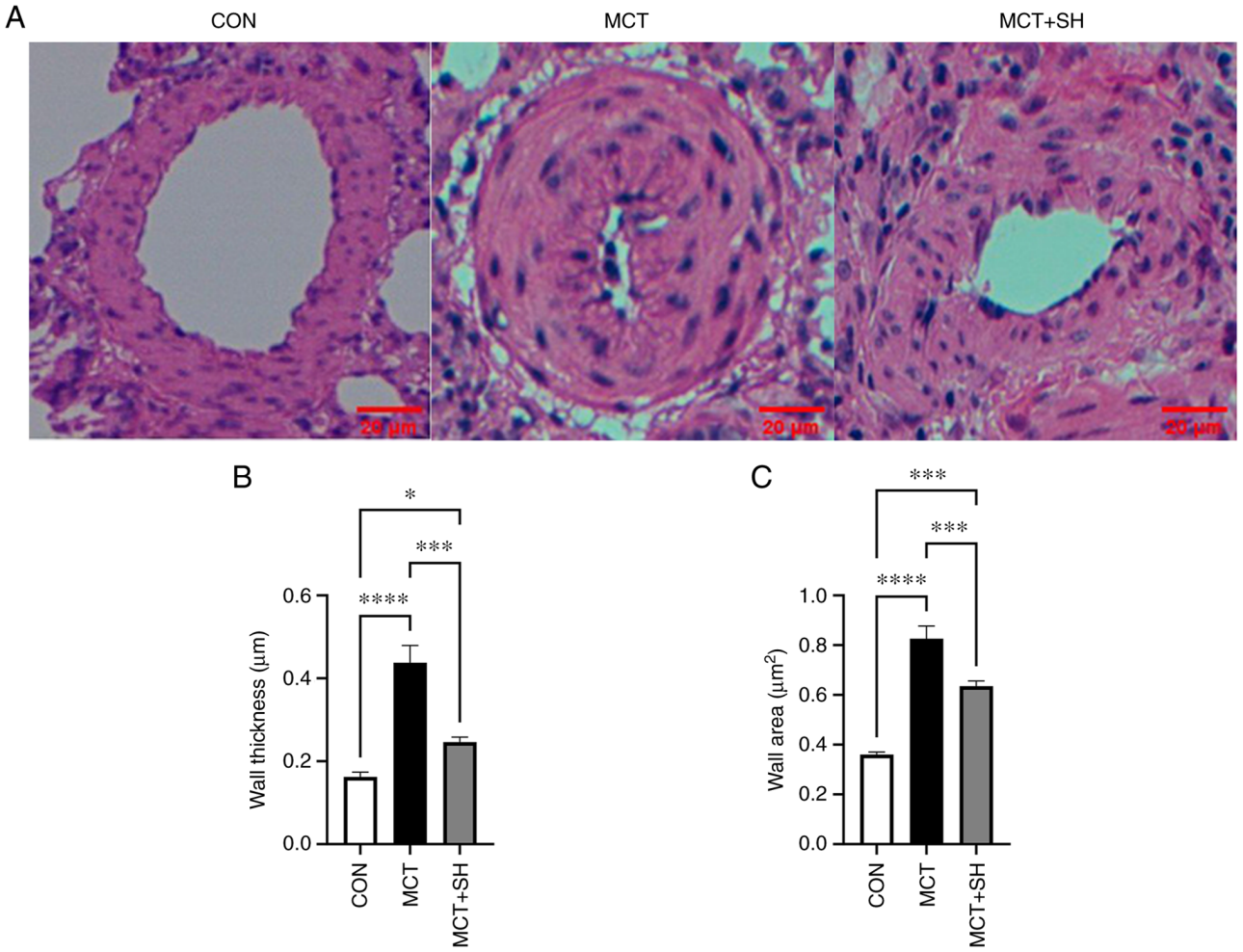

Figure 5. Shikonin suppresses pulmonary vascular remodeling of MCT-induced pulmonary arterial hypertension in rats. (A) Hematoxylin and eosin staining of pulmonary arterioles (x400) of the three groups of rats. (B) Shikonin treatment decreased pulmonary vessel wall thickening. (C) Shikonin treatment decreased pulmonary vessel wall area. Scale bar=50 μm. Data are presented as mean ± SD. Control, n=8; MCT, n=6; MCT + SH, n=7. *P<0.05, ***P<0.001 and ****P<0.0001. CON, control; MCT, monocrotaline; SH, shikonin; ns, not significant.

Shikonin inhibits PKM2 and downstream signaling protein expression. The expression of aerobic glycolysis enzymes in MCT-PAH rats was assessed using western blotting. To further investigate the connection between shikonin and aerobic glycolysis, the effect of shikonin on the protein expression levels of PKM2 and its downstream signaling proteins was evaluated, as these proteins have been reported to serve key roles in aerobic glycolysis (20). The results demonstrated, that compared with the control rats, the MCT-PAH rats exhibited significantly increased protein expression levels of PKM2, p-PKM2, p-ERK, GLUT1 and LDHA in lung tissue, which were significantly reversed by shikonin. However, significant changes in ERK1/2 protein expression levels were not detected in lung tissue from any experimental rats (Fig. 6).

Shikonin inhibits PKM2 expression in the pulmonary arteries of MCT-treated rats. Laser confocal microscopy was used to assess the PKM2 fluorescence intensity in pulmonary arteries and demonstrated that the fluorescence intensity was markedly increased in MCT-PAH rats compared with the control. Shikonin reduced the PKM2 fluorescence intensity in the pulmonary arteries of MCT-PAH rats compared with those not treated with shikonin (Fig. 7).

Shikonin decreases the Warburg effect in PASMCs. Extracellular glucose consumption, lactic acid production and cellular ATP levels were assessed to evaluate whether shikonin improved PAH through inhibition of the Warburg effect. The results demonstrated significant increases in glucose consumption and lactic acid generation and a significant decrease in ATP generation in PDGF-treated PASMCs compared with the control. Shikonin significantly suppressed the PDGF-induced Warburg effect *in vitro* (Fig. 8).

Discussion

The present study demonstrated that intraperitoneal injection of shikonin for 7 consecutive days exerted protective effects against MCT-induced PAH in rats. After shikonin treatment, PAAT, RVID and TAPSE values were significantly improved in MCT-induced PAH rats. Moreover, it was demonstrated that shikonin alleviated pulmonary vascular remodeling and significantly improved the RVSP and RVHI values of MCT-induced PAH rats. Western blotting demonstrated that shikonin also significantly decreased the upregulated protein expression levels of PKM2, p-PKM2, p-ERK, GLUT1 and LDHA in MCT-induced PAH rat lung tissues. Furthermore,

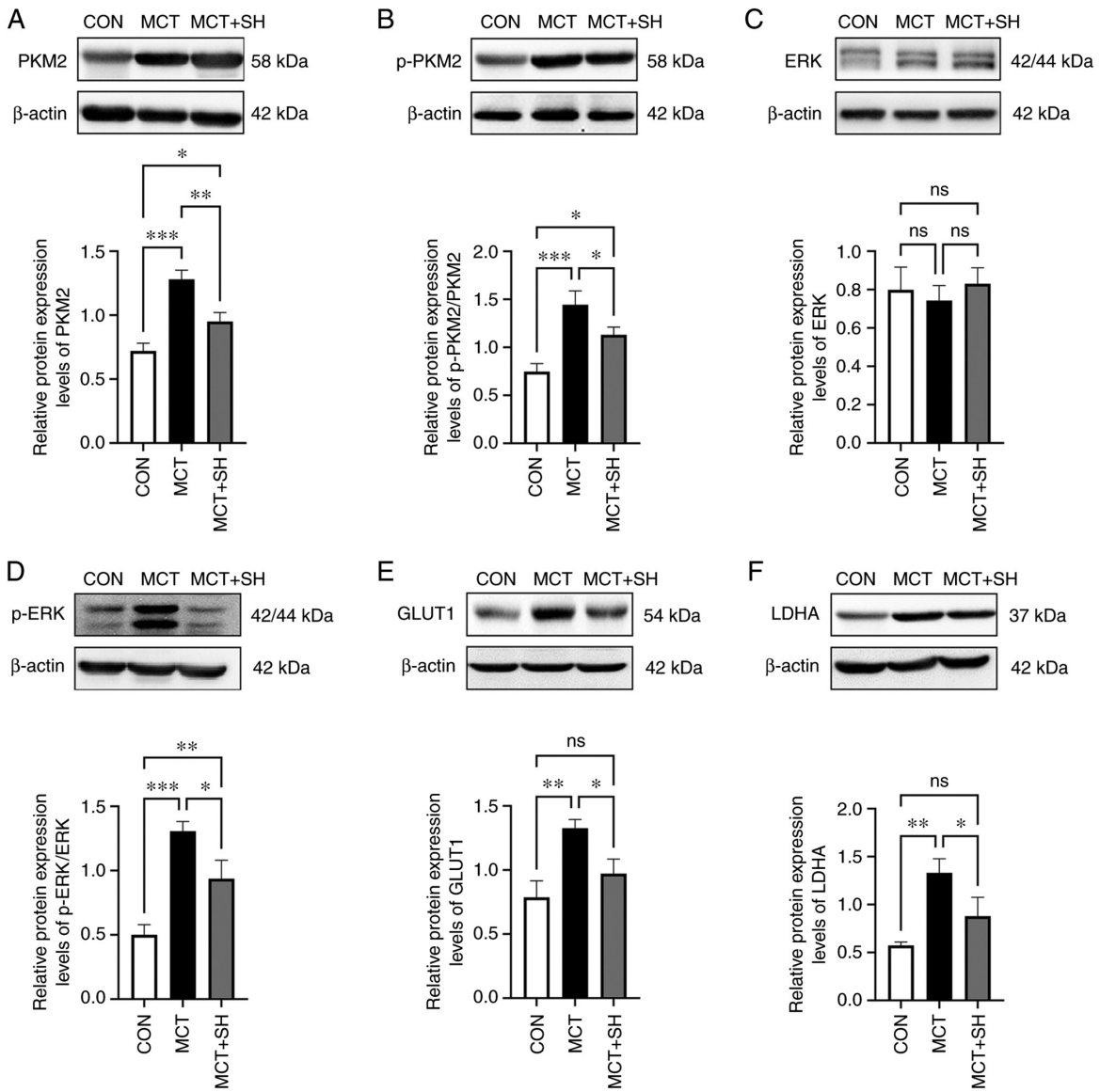

Figure 6. Expression of PKM2 signal pathway in the three groups of rat lung tissue. Representative western blot and corresponding densitometric analysis of (A) PKM2, (B) p-PKM2, (C) ERK, (D) p-ERK, (E) GLUT1 and (F) LDHA protein expression levels in the three groups of rat lung tissue. Data are presented as mean ± SD. Control, n=8; MCT, n=6; MCT + SH, n=7. *P<0.05, **P<0.01 and ***P<0.001. CON, control; MCT, monocrotaline; SH, shikonin; PKM2, pyruvate kinase M2; p, phosphorylated; GLUT1, glucose transporter 1; LDHA, lactate dehydrogenase A; ns, not significant.

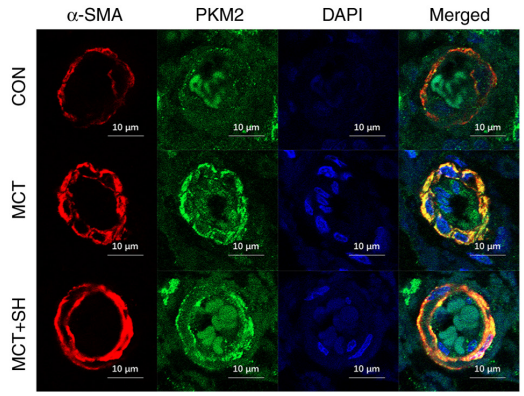

Figure 7. Effects of shikonin on PKM2 in PAH lung tissue expression. Immunofluorescence staining of α-SMA (red) and PKM2 (green) protein expression with DAPI (blue) counterstaining in the three groups of rat lung tissue. CON, control; MCT, monocrotaline; SH, shikonin; α-SMA, α-smooth muscle actin; PKM2, pyruvate kinase M2; DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride.

aerobic glycolysis was significantly inhibited by shikonin in PDGF-treated PASMCs. This result indicated that shikonin might improve MCT-induced PAH through downregulation of PKM2 expression and decreasing aerobic glycolysis. In summary, the present study demonstrated that shikonin exerted a protective effect against MCT-induced experimental PAH, partly through the reduction of aerobic glycolysis.

Pulmonary arterial hypertension has an insidious onset, rapid progression and high mortality. Therefore, elucidation of the pathogenic mechanism and improving pulmonary arterial vascular remodeling have important theoretical significance and application. The 'metabolic theory' of PAH pathogenesis centered on aerobic glycolysis and metabolism has become a popular research topic. It has been reported that aerobic glycolysis serves a core role in PAH and that blocking aerobic glycolysis can attenuate the proliferation of PASMCs in PAH (25).

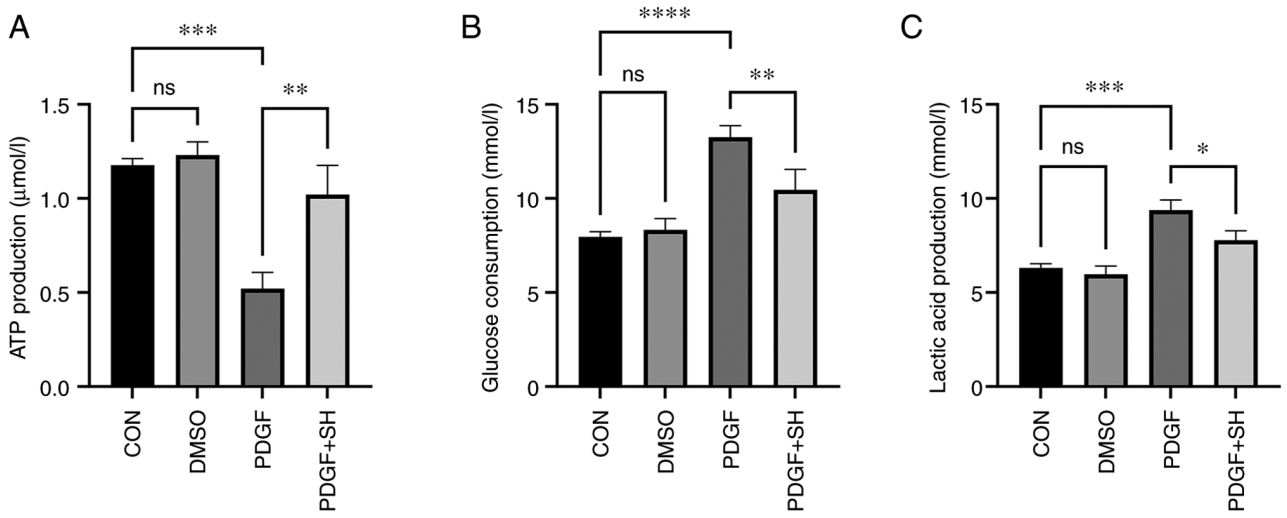

Figure 8. The cellular ATP, glucose consumption and lactic acid levels of primary pulmonary artery smooth muscle cells *in vitro*. Statistical graph of (A) ATP production, (B) glucose consumption and (C) lactic acid production. Data presented as mean ± SD. Control, n=8; DMSO, n=8; PDGF, n=8; PDGF + SH, n=8. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. CON, control; DMSO, dimethyl sulfoxide; PDGF, platelet derived growth factor; SH, shikonin; ns, not significant.

Shikonin is the biologically active component of a traditional Chinese medicine with marked antioxidant and anti-inflammatory effects (26,27). Shikonin depresses cancer by targeting certain aspects of this devastating disease, such as inhibiting cell development, migration and invasion and inducing cell death (28,29). Shikonin can improve the proliferation of endothelial cells and adventitial fibroblasts in a hypoxic pulmonary arterial hypertension mouse model through the microRNA-124/PTBP1/PKM signaling pathway and reduce pulmonary pressure in mice with PAH (8). pyruvate kinase (PK), especially PK type M2, generates pyruvate and ATP through control of the final step of glycolysis, dephosphorylating phosphoenolpyruvate and contributing to glycolytic flux in PKM2-expressing cells. Shikonin can inhibit cancer cell proliferation by modulation of the expression of PKM2. The present study evaluated whether shikonin could ameliorate MCT-induced PAH through the inhibition of PKM2 expression or activity and partly elucidated the mechanism of this.

The results of echocardiography, right heart catheterization and H&E staining demonstrated that shikonin significantly improved RVSP, pulmonary vascular remodeling and the RVHI in MCT-induced PAH rats. These results demonstrated the therapeutic effect of shikonin on MCT-induced experimental PAH, but the specific mechanism remained unclear.

After shikonin treatment, the protein expression levels of PKM2, downstream signaling pathway proteins and aerobic glycolysis-related proteins were assessed in rat lung tissue. A proliferation model of pulmonary artery smooth muscle cells was also developed *in vitro* with PDGF-BB and glucose consumption, lactic acid production and ATP production were assessed using assay kits. Shikonin significantly decreased the protein expression levels of PKM2, which was accompanied by a significant decrease in the ERK1/2 phosphorylation level and significant decreases in the protein expression levels of LDHA and GLUT1. These results indicated that the increase in PKM2 protein expression levels in PAH could also promote ERK1/2 and PKM2 phosphorylation and further upregulate the expression of critical enzymes in aerobic glycolysis, such as LDHA and GLUT1, thereby participating in PAH vascular remodeling. Furthermore, *in vitro* experiments demonstrated that after shikonin treatment, glucose consumption and lactic acid generation were significantly reduced and ATP production was significantly elevated, which indicated that shikonin suppressed the aerobic glycolysis induced by PDGF. These results provide preliminary evidence that there is upregulation of PKM2 expression in PAH and that upregulation of PKM2 promoted the phosphorylation and activation of ERK1/2, resulting in upregulation of GLUT1 and LDHA and positive feedback regulation of PKM2 expression, which enhances aerobic glycolysis and pulmonary vascular remodeling. Shikonin ameliorates PAH and pulmonary vascular remodeling in MCT-PAH rats. This mechanism may be related to the inhibition of PKM2 expression, ERK1/2 phosphorylation and aerobic glycolysis.

There were certain limitations to the present study. During the modeling process, 2 deaths occurred in the MCT group and 1 death occurred in the MCT + SH group. The two rats in the MCT group died of severe pulmonary hypertension and the rat in the MCT+SH group died of massive bloody ascites and severe peritonitis due to accidental puncture of the intestinal tube by the needle tip during intraperitoneal injection of shikonin. Moreover, in the present study, specific regulatory mechanisms between PKM2 and downstream signaling pathways were not evaluated. Therefore, the effects of shikonin on aerobic glycolysis and downstream signaling process require further evaluation in future studies.

In summary, shikonin treatment, in rats with PAH induced by MCT, exerted a significant protective effect. Shikonin treatment enhanced hemodynamics and right ventricular hypertrophy and decreased pulmonary artery remodeling. The protective effect of shikonin against PAH was associated with downregulation of PKM2, p-PKM2, p-ERK, GLUT1 and LDHA protein expression levels and inhibition of aerobic

glycolysis. These results suggested that shikonin may be a therapeutic option for patients with PAH.

Acknowledgments

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Author's contributions

WL and YX conceived and designed the experiments. WL, YZ and TH performed the experiments. WC, HP, ZX, JL, QS and XW acquired, analyzed and interpreted the data. WL and YX wrote the paper. WL and YX confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal protocols and experimental procedures were approved by The Hunan Children's Hospital Ethics Committee. (approval no. HCHLL-2020-44).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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