Canagliflozin inhibits PASMCs proliferation via regulating SGLT1/AMPK signaling and attenuates vascular remodeling in MCT-induced pulmonary arterial hypertension

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Research Article

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Abstract

Background: Pulmonary arterial hypertension (PAH) is a progressive, devastating vascular disease that eventually leads to right heart failure (RHF). Recent studies have shown that sodium-glucose cotransporter 2 inhibitors (SGLT2is) are effective in reducing cardiovascular events in patients with HF, but their efficacy in treating PAH remains uncertain. The aim of this study was to investigate the effects of canagliflozin (CANA), an SGLT2i with mild SGLT1 inhibitory effects, on rats with PAH, as well as its direct impact on pulmonary arterial smooth muscle cells (PASMCs).

Methods: PAH was induced in rats by subcutaneous injection of monocrotaline (MCT) (40 mg/kg), followed by 4 weeks of treatment with CANA by gavage (30 mg/kg/day) or saline alone. Echocardiography, hemodynamic measurements, and histological staining were performed to evaluate pulmonary vascular and right ventricular (RV) structure and function. The effect of CANA on cell proliferation was further investigated in PASMCs. Platelet-derived growth factor (PDGF)-BB, AMP kinase (AMPK) inhibitor compound C (CC) and siSGLT1 were utilized to explore the molecular regulation mechanism of CANA.

Results: Pulmonary artery and RV remodeling and dysfunction in PAH were alleviated with CANA, as assessed by echocardiography. Hemodynamic parameters, such as RV systolic pressure, and structural of pulmonary arteriole, including vascular wall thickness and wall area, were reduced by CANA treatment. RV hypertrophy index, cardiomyocyte hypertrophy, and fibrosis were decreased with CANA treatment. In vitro, PASMCs proliferation was inhibited by CANA, regardless of...
PDGF-BB stimulation. Activation of AMPK was induced by CANA treatment in cultured PASMCs in a time- and concentration-dependent manner. These effects of CANA were attenuated by treatment with CC. Abundant expression of SGLT1 was observed in PASMCs and pulmonary arteries of rats, while SGLT2 expression was undetectable. SGLT1 was increased in response to PDGF-BB stimulation, while PASMCs proliferation was inhibited and beneficial effects of CANA were counteracted by knockdown of SGLT1.

Conclusions: It is demonstrated for the first time that CANA inhibited the proliferation of PASMCs by regulating SGLT1/AMPK signaling and thus exerted an anti-proliferative effect on MCT-induced PAH. Our research revealed a novel mechanism for the beneficial effects of CANA on pulmonary vasculature.

Keywords: Canagliflozin, Pulmonary arterial hypertension, SGLT2 inhibitors, Proliferation, SGLT1, AMPK

Graphical Abstract
Background

Pulmonary arterial hypertension (PAH) was a rare and rapidly progressive vascular remodeling disease characterized by sustained elevation of pulmonary arterial pressure, resulting in right heart failure (RHF)[1]. Approximately 1% of the global population and 10% of adults aged over 65 suffered from PAH, and more than half of patients develop HF with only a median survival rate of 5 years[2, 3]. Abnormal proliferation of pulmonary arterial smooth muscle cells (PASMCs) led to pulmonary vasculature remodeling and was a key factor in the progression of PAH[4]. Current targeted drugs had limited effectiveness in preventing pulmonary vascular remodeling, highlighting the urgent need for novel treatment strategies that can efficiently target abnormal PASMCs proliferation in PAH[5].

Sodium-glucose cotransporter 2 inhibitors (SGLT2is) such as empagliflozin (EMPA), dapagliflozin (DAPA), and canagliflozin (CANA) were novel oral antidiabetic drugs that block urinary glucose reabsorption in the proximal tubule of the kidney[6]. Large cardiovascular outcome trials revealed significant and unexpected benefits of SGLT2is in different subgroups of HF[7-9]. The CHIEF-HF trial demonstrated for the first time that CANA reduced the symptom burden in HF patients at an early stage, regardless of ejection fraction (EF) or diabetes status[10]. EMBRACE-HF trial results showed that EMPA significantly and rapidly reduced pulmonary artery diastolic pressure in HF patients, with the effect observed during the first week and amplified over time[11]. Further studies are necessary to investigate the effects of CANA on structural and functional remodeling of the right ventricle.
(RV) in HF, especially in the context of PAH-induced RV remodeling.

The efficacy of SGLT2is in experimental PAH was highly controversial.

Monocrotaline (MCT)-induced rat model of experimental PAH was widely utilized in preclinical trials for the effective assessment of PAH pathophysiology and prediction of therapeutic efficacy[12]. EMPA was the first SGLT2i proven to prevent the progression of PAH in rats induced by MCT[13], whereas recent studies demonstrated that DAPA had no effect on PAH in rats induced by MCT and pulmonary trunk banding[14]. The role of CANA in MCT-induced PAH had not been investigated, and its specific mechanisms remain largely unknown. AMP kinase (AMPK) was a crucial energy sensor and regulator in eukaryotic cells[15], closely associated with the cardiovascular protective effects of SGLT2is[16]. However, it remained to be elucidated whether CANA can activate AMPK and inhibit PASMCs proliferation in PAH.

SGLT1 and SGLT2 are key members of the SGLT family. SGLT1 was located on the small intestine brush border membrane, facilitates rapid absorption of glucose and galactose from the gastrointestinal tract[17]. Growing evidence suggested that SGLT1 was closely implicated in the pathological of cardiovascular diseases, including oxidative stress, inflammation, fibrosis, proliferation[18, 19]. However, the precise involvement and contribution of SGLT1 and SGLT2 in the proliferation of PASMCs in pulmonary arteries remain uncertain. Notably, the available SGLT2is exhibit varying degrees of partial selectivity for SGLT1, which may explain their different pharmacological profiles and clinical effects[20]. Furthermore, the cardiovascular...
protective effects of SGLT2is may be associated with the inhibition of SGLT1[21].

For example, CANA exhibited stronger inhibitory effects on SGLT1 compared to EMPA[20]. This inhibition induced comprehensive anti-inflammatory and anti-apoptotic effects in the human myocardium via the SGLT1/AMPK/Rac1 signaling[22]. Therefore, further research was required to determine whether CANA exerts its effects through SGLT1 and SGLT2 in PAH.

In this study, we aimed to explore the potential cardiovascular benefits of CANA in an MCT-induced PAH rat model. We put forward a hypothesis that CANA could inhibit the proliferation of PASMCs via SGLT1/AMPK signaling, thereby reducing pulmonary vascular remodeling and alleviating RV hypertrophy and dysfunction. To test this hypothesis, we first established an MCT-induced PAH rat model and evaluated the protective effects of CANA. Next, we assessed the potential and effectiveness of CANA in inhibiting PASMCs proliferation by activating AMPK. Additionally, we investigated the expression profiles of SGLT1 and SGLT2 in PASMCs to elucidate their role in cell proliferation and their direct contribution to the effect of CANA.

Materials and methods

Animal model and treatment

Sprague-Dawley rats, weighing 200-230 g, were purchased from Shanghai SLACCAS Laboratory Animal Co. Ltd (certificate of quality SCXK 2022-0004) and housed in a common animal room with appropriate temperature and humidity. The rats were provided with unlimited access to food and water. A single subcutaneous
injection of 40 mg/kg MCT (Sigma-Aldrich, CA, USA) was administered on Day 1, following our previously established protocol, to establish the rat model of PAH[23]. The control rats received an equal volume of saline. Twenty-four rats were randomly divided into three groups (n = 8 for each group): the control group treated with vehicle (0.9% sterile saline) (Ctrl), the MCT group treated with vehicle (MCT), and the MCT group treated with CANA (30 mg/kg/day) (MCT + CANA). CANA (Janssen Pharmaceutical Co., NJ, USA) or the vehicle was administered by gavage for 4 weeks. Food intake, water consumption and body weight were measured and recorded weekly. The rats were anesthetized under 2% sodium pentobarbital (50 mg/kg) for hemodynamic and echocardiography examination. Parts of samples from lung and heart tissues were fixed in 4% paraformaldehyde for histological staining, while the rest were frozen at -80°C for further analysis (Fig. 1A).

**Echocardiography**

Transthoracic echocardiography was performed following the previously described protocol[24]. After the rats were anesthetized, their chest hair was shaved, and they were positioned in a supine position on operation board. A GE Vivid-E9 ultrasound device (General Electric Co., Fairfield, CT, USA) with a 12.0 MHz linear array transducer was used for M-mode, two-dimensional, and pulsed-wave Doppler measurements. The heart rate (HR) was recorded using synchronized electrocardiograph during the ultrasonic examination. Left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), interventricular septum thickness (IVST) and left ventricular posterior wall thickness (LVPWT) were
used to assess the structure of LV. Left ventricular ejection fraction (LVEF), cardiac output (CO), and left ventricular fractional shortening (LVFS) were calculated to assess the function of LV. Right ventricular end diastolic diameter (RVEDD), right ventricular end systolic diameter (RVESD) and right ventricular wall thickness (RVWT) were used to assess the structure of the RV. Right ventricular fractional shortening (RVFS), fractional area change (FAC) and tricuspid annular plane systolic excursion (TAPSE) were calculated to assess the function of RV. Pulmonary artery diameter (PAD) and pulmonary arterial acceleration time (PAAT) were used to assess the structure and function of the pulmonary artery in PAH. All parameters were acquired by a blinded echocardiologist who was unaware of the experimental grouping.

**Hemodynamic measurements**

Hemodynamic measurements were performed at the end of the echocardiography session, following the previously described protocol\[25, 26\]. The skin and subcutaneous tissue in the right neck of the rat were incised, and the jugular vein was isolated. A custom-made polyethylene catheter filled with heparin saline was connected to pressure transducer and inserted into the RV through the right external jugular vein. Then the RV systolic pressure (RVSP) was recorded, and the values were analyzed via a Powerlab-ML221 data acquisition and analysis system (AD Instruments Pvt Ltd, Bella Vista, New South Wales, Australia).

**Histopathological analysis**

The lungs and heart were harvested after the hemodynamic measurement, while the
weight of RV, LV and IVS were measured individually. The RV hypertrophy index (RVHI) was calculated as follows: RVHI = RV/ (LV + IVS). The lung and heart tissues were embedded in a paraffin block, sectioned into 5μm slices, and stained with hematoxylin-eosin (H&E) and Masson trichrome (MT) staining. Measurement of pulmonary arteriole wall thickness (WT), external diameter (ED), wall area (WA), and total area (TA) were determined by Image-Pro Plus 6.0 (IPP) software. The percentage of vascular wall thickness (WT%) and wall area (WA%) in pulmonary arterioles were calculated using the formulas: WT% = (2× WT/ED) × 100% and WA% = (WA/TA) × 100%. Cardiomyocyte cross-sectional area (CSA) in the RV tissue was evaluated by H&E staining. The degree of RV fibrosis was evaluated by MT staining, and the collagen volume fraction (CVF) was calculated using the following formulas: CVF% = blue area/total area × 100%.

**Immunohistochemical Staining**

The protocol was described in our previous work[27]. Briefly, the paraffin sections were deparaffinized, rehydrated, and treated with 3% BSA for 30 minutes at room temperature to saturate nonspecific bindings. Ki-67 antibody (1:500, cat. no:GB111141, Servicebio, China) and α-SMA (1:300, cat. no: GB111364, Servicebio, China) were added overnight at 4°C. Afterward, the sections were washed 3 times with phosphate buffer saline (PBS). The secondary antibody was then incubated for 1 hour at room temperature, followed by 3 times washes with PBS. Finally, the freshly prepared DAB color developing solution was added. The sections were observed under the light microscope Nikon 80i Microscope (Nikon, Japan).
Cell culture and treatment

PASMCs were isolated from adult rat pulmonary arteries tissues following the previously described protocol[28]. Briefly, the tissues were cut into small pieces and cultured in Dulbecco’s modified Eagle medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cultured dishes were maintained in a humidified atmosphere with 5% CO2 at 37°C. The well-grown PASMCs were subsequently passaged using 0.25% trypsin after approximately one week, during which they exhibited a typical "peak-valley" distribution. When PASMCs reached approximately 70-80% confluence, the culture medium was replaced with serum-free DMEM, and the cells were starved for 24 hours. Subsequently, the PASMCs were exposed to PDGF-BB (20 ng/ml, cat. no: HY-P7055, MCE, USA) with or without CANA (20 µM, cat no: HY-10451, MCE, USA) and compound C (CC, 10 µM, cat. no: HY-13418A, MCE, USA). The PASMCs were visualized and photographed under an inverted light microscope (Nikon, Japan) after a certain period of incubation. Subsequently, the cells were collected for further measurements as described below.

Cell transfection

PASMCs were cultured at 37°C and transfected overnight with siRNA (5 µmol) specific for SGLT1 (GenePharma, China), or negative siRNA as a control, using OPTI-MEM™ (GIBCO, USA) and Lipo8000™ Transfection Reagent (Beyotime Biotechnology, China) according to the previous established protocol[27]. Following transfection, the OPTI-MEM™ was replaced with a complete medium, and the
PASMCs were incubated for 24 hours. Expression of SGLT1 was then detected by quantitative RT-PCR and western blot analysis. This experiment was independently repeated five times.

**Transmission electron microscope (TEM)**

To detect the ultrastructure of PASMCs under CANA treatment, cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at room temperature and avoid light for 5 minutes, followed by gently scraped, centrifuged and postfixed in 1%OsO4 for 2 hours at room temperature. Subsequently, PASMCs were embedded in resin for ultrathin sectioning and subjected to staining with 2% uranium acetate and 2.6% lead citrate. Images were acquired using TEM (HT7800, HITACHI, China).

**Cell immunofluorescence staining**

Immunofluorescence staining of PASMCs was conducted following the previously described protocol[27]. PASMCs were dissociated using 0.25% trypsin and cultured in confocal dishes. After a single wash with PBS, the cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. Subsequently, they were rinsed with PBS and treated with 0.2% Triton X-100 on ice for 10 minutes. The cells were then blocked with 5% nonfat milk for 30 minutes at room temperature. Following the blocking step, the cells were incubated overnight at 4°C with anti-SGLT1 antibody (1:100, cat. no: PA5-88282, Invitrogen, USA). After washing 3 times with PBS, they were incubated with Alexa Fluor® 488-labeled goat anti-rabbit IgG (H + L) secondary antibody (1:200, cat. no: ZF-0511, ZSGB-BIO, China) in PBS for 2 hours at room temperature. Subsequently, the cells were washed 3 times with
PBS and counterstained with DAPI (cat. no: P0131, Beyotime, China) for 5 minutes.

Finally, the stained specimens were visualized under a Zeiss LSM780 confocal microscope (Zeiss, Germany).

**Cell proliferation assay**

The expression of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, was detected by western blot analysis to evaluate cell proliferation. At the same time, Cell Counting Kit-8 (CCK8) reagent (GlpBio, USA) was also utilized to determine cell proliferation. Specifically, PASMCs were seeded into 96-well plates in triplicates and cultured for 24 hours. Subsequently, different treatments and interventions were administered to the cells, respectively. At the end of the experiments, CCK8 reagent (10 µL) was added to each well and incubated at 37°C for 3 hours. The optical density was measured at 450 nm using a spectrophotometer (Biotek, USA). This experiment was independently repeated five times. Furthermore, the EdU Proliferation Assay Kit (Beyotime Biotechnology, China) was employed to assess cell proliferation. PASMCs were seeded into 12-well plates, and transfected or treated with different interventions after 24 hours. A cell proliferation assay was performed using the Edu-labeled proliferative cells (red) and Hoechst33342-stained nuclei (blue), which were visualized and photographed under a fluorescent microscope (Nikon, Japan).

**Reverse transcription (RT)-PCR amplification and quantitative (q) PCR**

Total RNA from pulmonary artery and PASMCs was isolated, and cDNA synthesis was performed as described previously[29]. Briefly, RNA extraction was carried out
using the FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, Nanjing, China), and cDNA synthesis was performed using Hifair® III 1st Strand cDNA Synthesis Kit (Yeasen Biotech, Shanghai, China) following the manufacturer’s instructions. The specific rat primers utilized for PCR amplification are provided in Table 1.

Quantitative RT-PCR was conducted using the Hieff® qPCR SYBR Green Master Mix (Yeasen Biotech, Shanghai, China) on the LightCycler® 96 System (Roche Diagnostics, Mannheim, Germany). The amplified products were separated on 2% agarose gels with TAE buffer and visualized using 0.5μg/ml ethidium bromide. The relative gene expression was calculated using the $2^{-ΔΔCt}$ method, and normalized to β-actin.

Table 1 Primer sequence for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGLT1</td>
<td>Forward: GGGACTGATTCTCGGCTTCC Reverse: ATGACCAAGACGGTGACGAC</td>
<td>171 bp</td>
</tr>
<tr>
<td>SGLT2 pair 1</td>
<td>Forward: CTTCGCCATCATTCTCTTTCTTGT Reverse: GACTCCCTGACC CACTCTTG</td>
<td>293 bp</td>
</tr>
<tr>
<td>SGLT2 pair 2</td>
<td>Forward: CTGGTCATTGGTGTTGGCTTG Reverse: CGATGTTGCTGGCGAACAGA</td>
<td>127 bp</td>
</tr>
<tr>
<td>SGLT2 pair 3</td>
<td>Forward: ATATCTACACAGCCTGCGG Reverse: GGTAGTGTACCAGGCAAG</td>
<td>357 bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: CGCGAGTACAACCT TCTTGC Reverse: CCTTCTGACCACCCATACCCA</td>
<td>211 bp</td>
</tr>
</tbody>
</table>

Western blot analysis

Western blot analysis was carried out as previously described[27]. Briefly, PASMCs and pulmonary artery tissues were thoroughly homogenized in lysis buffer (Beyotime, Shanghai, China) supplemented with DTT (200 µl/ml), Cock tail (10 µl/ml), and PMSF (10 µl/ml) at 4 °C, followed by centrifugation at 12,000 g for 15 minutes. The
supernatants were mixed with equal volumes of 2× SDS sample buffer. Samples were separated on 10% SDS-PAGE gel and transferred onto polyvinylidene fluoride (PVDF) membranes. Subsequently, the membranes were blocked with 5% nonfat milk for 2 hours, and then incubated overnight at 4°C with primary antibodies, including anti-SGLT1 (1:1000, cat. no: A11976, ABclonal, China), anti-PCNA (1:1000, cat. no: ab29, Abcam, USA), anti-phospho-AMPK (1:1000, cat. no: #50081, Cell Signaling Technology, USA), anti AMPK (1:1000, cat. no: #5832, Cell Signaling Technology, USA) and anti-β-actin (1:2000, cat. no: GB12001, Servicebio, China). The membranes were then washed 3 times for 10 minutes each with Tris Buffered Saline Tween (TBST) and incubated with corresponding secondary antibodies for 2 hours at 37.0°C. Finally, the protein bands were visualized using an Enhanced Chemiluminescence Detection Kit (Proteintech, Wuhan, China), captured with iBright 1500 (Thermo Fisher Scientific, Waltham, USA), and quantified with Image J software. The protein expression was normalized to β-actin, and the experiment was independently repeated five times.

**Statistical analysis**

The values were presented as the mean ± standard deviation (SD). Statistical significance between multiple experimental groups was determined using one-way ANOVA followed by Tukey’s multiple comparisons test with GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA). Only differences with $P<0.05$ were considered statistically significant.

**Results**
1. Effects of CANA on body weight and dietary intake in MCT-induced PAH rats

The effects of CANA on body weight and dietary intake were examined in MCT-induced PAH rats, with consideration of the promotion of sugar excretion through urine by CANA, resulting in glucose-induced osmotic diuresis and weight reduction[30]. As shown in Fig. 1B, the baseline body weight prior to treatment was similar among the SD rats. Lower body weight was observed in the MCT group animals compared to the Ctrl group, which was attributed to decreased food and water intake as PAH progressed. Although more food and water were consumed by rats treated with CANA (30 mg/kg/day) compared to the MCT group throughout the

**Fig. 1** Physical parameters in Ctrl and MCT-induced PAH rats treated with vehicle or CANA. A Experimental approach. Rats were randomized to receive MCT (40 mg/kg) or equal volume of saline on Day 1, followed by intragastric administration of CANA (30 mg/kg/day) or equivalent normal saline. B Body weight measured once per week in Ctrl and MCT-induced PAH rats. C, D Quantification of water intake and food intake within four weeks. Values are expressed as mean ± SD (n = 8). *P<0.05, **P<0.01, ***P<0.001 relative to Ctrl group. ###P<0.001 relative to MCT group.
4-week treatment period (Fig. 1C, D), there was a slightly decrease in the body weight of MCT-induced PAH rats when treated with CANA as compared to those without treatment (Fig. 1B).

2. Effects of CANA on RVSP and pulmonary vascular remodeling in MCT-induced PAH rats

The severity of PAH development was assessed by measuring RVSP through right heart catheterization at the fourth week. As shown in Fig. 2A, RVSP significantly increased in the MCT group, confirming the successful induction of PAH using MCT. RVSP was significantly reduced in CANA-treated rats with PAH compared to the MCT group (Fig. 2D). Echocardiographic measurements of PAD and PAAT, which are correlated with invasive pulmonary artery pressur, are commonly utilized for estimating the structure and function of pulmonary arteries[31]. In comparison to the Ctrl group, a noticeable widening in PAD was exhibited in the MCT group, but following CANA treatment, the severity of PAD was alleviated (Fig. 3H). The PAAT was found to be lower in the MCT group compared to the Ctrl group, and the down-regulation caused by PAH was significantly reversed by CANA treatment (Fig. 3I). The pulmonary structure of the MCT group was characterized by thickened arterial walls accompanied by massive infiltration of inflammatory cells, as revealed by H&E staining of lung sections, compared to the Ctrl group. However, the thickness of the arterial wall and the infiltration of inflammatory cells were significantly reduced by CANA treatment (Fig. 2B). The degree of muscular activity in pulmonary arterioles was increased in the MCT group, as demonstrated by immunohistochemistry with α-
SMA. However, this effect was attenuated by CANA treatment (Fig. 2C). The WT% and WA% of pulmonary arterioles were significantly higher in the MCT group compared to the Ctrl group, as confirmed by quantitative assessments. Notably, these increases were significantly attenuated by CANA treatment (Fig. 2E, F). Collectively,

**Fig. 2** CANA mitigates pulmonary vascular remodeling in MCT-induced PAH rats. A Representative image of RVSP in each group of animals. B Representative photomicrographs showing pulmonary vascular remodeling detected by H&E staining. Scale bar = 25 µm. C Expression of α-SMA in lung tissues evaluated with immunohistochemistry staining. Scale bar = 25 µm. D Quantification of RVSP. E, F Quantification of pulmonary arterioles WT% and WA%. Values are expressed as mean ± SD (n = 8). ***P<0.001
these findings indicate that CANA alleviates MCT-induced pulmonary vascular remodeling during PAH development.

3. Effects of CANA on RV structure and function in MCT-induced PAH rats

Transthoracic echocardiography was conducted to evaluate the effects of CANA on cardiac structure and function in MCT-induced PAH rats. As shown in Table 2, parameters such as LVEDD and LVESD, which are crucial indicators of LV structure, were found to be significantly decreased following MCT injection. However, these values were significantly ameliorated in the MCT-induced PAH rats by CANA treatment. Parameters such as RVEDD, RVESD, and RVWT were crucial indicators of RV structure, and their values were found to be significantly increased following

Table 2 Structural and functional parameters of LV in transthoracic echocardiography in MCT-induced PAH rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ctrl</th>
<th>MCT</th>
<th>MCT+CANA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>425.10±30.41</td>
<td>384.50±61.59</td>
<td>380.90±31.06</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>7.13±0.63</td>
<td>6.05±0.99*</td>
<td>7.17±0.62*</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>4.60±0.31</td>
<td>3.85±0.69*</td>
<td>4.58±0.52*</td>
</tr>
<tr>
<td>IVST (mm)</td>
<td>1.66±0.10</td>
<td>1.62±0.08</td>
<td>1.56±0.09</td>
</tr>
<tr>
<td>LVPWT (mm)</td>
<td>1.69±0.16</td>
<td>1.62±0.17</td>
<td>1.56±0.08</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>63.19±3.44</td>
<td>65.49±3.67</td>
<td>64.25±4.34</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>35.35±2.74</td>
<td>36.64±2.58</td>
<td>36.18±3.20</td>
</tr>
<tr>
<td>CO (ml/min)</td>
<td>72.15±15.39</td>
<td>46.87±13.55**</td>
<td>63.70±12.21</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SD

HR: heart rate; LVEDD: left ventricular end diastolic diameter; LVESD: left ventricular end systolic diameter; IVST: interventricular septum thickness; LVPWT: left ventricular posterior wall thickness; LVEF: left ventricular ejection fraction; LVFS: left ventricular fractional shortening;

CO: cardiac output

*P<0.05, **P<0.01 relative to Ctrl group. *P<0.05 relative to MCT group.
MCT injection (Fig. 3 B, C, G). In addition, RVFS, TAPSE, and FAC, which reflect RV function, were found to be significantly reduced in the MCT group compared to the Ctrl group, indicating severe disorder in the RV function of PAH rats (Fig. 3D-F). However, these abnormal indicators were significantly ameliorated by CANA treatment in the MCT-induced PAH rats (Fig. 3B-G). The results of RV echocardiography in rats suggest that CANA significantly alleviates the structure and function of the RV in PAH rats.
**Fig. 3** CANA attenuates pulmonary artery and RV remodeling in MCT-induced PAH rats by echocardiography. A Representative images of RVESD, RVWT, PAD, PAAT and TAPSE echocardiography images; B-I Quantitative analysis of the RVEDD, RVESD, RVFS, TAPSE, FAC, RVWT, PAD and PAAT. Values are expressed as mean ± SD (n = 8). *P<0.05; **P<0.01; ***P<0.001

4. Effects of CANA on cardiomyocyte hypertrophy and interstitial fibrosis in MCT-induced PAH rats

Histopathological analysis was performed to evaluate the effects of CANA on RV structural remodeling, specifically assessing cardiomyocyte hypertrophy and interstitial fibrosis. Cardiomyocyte CSA exhibited a substantial increase in the MCT group as evidenced by H&E staining of the RV cross-section. However, CANA treatment effectively mitigated this effect (**Fig. 4A, C**). RV fibrosis, induced by PAH, was observed via MT staining, revealing an increased presence of blue-stained fibers in the myocardial tissues of rats after 4 weeks. However, the fibrotic changes were significantly mitigated by CANA treatment (**Fig. 4B**). The deposition of collagen...
fibers, as indicated by the higher CVF in the MCT group compared to the Ctrl group, was notably attenuated by CANA treatment (Fig. 4D). These observations strongly suggest that a notable inhibitory effect on the progression of RV fibrosis induced by

![Image of H&E staining and MT staining with quantification of CSA, CVF, and RVHI](image)

**Fig. 4** CANA alleviates cardiomyocyte hypertrophy and interstitial fibrosis in MCT-induced PAH rats. **A** The cardiomyocyte CSA was stained with H&E staining. Scale bar = 25 µm. **B** Representative images of MT staining of RV free wall cardiomyocyte sections. Blue staining represents collagen. Scale bar = 100 µm and 25 µm. **C** Quantification of CSA of RV. **D** Quantification of interstitial CVF. **E** Quantification of RVHI. RVHI = RV/(LV + IVS). Values are expressed as mean ± SEM (n = 8). ***P<0.001
PAH was exerted by CANA treatment. Consistent with the above results, RVHI was significantly higher in the MCT group compared with the Ctrl group, while noticeably lower RVHI values were observed in the CANA-treated group compared to the MCT group (Fig. 4E). These histopathological findings further confirm the protective effects of CANA on RV structural remodeling induced by PAH in rats.

5. CANA attenuates PDGF-BB-induced proliferation in PASMCs

Primary rat PASMCs were selected to investigate the mechanism by which CANA alleviates pulmonary vascular remodeling in MCT-induced PAH rats. The purity of PASMCs was identified through cell immunofluorescence staining utilizing an antibody against \( \alpha \)-SMA (Fig. 5A). To simulate pathological conditions in vivo, PASMCs were treated with PDGF-BB at a concentration of 20 ng/ml for 24 hours. As illustrated in Fig. 6A, C, the proliferation of PASMCs was markedly suppressed under a light microscope by the treatment with CANA, regardless of PDGF-BB stimulation. Ultrastructural changes were observed in PASMCs following treatment with CANA, as visualized using TEM (Additional file 1: Fig. S1A). Mitochondrial (M) swelling, matrix vacuolation and dissolution, cristae fragmentation and reduction, as well as an increase in the rough endoplasmic reticulum (RER) characterized by noticeable expansion and retention, were observed. Additionally, the presence of autolysosome (ASS) and autophagosomes (AP) was detected. The protein expression levels of PCNA in PASMCs were detected by western blot analysis, which revealed an upregulation following treatment with PDGF-BB. However, the upregulations were effectively reduced by CANA, regardless of PDGF-BB stimulation (Fig. 5E).
results of the CCK8 assay showed that the relative absorbance was significantly increased by PDGF-BB, which was subsequently attenuated by CANA after 24 hours (Fig. 5F). Similar outcomes were also observed in the EdU assay (Fig. 6E). Consequently, these findings suggest that CANA inhibits the proliferation of PASMCs, irrespective of PDGF-BB stimulation.

6. CANA promotes AMPK activation in PASMCs

As illustrated in Fig. 5B, rapid morphological changes were observed in PASMCs under a light microscope following treatment with CANA for different time points. The activation of AMPK induced by CANA in a time-dependent manner was investigated by western blot analysis. A significant enhancement in AMPK phosphorylation levels was observed after just 30 minutes of incubation with CANA, and this effect persisted for at least 120 minutes. However, no alteration in the expression of AMPK in PASMCs was observed within 120 minutes (Fig. 5C).

Meanwhile, the concentration-dependent effect of the activation of AMPK induced by CANA was investigated. The expression of p-AMPK in response to the stimulation of CANA began at a concentration of 10 µM after 30 minutes and peaked at 40 µM. However, no alteration in the expression of AMPK in PASMCs was observed with 40 µM (Fig. 5D). Furthermore, the expressions of p-AMPK significantly increased following a 24-hour treatment with CANA, irrespective of PDGF-BB stimulation (Fig. 5E, F). These observed results reveal that CANA promotes AMPK activation in cultured PASMCs in a time- and concentration-dependent manner.
Fig. 5 CANA promotes AMPK activation and attenuates PDGF-BB-induced proliferation in PASMCs. A The bright field and immunofluorescence staining of PASMCs. Scale bar = 100 µm and 25 µm. B Morphological changes of PASMCs by CANA in bright field. Scale bar = 100 µm. C PASMCs were stimulated with CANA (20 µM) at different time points (0, 15, 30, 60, 90, 120
minutes), then p-AMPK, AMPK and β-actin expression were detected by western blot. D PASMCs were stimulated with different concentrations of CANA (0, 5, 10, 20, 40 µM) for 30 minutes, then p-AMPK, AMPK and β-actin expression were detected by western blot. E PASMCs were treated with PDGF-BB (20 ng/mL) and CANA (20 µM) for 24 hours, then p-AMPK, AMPK, PCNA and β-actin expression were detected by western blot. F CCK8 assay revealed CANA inhibit PDGF-BB-induced proliferation in PASMCs. Values are expressed as mean ± SD (n = 5). ns no significant; *P<0.05; **P<0.01; ***P<0.001

7. AMPK inhibition abrogates the anti-proliferation effect of CANA in PASMCs

To determine the molecular significance of AMPK activation in CANA-treated PASMCs, cells were pretreated with CC, an AMPK inhibitor, before CANA administration. As illustrated in Fig. 6A, the morphological alterations caused by CANA in PASMCs were significantly inhibited by the treatment with CC, as observed under a light microscope. CC was found to inhibit the CANA-activated phosphorylation of AMPK in a concentration-dependent manner, as demonstrated through western blot analysis. Moreover, the expression levels of PCNA were significantly upregulated when co-treated with CC, in comparison to treatment with CANA alone (Fig. 6B). In subsequent experiments, PASMCs treated with PDGF-BB were pretreated with CC to block the activation of AMPK induced by CANA. As illustrated in Fig. 6C, the morphology of PDGF-BB treated PASMCs was altered by both CANA and CC. Moreover, the morphological changes induced by CANA in PDGF-BB treated PASMCs were restored by CC. The expression level of PCNA was significantly decreased in PDGF-BB-induced proliferation by CC through the inhibition of the phosphorylation of AMPK. Additionally, the expression of PCNA protein levels in PASMCs was stimulated by PDGF-BB, but significantly
downregulated after CANA treatment. However, this phenomenon was effectively reversed by the intervention of CC (Fig. 6D). Similar outcomes were observed in the EdU and CCK8 assays (Fig. 6E, F). Collectively, these results indicate that CANA exerts anti-proliferative effect through targeted activation of AMPK in PASMCs.
(See figure on previous page.)

**Fig. 6** AMPK inhibition abrogates the anti-proliferation effect of CANA in PASMCs. **A, C** Morphological changes of PASMCs by CANA, CC and PDGF-BB in bright field. Scale bar = 100 µm. **B** PASMCs were pretreated with different concentrations of CC (0, 5, 10, 20 µM) for 2 hours and then incubated with CANA (20 µM) for 24 hours, p-AMPK, AMPK, PCNA and β-actin expression were detected by western blot. **D** PASMCs were pretreated with CC (10 µM) for 2 hours and then incubated with PDGF-BB (20 ng/mL) or CANA (20 µM) for 24 hours, p-AMPK, AMPK, PCNA and β-actin expression were detected by western blot. **E, F** EdU and CCK8 assays revealed AMPK inhibition reduced the anti-proliferative effect of CANA in PASMCs. Scale bar = 100 µm.

Values are expressed as mean ± SD (n = 5). ns no significant; *P<0.05; **P<0.01; ***P<0.001

8. Role of SGLT1 in the proliferation of PASMCs in response to PDGF-BB stimulation

The expression of SGLT1 and SGLT2 in rat PASMCs was evaluated by RT-PCR analysis, as showed in **Fig. 7A**. SGLT1 mRNA was positively detected at 171 bp, while SGLT2 expression was undetectable at 293 bp. The high expression of SGLT1 [green] in PASMCs was further confirmed by immunofluorescence staining using the confocal microscope with 20×, 40× or oil-immersion 63× objective lens (**Fig. 7B** and Additional file 1: **Fig. S2A**). Consistently, a band with an approximate molecular weight of about 73 kDa, as expected, was observed for the SGLT1 protein (**Fig. 7C**). What's more, the expression of SGLT1 was increased in response to PDGF-BB after a 24-hour period, which correlated with elevated PCNA protein levels (**Fig. 7C**). As shown in **Fig. 7D**, siRNA was used to knock down the expression of SGLT1 to further explore its role in regulating the proliferation of PASMCs. Both SGLT1 mRNA and protein levels were significantly decreased upon knockdown of SGLT1 compared with siCtrl (**Fig. 7E, F**). The expressions of PCNA in PASMCs were also down-
Fig. 7 Role of SGLT1 in the PDGF-BB-induced proliferation response in PASMCs. A RT-PCR analysis showing the expression of SGLT1 and SGLT2 in PASMCs. B Representative images of immunofluorescence staining obtained using the confocal microscope with 20× and 40× objective lens reveal the expression of SGLT1 (green) in PASMCs. Scale bar = 50 µm and 20 µm. C PASMCs were incubated with PDGF-BB (20 ng/mL) for 12 and 24 hours, the expression of SGLT1, PCNA and β-actin were detected by western blot. D Fluorescent labeling confirmed low toxicity and efficiency of transfected with an siRNA knocking down SGLT1. Scale bar = 250 µm. E Western blot revealed knocking down SGLT1 down-regulated the expressions of SGLT1 and PCNA protein in PASMCs. F RT-qPCR revealed down-regulation of SGLT1 mRNA. G CCK8 assay revealed knocking down SGLT1 reduced PASMCs proliferation. Values are expressed as mean ± SD (n = 5). ns no significant; **P<0.01; ***P<0.001
regulated when SGLT1 was knocked down (Fig. 7E). Similar results were observed in EdU and CCK8 assays, demonstrating that the proliferation of PASMCs was reduced in cells knockdown of SGLT1 compared with siCtrl (Fig. 8C, D). These results highlight the crucial role of SGLT1 in PASMCs proliferation.

**9. CANA inhibits the proliferation of PASMCs by activating AMPK through SGLT1**

To further assess whether the activation of AMPK and the suppression of proliferation by CANA were mediated through SGLT1, knockdown of SGLT1 in PASMCs was performed. As demonstrated in Fig. 8A, the morphological alterations caused by CANA in PASMCs were significantly inhibited by knockdown of SGLT1, as observed under a light microscope. Importantly, the activation of AMPK by CANA treatment was inhibited following the knockout of SGLT1, and an upregulation of PCNA expression was observed in PASMCs (Fig. 8B). Additionally, the anti-proliferative effect of CANA was significantly decreased by the knockdown of SGLT1, as demonstrated by EdU and CCK8 assays (Fig. 8C, D). These results strongly suggest that CANA directly suppresses proliferation via AMPK activation through SGLT1 in PASMCs.

**10. CANA ameliorates pulmonary vascular remodeling in rats with PAH through SGLT1/AMPK signaling**

The expression of SGLT1 and SGLT2 was determined by agarose gel electrophoresis, using kidney tissues as the positive control (Fig. 9A). Abundant expression of SGLT1 was observed in lung and pulmonary artery tissues, whereas SGLT2 was found to be
nearly undetectable. Furthermore, the absence of SGLT2 expression in these tissues was demonstrated using two additional pairs of SGLT2 primer sequences (Additional file 1: **Fig. S3A, B**). Interestingly, an increase in SGLT1 expression was observed in

**Fig. 8** CANA inhibits the proliferation of PASMCs by activating AMPK through SGLT1. **A** Morphological changes of PASMCs by CANA and siSGLT1 in bright field. Scale bar = 100 µm. **B** Cells were transfected with siRNA overnight and then incubated with CANA (20 µM) for 24 hours, p-AMPK, AMPK, PCNA and β-actin expression were detected by western blot. CANA induced AMPK activation was attenuated by knocking down SGLT1 compared with siCtrl in PASMCs. **C, D** EdU and CCK8 assays revealed knocking down SGLT1 reduced the anti-proliferative effect of CANA. Scale bar = 100 µm. Values are expressed as mean ± SD (n = 5). ns no significant; *P<0.05; **P<0.01; ***P<0.001
**Fig. 9** CANA ameliorates pulmonary vascular remodeling in rats with PAH through SGLT1/AMPK signaling. **A** RT-PCR analysis showing the expression of SGLT1 and SGLT2 pair (293bp) in kidney, lung and pulmonary artery (PA) tissues. **B** Effects of CANA treatment on the expression of SGLT1, p-AMPK, AMPK, PCNA and β-actin in rats with PAH were examined by western blot. **C** Representative images of Ki-67 immunohistochemical staining. Scale bar = 25 µm. Values are expressed as mean ± SD (n = 8). ns no significant; *P<0.05; **P<0.01; ***P<0.001.
the pulmonary artery of rats with PAH. However, the expression was subsequently
downregulated following treatment with CANA (Fig. 9B). The expression levels of p-
AMPK, AMPK, and PCNA in rat pulmonary artery were explored by western blot to
investigate the role of the SGLT1/AMPK signaling and proliferation in PAH rats.
Compared with the Ctrl group, the decreased levels of p-AMPK were associated with
a significant upregulation of PCNA expression in MCT-induced PAH rats. However,
these changes were effectively reversed by CANA intervention (Fig. 9B). In the
pulmonary arteries of rats with PAH, a significant increase in the expression of Ki-67,
a marker of cell Proliferation, was shown compared to the Ctrl group. Conversely, in
the rats treated with CANA, there was a significant decrease in the expression of Ki-67
compared to the MCT group (Fig. 9C). Collectively, these findings suggest that
CANA therapy effectively mitigates pulmonary vascular remodeling in MCT-induced
PAH via modulation of the SGLT1/AMPK signaling.

Discussion
In this work, we investigated the effects and molecular mechanisms of CANA treatment
in MCT-induced PAH rats. Our findings revealed that CANA was effective in
preventing pulmonary vascular remodeling, RV hypertrophy and dysfunction in MCT-
induced PAH rats. A significant inhibitory effect of CANA was found in PDGF-BB-
induced PASMCs proliferation by activating AMPK in vitro. Importantly, it was found
that SGLT1 was the main SGLT isoform in rat PASMCs, and expression of SGLT1 was
positively correlated with PASMCs proliferation. Furthermore, it was demonstrated that
CANA directly target SGLT1, by activating AMPK and inhibiting PASMCs
proliferation. Taken together, these results provided a novel mechanism underlying the beneficial effects of CANA on pulmonary vascular remodeling in PAH and offered new insights into the pathophysiology of this condition and potential innovative therapies.

In this study, we reported for the first time the protective effects of CANA on pulmonary vascular remodeling in a rat model of MCT-induced PAH. The results were consistent with previous findings of EMPA in MCT-induced PAH rats[13]. EMPA, the first proven SGLT2i, effectively mitigated pulmonary vascular remodeling, reduced RVSP, RV hypertrophy, and fibrosis in MCT-induced PAH rats.

It was reported that RV function is of significant predicting value of clinical prognosis and survival in patients with PAH[32]. Transthoracic echocardiography was utilized to evaluate the therapeutic effect of CANA on RV function. Our research indicated that RV function was significantly improved by CANA treatment and pulmonary trunk size was normalized. These findings suggested that CANA ameliorates the severity of illness and has a positive impact on the prognosis and survival of rats with MCT-induced PAH. However, the efficacy of DAPA in the treatment of experimental PAH remained controversial. An early study reported that DAPA neither reversed RVSP, nor pulmonary vascular remodeling in MCT-induced PAH rats, nor ameliorated RV remodeling and dysfunction under pressure overload[14]. Recently, on the contrary, it was reported that DAPA reduced RVSP and attenuated pulmonary vascular remodeling in MCT-induced PAH rats[33]. The discrepancy may be attributed to the severity of PAH, the duration of treatment or the pleiotropic effects of SGLT2is[34]. Several animal studies confirmed the cardiovascular protective effects of SGLT2is,
regardless of their glucose-lowering properties. For instance, CANA had been proved to alleviate aortic calcification in chronic kidney disease rats and VitD3-overloaded mice[35], and also effectively ameliorate cardiac inflammation and cardiac function in experimental autoimmune myocarditis[36]. Additionally, recent studies revealed that DAPA attenuated endothelial-mesenchymal transition induced by isoproterenol in non-diabetic HF mice through SIRT1-mediated deacetylation and degradation of Notch intracellular domain[37]. Our data provided strong support for the previously unrecognized protective effects of CANA in MCT-induced PAH rats, suggesting its potential as a therapeutic option for human PAH. Nonetheless, the animal models used in this study, specifically the MCT-induced PAH rat model, may not precisely replicate all five types of human pulmonary hypertension (PH). Therefore, further rigorous clinical trials involving human subjects are needed.

After observing the protective effects of CANA on PAH, we investigated its impact on PASMCs proliferation. Our findings obviously provided evidence that CANA effectively suppressed cell proliferation, reduced PDGF-BB-induced PCNA expression, and also inhibited PCNA expression in the pulmonary artery tissues of PAH rats. These findings provided strong evidence for the therapeutic potential of CANA in treating PAH by targeting PASMCs proliferation. Previous studies demonstrated that abnormal proliferation of PASMCs was central to the pathological feature of PAH[4]. PDGF-BB, a chemoattractant and potent mitogen of PASMCs, was associated with increased cell proliferation and migration, and involved in vascular remodeling during the development of PAH[38, 39]. Our previous study
demonstrated elevated levels of PDGF-BB and PCNA in the lungs of PAH rats[28].

Consistent with previous studies, in which CANA inhibited aortic smooth muscle cell proliferation[34], and DAPA inhibited angiotensin II (Ang II) induced collagen production in cardiac fibroblasts[40]. These findings indicated that SGLT2is exerted cardiovascular protective effects by suppressing proliferation in various cell types within the cardiovascular system. In PAH, it was found that PASMCs exhibit a cancer-like pro-proliferative state, whereas SGLT2is effectively inhibit proliferation in numerous cancers, including thyroid cancer[41], breast cancer[42], liver cancer[43], and glioblastoma[44]. In this study, we specifically investigated the anti-proliferative effect of CANA in PASMCs. Notably, CANA played multiple roles in anti-inflammatory and autophagy regulation, which were also implicated in the pathogenesis of PAH and deserved further exploration.

In terms of the mechanism underlying the inhibition of PASMCs proliferation by CANA, the phosphorylation of AMPK at Thr172, located at the N terminal of the α subunit, was assessed using western blot analysis. Our study revealed that CANA activated AMPK in PDGF-BB stimulated PASMCs and pulmonary artery tissues of MCT-induced PAH rats. Additionally, morphological characteristics of autophagy were observed in PASMCs following CANA treatment using TEM. Previous studies reported a strong association between autophagy and AMPK[15]. These findings strongly suggested that the inhibitory effect of CANA on PASMCs proliferation may be mediated through the activation of AMPK. Furthermore, CC, an AMPK inhibitor, effectively blocked AMPK activation and reversed the anti-proliferative effect of
CANA, providing believable evidence for the inhibitory role of CANA on PDGF-BB-induced PASMCs proliferation through AMPK signaling. AMPK was a critical regulator of various biological processes, including cell growth, proliferation, autophagy and metabolism. Previous studies demonstrated the involvement of AMPK in the abnormal proliferation of PASMCs during PAH development[45, 46]. Specifically, our research and others observed a significant inhibition of AMPK activity in PDGF-BB-induced PASMCs proliferation and lung tissues of PAH rats[28, 47, 48]. Increasing evidence supported the beneficial effects of pharmacological AMPK activation in preventing the development and progression of PAH. AICAR, a direct AMPK activator, inhibited PASMCs proliferation and prevented PAH development[49]. Metformin, a canonical AMPK activator, was also demonstrated to be effective in ameliorating PAH via suppressing PASMCs proliferation[50, 51] and mitigating vasoconstriction[52]. Our study further confirmed the importance of AMPK activation in inhibiting PASMCs proliferation and MCT-induced pulmonary hypertension in rats. Furthermore, new evidence suggested that SGLT2is exerted cardiovascular benefits through AMPK activation[16]. EMPA protected the heart from inflammation and energy depletion by activating AMPK[53], and CANA exhibited anti-inflammatory and anti-apoptotic effects in the human myocardium through AMPK activation[22]. Bibliometric and visual analysis suggested that further research in the future regarding SGLT2is would primarily investigate their molecular mechanisms, especially their specific interconnection with AMPK[54]. Previous experimental results indicated that AMPK activation by SGLT2is inhibits the
proliferation of various cancer cells[42, 55]. Activation of AMPK/FOXA1 signaling
by EMPA inhibited migration and induced apoptosis in cervical cancer cells[56].

To elucidate the mechanism of CANA activation of AMPK in PASMCs, we
initially focused on the direct target of SGLT2is. Interestingly, our study clearly
showed abundant SGLT1 expression in rat PASMCs, pulmonary artery, and lung
tissues, while SGLT2 expression was undetectable. Based on these findings, it is
worthwhile further exploration of SGLT1 involvement in PASMCs and its potential
relevance to PAH. The expression of SGLT isoforms in the cardiovascular system was
controversial, with some studies confirming SGLT2 expression in endothelial
cells[57] and cardiomyocytes[58], while most research reported its absence in human
hearts[22, 59]. Notably, we observed an increase in SGLT1 expression during PDGF-
BB-induced PASMCs proliferation, and siSGLT1 inhibited PASMCs proliferation. In
addition, the down-regulation of SGLT1 and PCNA expression was observed in PAH
rats treated with CANA. Our results support the association of SGLT1, rather than
SGLT2, with cardiovascular disease, aligning with recent research[18, 19]. SGLT1
expression was upregulated in the LV of HF patients, regardless of diabetes status[60].

Increased SGLT1 expression was associated with enhanced cell proliferation and
migration in cardiac fibroblasts under high glucose conditions, which can be
effectively counteracted by employing siSGLT1[61]. Additionally, upregulation of
SGLT1 by Ang II-induced persistent oxidative stress in endothelial cells. Inhibiting
SGLT1 can enhance protective endothelial function[57]. Recently, studies supported
the involvement of SGLT1 in diverse cancer types[17]. High expression of SGLT1
was a poor prognostic factor in breast cancer patients, while SGLT1-specific inhibitors, such as mizagliflozin and KGA-2727, had demonstrated inhibitory effects on the proliferation of breast cancer cells[62, 63]. CRISPR/Cas9 mediated knockout of SGLT1 had been shown to suppress proliferation and alter the metabolism of gastric cancer cells[64]. Our findings suggested a significant regulatory role of SGLT1 in PASMCs proliferation and its contribution to PAH pathogenesis. Therefore, further investigations were required to validate the expression and functionality of SGLT1 in human PASMCs through the collection of pulmonary artery tissues from PAH patients.

Further studies are ongoing to elucidate the role of SGLT1 in mediating the anti-proliferative effect of CANA. Remarkably, siSGLT1 blocked CANA induced AMPK activation and subsequent PCNA expression in PASMCs. Similarly, CANA downregulated SGLT1, activated AMPK, and reduced PCNA expression in the pulmonary arteries of PAH rats. These findings supported the role of CANA in alleviating PASMCs proliferation in PAH through the activation of AMPK by targeting SGLT1. Our findings were consistent with a recent study demonstrating the specific targeting of SGLT1 by CANA in human cardiomyocytes[22]. CANA had been reported to inhibit nicotinamide adenine dinucleotide phosphate oxidase activity, restored nitric oxide synthase coupling, and exerted anti-inflammatory and anti-apoptotic effects in myocardial tissue via SGLT1/AMPK/Rac1 signaling. Evidence showed distinct cellular responses to different SGLT2is. Clinical SGLT2is exhibited varying selectivity for SGLT1, which may account for the diverse cellular
responses[20]. For instance, CANA demonstrated stronger inhibition of SGLT1 than DAPA and EMPA, and robustly activated AMPK in HEK293 cells, human endothelial cells, and cancer cells[55, 65, 66]. Our findings revealed that the cardiovascular protective effect of CANA was primarily due to direct SGLT1 targeting. While previous studies showed that cardiac benefits achieved by SGLT2is can be attributed to their direct inhibition of sodium transporters in the plasma membrane, including Na⁺/H⁺ exchanger-1 (NHE1) and Nav1.5[67], it was still crucial to further investigate whether CANA affected other targets to reduce PASMCs proliferation in PAH.

Additionally, SGLT2is without SGLT1 affinity also demonstrated cardiovascular protective effects, suggesting the potential for multi-target effects of systemic SGLT2is requiring additional exploration.

**Conclusions**

Our study proved for the first time that SGLT1 was involved in the proliferation of PASMCs, which contributed to the pathogenesis of PAH. Importantly, we demonstrated that CANA inhibited PASMCs proliferation by specifically targeting SGLT1 and activating AMPK. Our research strongly indicated that targeting SGLT1/AMPK signaling was promising as a therapeutic strategy against PAH.

Furthermore, our findings provided a new perspective on promoting the clinical application of CANA in the intervention of PAH.

**List of abbreviations**

- AMPK: AMP kinase
- BSA: Bovine serum albumin
- CANA: Canagliflozin
- CC: Compound C
CCK8  Cell Counting Kit-8  
CO    Cardiac output  
CSA   Cross-sectional area  
Ctrl  Control  
CVF   Collagen volume fraction  
DAPA  Dapagliflozin  
DAPI  4’,6-diamidino-2-phenylindole  
DMEM  Dulbecco’s modified Eagle medium  
ED    External diameter  
EF    Ejection fraction  
EMPA  Empagliflozin  
FAC   Fractional area change  
H&E   Hematoxylin-eosin  
HR    Heart rate  
IVS   Interventricular septum  
IVST  Interventricular septum thickness  
LV    Left ventricle  
LVEDD Left ventricular end diastolic diameter  
LVEF  Left ventricular ejection fraction  
LVESD Left ventricular end systolic diameter  
LVFS  Left ventricular fractional shortening  
LVPWT Left ventricular posterior wall thickness  
MCT   Monocrotaline  
MT    Masson trichrome  
PAAT  Pulmonary arterial acceleration time  
PAD   Pulmonary artery diameter  
PAH   Pulmonary arterial hypertension  
PASMCs Pulmonary artery smooth muscle cells  
PBS   Phosphate buffer saline  
PCNA  Proliferating cell nuclear antigen  
PDGF-BB Platelet-derived growth factor (PDGF)-BB  
PVDF  Polyvinylidene fluoride  
RHF   Right heart failure  
RV    Right ventricular  
RVEDD Right ventricular end diastolic diameter  
RVESD Right ventricular end systolic diameter  
RVFS  Right ventricular fractional shortening  
RVHI  Right ventricular hypertrophy index  
RVSP  Right ventricular systolic pressure  
RVWT  Right ventricular wall thickness  
SD    Standard deviation  
SGLT1 Sodium-glucose cotransporter 1  
SGLT2 Sodium-glucose cotransporter 2
Declarations

Ethics approval and consent to participate

The study was approved by the Laboratory Animal Welfare and Ethics Committee of Fujian Medical University (Approval No. 2022–0842, Fuzhou, China) and conducted in accordance with the guidelines outlined in the “Guide for the Care and Use of Laboratory Animals” published by the National Academy of Sciences.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional files.

Competing interests

The authors have declared no competing interests.

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Authors' contributions

LX and XC designed the research. XC and XY conducted the research, collected data, and wrote the manuscript. HT, YY, GG, BH analyzed and interpreted data. LX, LL and
GL guided the overall experiment and revised the manuscript. All authors contributed to the article and approved the submitted version.

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References


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