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Original Research Article

Perillyl alcohol and quercetin modulate the expression of non-coding RNAs MIAT, H19, miR-29a, and miR-33a in pulmonary artery hypertension in rats

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ABSTRACT

Background: Non-coding RNAs, including long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), play critical roles in the pathogenesis and progression of pulmonary artery hypertension (PAH). LncRNA H19, myocardial infarction-associated transcript (MIAT), miR-29a, and miR-33a have been suggested as potential targets for treating arterial hypertension. We explored the expression pattern of non-coding RNAs H19, MIAT, miR-29a, and miR-33a in monocrotaline (MCT)-induced PAH rats. Moreover, we investigated whether perillyl alcohol (PA) and quercetin (QS), two plant derivatives with beneficial effects on PAH-induced abnormalities, act through regulating the expression of these non-coding RNAs.

Methods: Male Wistar rats ($n = 30$) were divided into five groups. MCT (60 mg/kg) was injected subcutaneously to induce PAH. PA (50 mg/kg daily) and QS (30 mg/kg daily) were administered three weeks after induction of PAH. H&E staining and qRT-PCR were performed to assess arteriole wall thickness and gene expression, respectively.

Results: Right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (RVH) increased in MCT and MCT + Veh. groups compared to the control group (in both $P < 0.001$). QS and PA decreased RVSP and RVH significantly. Wall thickness and fibrosis score in the MCT group (score 3) increased compared to the control group (score 0). PA and QS ameliorated wall thickness and fibrosis to score 1 (mild). Also, the expression of miR-29a and miR-33a decreased in the PAH group (in both, $P < 0.001$). Treatment with PA and QS decreased the expression of H19 ($P < 0.001$) and MIAT ($P < 0.01$) and increased the expression of miR-29a ($P < 0.01$) and miR-33a significantly ($P < 0.05$ for QS and $P < 0.001$ for PA).

Conclusions: The beneficial effects of PA and QS on PAH-induced abnormalities were exerted through returning the dysregulated expression of H19, MIAT, miR-29a, and miR-33a to normal levels in rats with MTC-induced PAH. This study emphasized the therapeutic potential of PA and QS in PAH. However, more detailed investigations are needed to clarify the underlying molecular mechanisms.

1. Introduction

Pulmonary artery hypertension is a fatal vascular disease in which the pulmonary artery cells undergo remodeling as a result of increased proliferation and decreased apoptosis, resulting in increased resistance and pressure. Increased pressure creates an afterload on the right

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ventricle, inducing adaptive hypertrophy and, finally, cardiac failure, which leads to death [1]. The prevalence of PAH in adults ranges from 15 to 25 cases per million [2]. Although the survival rate of PAH patients has increased in the last two decades, because treatments are more vasodilators in common and cannot directly eliminate vascular remodeling and improve right ventricular function, thus this disease is still incurable [3]. Therefore, any effort for improvement in current treatment methods of PAH would be helpful. A few studies have focused on adjuvant or alternative drugs, especially plant derivatives, to achieve better clinical outcomes in incurable diseases It has been reported that phytochemicals have the potential to improve pathologic features in PAH [4].

Quercetin (QS) and perillyl alcohol (PA) are phytochemical derivatives with antioxidant, anti-inflammatory, and anti-cancer properties [4–6]. The inhibitory role of QS and PA on MCT-induced PAH in rats has been previously reported [7,8]. QS exerts its inhibitory effect through reversing abnormal migration, proliferation, and apoptosis of pulmonary arterial smooth muscle cells (PASMCs) in hypoxia-induced PAH [9]. In our previous studies on the lungs and hearts of rats with MCT-PAH, we have also shown that these two plant derivatives improve vascular remodeling by affecting miRNA-204 and reducing the effect of inflammation, oxidative stress, and apoptosis, leading to the reversed progression of the disease [7].

Since 2001, advances in transcriptome analysis have revealed that the previously assumed "junk DNA" encodes a group of non-coding RNAs (ncRNAs) as a new layer of gene regulation [10,11]. Non-coding RNAs are classified according to the length of the oligonucleotides into small (20–25 nt) (microRNAs) and long (>200 nt) non-coding RNAs (lncRNAs). MicroRNAs, which account for 8% of all ncRNAs, are well-known for inhibiting post-transcriptional gene expression by binding to the 3′ UTR of mRNAs [12]. LncRNAs make up around 70% of all ncRNAs, and their regulatory role is being discovered in various genomic and cellular processes such as chromatin remodeling, genomic imprinting, post-transcriptional regulation, cell differentiation, and invasion [13]. Variations in lncRNA expression have been linked to acute lung damage, idiopathic pulmonary fibrosis, lung cancer, COPD, pulmonary hypertension, and PAH [14]. LncRNAs and miRNAs have been demonstrated to alter molecular pathways involved in endothelial dysfunction and abnormalities of PASMCs (pulmonary artery smooth muscle cells) in PAH [14,15]. Although the exact mechanism of lncRNA activity is uncertain, therapy-induced changes in lncRNA expression can suggest that disease conditions are improving.

LncRNA H19 was the first reported lncRNA [16,17] located on chromosome 1q41 in rats and transcribed as a 2.369-kb spliced and polyadenylated RNA. Even though H19 RNA levels in vertebrate tissues decline after embryonic development, its dysregulation has been identified in diseases such as lung cancer [17] and PAH [18]. MIAT (myocardial infarction-associated transcript) was first discovered in 2000 and has since been proven to be expressed in various disorders, such as myocardial infarction, cancers, diabetes, and, recently, in hypoxia-induced hypertension [19,20]. Although no link has been established between MIAT and PAH, it has been suggested that MIAT stimulates proliferation and migration in hypoxic HPAEC cells, exacerbating the development of HPH (hypoxic pulmonary hypertension), similar to what occurs in PASMCs in PAH [21]. The mir-29 family (miR-29s) has the highest expression of microRNAs in the lungs. PASMC differentiation and function are both influenced by miR-29. Furthermore, by targeting more than 16 genes linked to the ECM, miR-29 plays a significant role in collagen vascular disorders, which are increasingly identified as possible causes of PAH development [22]. The importance of miR-33a in artery thickness in atherosclerosis is well documented [23]. miR-33a has also been linked to a higher chance of carotid intima-media thickness in hypertensive patients [24]. However, it is unclear whether miR-33a has a role in arterial thickness in PAH.

The association between the administration of QS and PA and the expression of non-coding RNAs, particularly lncRNAs, in arterial hypertension remains largely unknown. Considering the improving role of PA and QS on PAH, unraveling mechanisms of action of PA and QS would be helpful for future clinical application. Here, we hypothesized that PA and QS might work through influencing the expression of non-coding RNAs such as H19, MIAT, miR-29a, and miR-33a, which have been associated with the etiology and progression of arterial hypertension.

2. Methods

2.1. Animals and experimental protocol

Male rats (Wistar, $n = 30$), weighing 230 ± 30 g, were kept on a 12-h light/12-h dark cycle with unrestricted access to water and food. Kerman University of Medical Sciences Ethics Committee authorized the experiment protocol (permission code. IR.KMU.REC.1400.486). For induction of PAH, rats received a subcutaneous (SC) single-dose injection of monocrotaline (MCT) (60 mg/kg) (Sigma-Aldrich Co., St. Louis, MO, USA) on day 0 [25]. Rats in the control group received normal saline 0.8 ml/kg on day 0, SC. After 21 days of MCT injection, when rats developed PAH, they received daily intraperitoneal injections of 5% ethanol (Veh.), 50 mg/kg PA or 30 mg/kg QS for 3 weeks (from day 22 to day 42). The studied groups ($n = 6$) were control (CTL), monocrotaline (MCT), MCT + Veh., MCT + PA, and MCT + QS.

2.2. RVH and RVSP measurements

The rats were anesthetized with IP injections of ketamine at 80 mg/kg and xylazine at 20 mg/kg at the end of the 42 days, on day 43. A polyethylene catheter (PE 50) filled with heparinized saline (7 units/ml) was inserted into the right ventricle (RV) via the right jugular vein to assess right ventricular systolic pressure (RVSP) as an indicator of pulmonary arterial systolic pressure. Pressure transducers and then a power lab system was used to connect the catheter (AD instrument, NSW, Australia). The RV hypertrophy index was determined as the ratio of the right ventricle weight to that of the left ventricle plus septum [RV/(LV + S)] (RVH). For histological research, a portion of the lung was soaked in 10% formalin, while the rest was snap-frozen in liquid nitrogen and kept at 80 °C for RNA expression evaluation.

2.3. Lung tissue preparation and histopathological evaluation

At the end of the experiments (day 43), the rats were euthanized with ketamine (80 mg/kg) and xylazine (10 mg/kg). The lungs were removed immediately and stored at −80 °C. A small part of the lung tissue was fixed with 10% buffered formalin (pH 7.4) and then embedded in paraffin and cut into 5-μm-thick sections. H&E was used to stain the sections, and arteriole wall thickness was examined using a computerized light microscope (Olympus, Japan) by taking the internal diameter and subtracting it from the external diameter. Ten pulmonary arteries with an external diameter of 50–120 μm were randomly chosen for observation in each rat. Masson's trichrome staining was used for fibrosis assessment in the lung. Six fields in each sample were investigated. The quantitative scoring system was as follows: 0 (normal), 1 (<20%), 2 (20–50%), and 3 (>50%) [26].

2.4. Extraction of total RNA and quantitative real-time PCR

Total RNA, including short and long RNAs, was extracted using the total RNA Mini-Preps Kit (Bio Basic, Canada) after homogenization according to the manufacturer's instructions. RNA concentration and purity were quantified using NanoDrop 2000c (Thermo Fisher Scientific, USA). The PrimeScript 1st cDNA Synthesis Kit was used to make complementary DNA (cDNA) (Takara Bio, Japan). The specific RT primer (Table 1) was added to the reaction mixture for miRNA cDNA synthesis.

Expressions of miRNA-29a, H19, MIAT, and miR-33a genes (Table 1) were quantified by StepOnePlus instrument (Applied Biosystems, USA)

Table 1
Sequences of primers for H19, MIAT, miRNA-29a and miR-33a.

miRNA-29a	RT	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAG TTGAGTAACCGAT-3′	[27]
	Forward	5′-CCGTCCTCCGTAGCACCATCTGAAAT-3′	
	Reverse	5′-CTCAACTGGTGTCGTGGAGTCGGC-3′	
miRNA-33a	RT	TGGATATCCACACCAGGGTCCGAGGTATTCGGTGTGGATATCCATGCAAG	[28]
	Forward	CGCGCGTGCATTGTAGTTG	
	Reverse	CACCAGGGTCCGAGGT	
H19	Forward	5′-GATGGAGAGGACAGAAGGACAGT-3′	[29]
	Reverse	5′-GAGAGCAGCAGAGATGTGTTAGC-3′	
MIAT	Forward	GAGGGAAGTTCTGAGCTTGG	[30]
	Reverse	CCTTTCTTCTGGGCTGAGAC	
RNU6	Forward	5′-CTCGCTTCGGCAGCACA-3′	[25]
	Reverse	5′-AACGCTTCACGAATTTGCGT-3′	
18s	Forward	5′-AGTCCCTGCCCTTTGTACACA-3′	[26]
	Reverse	5′-CGATCCGAGGGCCTCACTA -3′	

using RealQ Plus 2x Master Mix Green (Amplicon, Denmark). Small nucleolar RNA U6 (RNU6) [27] was used as the internal control for miR-29a and miR-33a, and 18S rRNA [28] was used for the H19 and MIAT genes. Fold change expressions of H19, MIAT, miR-29a, and miR-33a were calculated using $2^{-\Delta\Delta CT}$, in which ΔCT is the difference between the CT of the gene and the CT of the internal control and ΔΔCT is the ΔCT of each group minus the ΔCT of the CTL group. Therefore, $\Delta\Delta CT = [(CT\ gene - CT\ RNU6)_{treatment} - [CT\ gene - CT\ RNu6)]_{CTL}$.

2.5. Statistical analysis

Data are expressed as mean ± SEM. The Kolmogorov-Smirnov test was used to ensure that the data were distributed normally. Statistical analysis was performed using one-way ANOVA to assess the differences between the groups. Tukey's post hoc test was performed to investigate the significantly different groups. A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of PA and QS on RVSP and RVH

In pulmonary arterial hypertension (PAH), the right ventricle (RV) is the most important indicator of functional condition and prognosis. RV hypertrophy (RVH) is a compensatory response to pressure overload even though it frequently leads to RV failure [8]. The MCT-induced PAH model was successfully constructed according to prior research [29]. PAH is characterized by high right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (RVH). RVSP is important because it is used to calculate the pressure inside the artery that supplies blood to the lungs. Injection of a single dose of MCT (60 mg/kg) significantly increased pulmonary artery remodeling and pulmonary hypertension (Table 2), with the mean RVSP rising to 86.5 ± 3.6 mmHg (MCT) and 83.78 ± 4.2 mmHg (MCT + Veh.), which was significantly higher ($P < 0.001$) than the control group (27.35 ± 1.02 mmHg). According to the increase in the ratio of RV/(LV + septum), RVH significantly increased in the MCT (0.55 ± 0.36) and MCT + Veh. (0.52 ± 0.03) groups compared with the CTL group (0.27 ± 0.03). Since RVSP and RVHI increased mainly during the third and fourth weeks after MCT administration, we started daily intraperitoneal injections of PA (50 mg/kg) and QS (30 mg/kg) 21 days after MCT administration. PA and QS treatment lasted for three weeks. According to the results, PA and QS significantly reduced RVSP and RVH ($P < 0.001$) compared to the MCT and MCT + Veh. rats. There was no significant difference between the control group RVH and RVSP rates with those of the QS and PA groups. Overall, it seems that PA and QS reversibly improved RVH and RVSP.

Table 2
RVH and RVSP measurements in monocrotaline (MCT)-induced pulmonary hypertension, and the effect of treatment with quercetin (QS) and perillyl alcohol (PA).

Groups	RVSP (mmHg)	RV/(LV + septum) Ratio
CTL	27.35 ± 1.02	0.27 ± 0.03
MCT	86.5 ± 3.6[a]	0.55 ± 0.36[a]
MCT + Veh	83.78 ± 4.2[a]	0.52 ± 0.03[a]
MCT + QS	48.5 ± 9.6[b]	0.33 ± 0.04[b]
MCT + PA	33.23 ± 3.8[b]	0.29 ± 0.038[b]

[a] $p < 0.001$ compared to CTL, and.
[b] $p < 0.001$ compared to MCT + Veh group. n = 6 in each group.

3.2. Histopathology of the lung

Vascular remodeling leads to vessel walls thickness and vasoconstriction, resulting in a significant increase in pulmonary arterial resistance. Fig. 1 shows a representative segment of the lung of rats in the study groups. The thickness of the walls is normal in the control group (22.5 ± 1.3) (A), in contrast to severe thickening in the MCT (57.13 ± 3.22) (B) and MCT + Veh. (58.78 ± 2.8) (C) groups. Mild thickening of arteriole wall thickness was observed in rats treated with QS (35.2 ± 1.7) and PA (29.23 ± 2.47), which was not significant in comparison with the control group (Fig. 1). Fibrosis level was also investigated in the studied groups. Fibrosis was severe in the MCT and MCT + Veh. groups and it decreased to a mild level after treatment with QS and PA (Fig. 2). Overall, it seems that daily administration of PA and QS reversed the histopathologic features of MCT-induced PAH, including vessel wall thickness and fibrosis, to a large extent.

3.3. The expression of MIAT and H19 changed in response to PA and QS treatment

According to the real-time PCR data (Fig. 3), the expression level of lncRNA MIAT increased significantly in the lung tissue of rats in the MCT ($P < 0.01$) and MCT + Veh. ($P < 0.05$) groups compared to the normotensive control group (Fig. 3A). Administration of PA ($P < 0.01$) and QS ($P < 0.05$) successfully decreased the expression of MIAT to normal levels. The changes in the expression of MIAT were the same in the PA and QS groups. On the other hand, the effects of PA and QS on the expression of MIAT in the lung of rats with MCT-induced PAH were similar. The expression of H19 (Fig. 3B), the other lncRNA, increased in the MCT and MCT + Veh. groups significantly ($P < 0.001$), and PA and QS returned H19 to normal levels ($P < 0.001$). The effects of PA and QS on the expression of H19 were identical. Overall, the results suggest the lncRNAs MIAT and H19 as potential markers for MCT-induced PAH in rats. The effects of PA and QS on the improvement of MCT-induced PAH are partially attributed to changes in lncRNA expression.

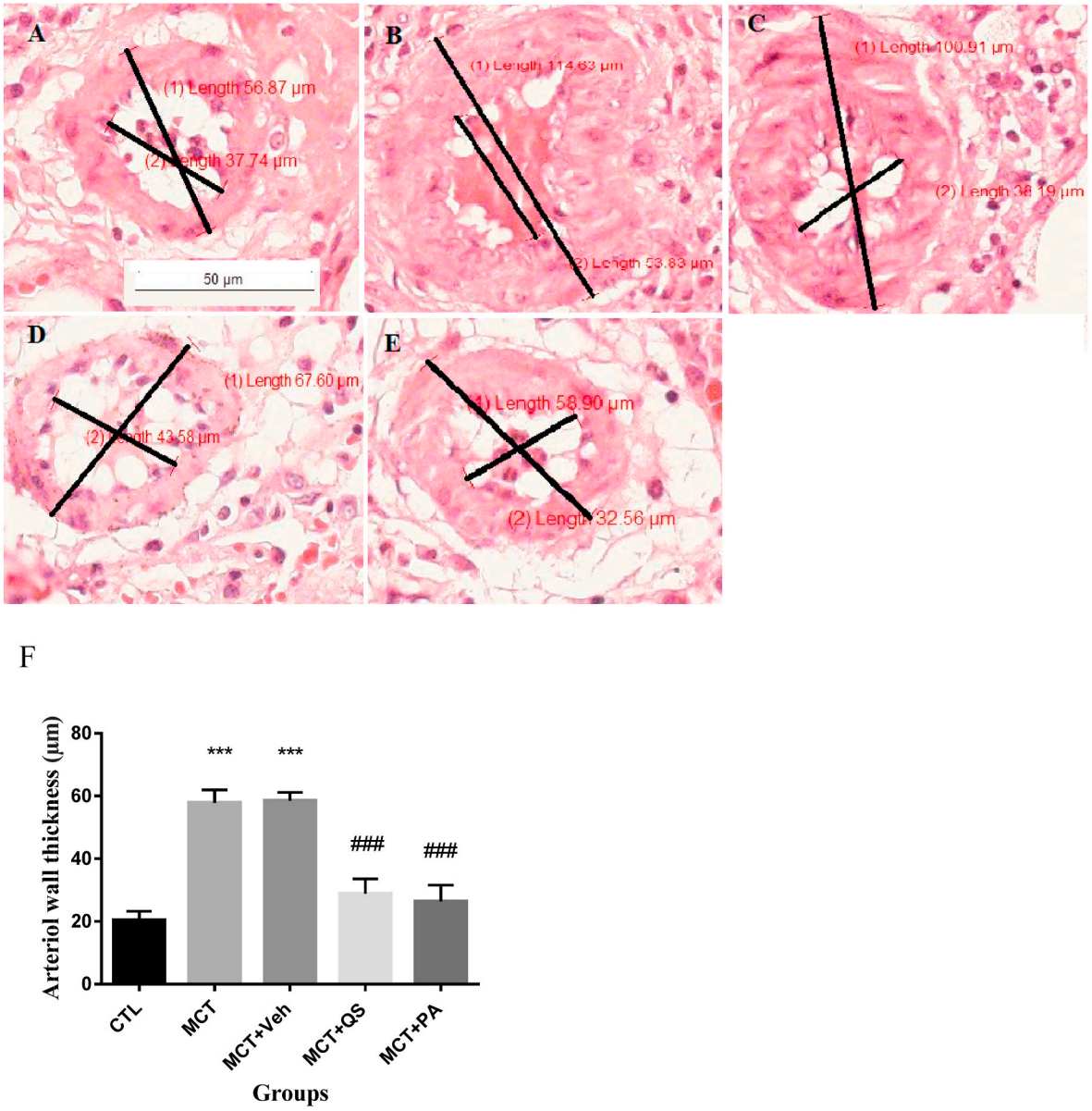

Fig. 1. Representative section of the small pulmonary arteries of rats in studied groups (A–D) and relative quantitative analyses (mean ± SEM) in groups (F). H&E staining; Magnification × 400. $n = 6$ in each group. Scale bars are 50 µm ***$P < 0.001$ *vs.* control, ###$P < 0.001$ *vs.* MCT + Veh. CTL: control; MCT: monocrotaline; Veh.: vehicle; QS: quercetin; PA: perillyl alcohol.

3.4. The expression of miR-33a and miR-29a changed in response to PA and QS treatment

We also evaluated the expression of miR-33a and miR-29a in the lung tissues of rats with PAH (Fig. 4). Our results showed significant decrease in the expression of miR-33a (MCT: $P < 0.001$; MCT + Veh.: $P < 0.01$) (Fig. 4A) and miR-29a (MCT and MCT + Veh.: $P < 0.01$) (Fig. 4B) compared to the associated controls. Administration of PA (miR-33a and miR-29a: $P < 0.05$) and QS (miR-33a: $P < 0.001$; miR-29a: $P < 0.01$) significantly returned the expression of miR-33a and miR-29a to normal levels. The effect of QS on the expression of miR-33a is slightly more than that of PA. PA and QS had identical effects on the expression of miR-29a. Overall, the results suggest miR-33a and miR-29a as potential markers for MCT-induced PAH in rats. The effects of PA and QS on the improvement of MCT-induced PAH are partially attributed to changes in miRNA expression.

4. Discussion

In the present study, PA and QS treatment influenced the expression of the lncRNAs H19 and MIAT, and the microRNAs miR-29a and miR-33a in MCT-induced PAH in rats. The expression of long non-coding RNAs H19 and MIAT was reduced by these two plant derivatives, whereas the expression of miRNAs 29a and 33a was increased.

It has been confirmed that the effects of phytochemicals on inflammation, oxidative stress, drug resistance, cell proliferation, and apoptosis are mediated through alterations in the expression of non-coding RNAs [30–32]. We showed in a prior study that QS and PA improved pulmonary artery hypertension by acting on miR-204, a critical miRNA in PAH, and its target proteins HIF1α and NFATc2 [7]. PA and QS also improved right ventricular (RV) function by modulating miR-204 expression. We also found a relation between PA and QS treatment, improved right ventricular function, and miR-204 expression [33].

It seems that changes in the expression of H19, MIAT, miR-29a, and

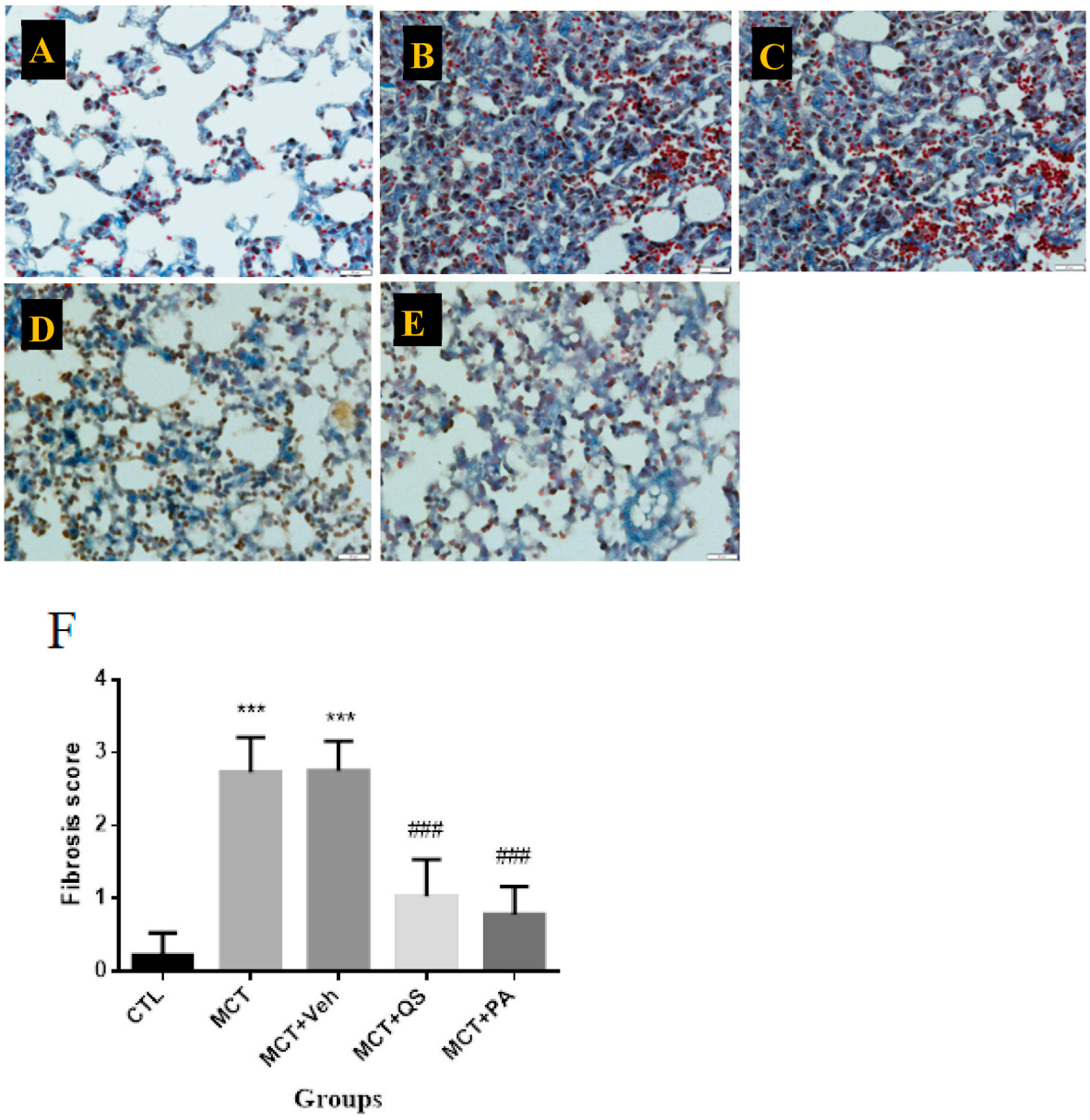

Fig. 2. Representative section of the lung of a normal rat (A), MCT (B), vehicle (C), QS-treated (D), and PA-treated (E). No significant abnormality is observed in A, but severe fibrosis was observed in B and C. The lung of rats treated with PA and QS shows mild fibrosis. Masson's trichrome staining; magnification × 100.

miR-33a might be involved in QS- and PA-induced improvements in lung pathology in rats with MTC-induced PAH. In previous studies, a decrease in the expression of H19 and MIAT and an increase in the expression of miR-29a and miR-33a have been proposed as treatment options for arterial hypertension diseases [14,19,24]. LncRNA H19 is one of the lncRNAs most involved in PAH [34,35]. Hua et al. showed the proliferation of smooth muscle cells in the pulmonary arteries in MCT-induced rats are promoted by the H19-let-7b-AT_1R axis [36]. LncRNA H19 competitively sponges let-7b and inhibits binding to its target AT1R, which belongs to the RAS GTPase family, and enhances vascular proliferation via activation of the MAPK and RhoA pathway [37]. Similarly, in another study, it has been confirmed that the H19-miR-200a-PDCD4 axis is involved in melatonin-mediated relief of MCT-induced PAH in rats [18]. Omura et al. confirmed that high serum levels of H19 in 70 PAH patients are associated with disease severity and prognosis [35]. Furthermore, inhibiting H19 reduces pathological RV hypertrophy, fibrosis, and capillary rarefaction in PAH [34]. MIAT expression was found to be reduced in MCT-induced PAH in rats in this study, which has never been reported before. Upregulation of MIAT increases the proliferation of vascular smooth muscle cells (VSMCs) and human pulmonary artery endothelial cells (HPAECs) in atherosclerosis and hypoxic pulmonary hypertension (HPH) [21,38,39]. MIAT may be implicated in PASMC proliferation, which leads to pulmonary vasoconstriction similar to HPH and atherosclerosis, considering the high levels of MIAT in PAH. The exact mechanisms, however, must be investigated.

MiR-29s are frequently dysregulated in PAH [14]. We found a lower level of miR-29a in the lung of MCT-induced PAH rats, which returned to normal after treatment with QS and PA. Smooth muscle actin (a-SMA) expression, the collagen type I alpha I chain, and fibroblast activation is affected by low miR-29 levels [40]. In addition to restoring miR-29a, QS and PA also restored normal miR-33a levels in the lung tissues of MCT-induced PAH patients. miR-33a was one of the 39 miRNAs downregulated in a surgical model of PAH; according to Rothman et al., researchers have found that miR-33a is predicted to target more than 30 PAH-related genes [40].

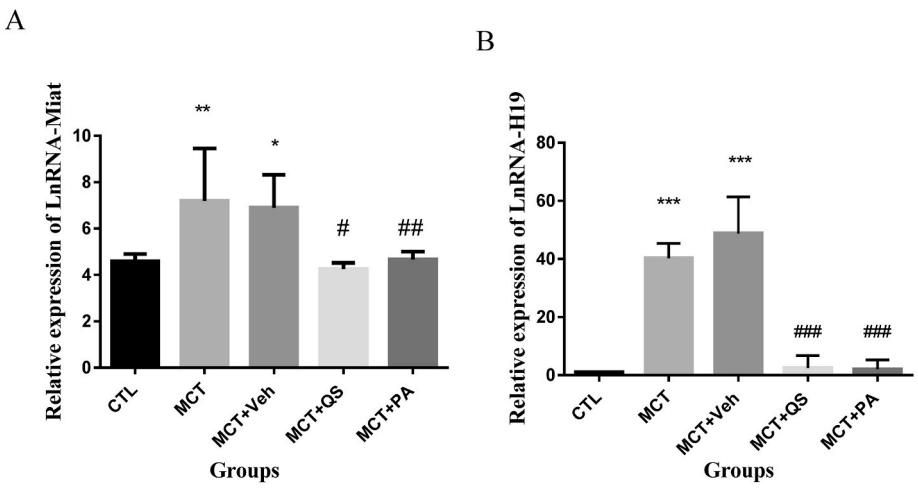

Fig. 3. Relative expression (Mean ± SEM) of MIAT (**A**) and H19 (**B**) in the lungs of studied groups. $n = 6$ in each group * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ *vs.* control, # = $P < 0.05$, ## = $P < 0.01$, ### = $P < 0.001$ MCT + Veh. CTL: control; MCT: monocrotaline; Veh.: vehicle; PA: perillyl alcohol; QS: quercetin.

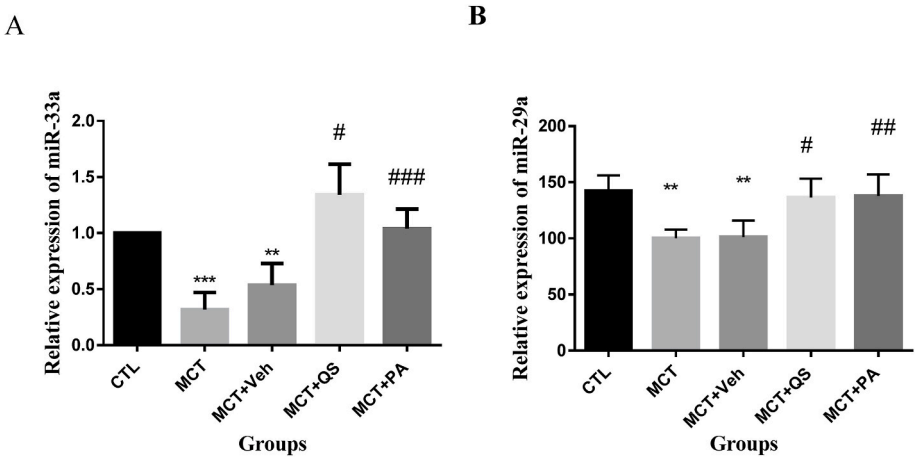

Fig. 4. Relative expression (Mean ± SEM) of miR-33a (A) and miR-29a (B) in the lungs of PAH rats, considering the impact of PA and QS therapy. $n = 6$ in each group. *and # means significantly different compared tp control and MCT + Veh., respectively. * and # = $P < 0.05$, ** and ## = $P < 0.01$, *** and ### = $P < 0.001$. CTL: control; MCT: monocrotaline; Veh: vehicle; PA: perillyl alcohol; QS: quercetin.

5. Conclusion

PA and QS influenced the expression of lncRNAs H19 and MIAT, and miRNAs miR-29a and 33a in MCT-induced PAH in rats. Given the previously established regulatory role of these ncRNAs in pulmonary hypertension, we propose that QS and PA reduce MIAT and H19 expression while raising miR-29a and miR-33a expression to partially mediate their positive effect on PAH. More research is needed to fully understand the involvement of these non-coding RNAs and MCT-induced fibrosis in PAH.

Author contributions

Soodeh Rajabi: data acquisition and drafting of the paper; Hamid Najafipour: conception of the work, interpretation of the data, and critical revision of the paper; Mozhgan Sheikholeslami and Zahra Miri Karam: molecular work and software; Saeideh Jafarinejad Farsangi: conception of the work and interpretation of the data and responsible for the molecular work; Ahmad Beik: data acquisition and drafting of the paper; Majid Askarpour: data acquisition and drafting of the paper.

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Data availability statement

Data available on request.

Declaration of competing interest

Soodeh Rajabi, Hamid Najafipour, Mozhgan Sheikholeslami, Saeideh Jafarinejad Farsangi, Ahmad Beik, and Majid Askarpour and Zahra Miri Karam declare that they have no conflict of interest. This study was approved by the Ethics Committee of Kerman University of Medical Sciences (IR.KMU.REC.1400.486) and followed the principles of the Declaration of Helsinki (October 2008).

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