Protection against monocrotaline-induced pulmonary arterial hypertension and caveolin-1 downregulation by fluvastatin in rats

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Abstract. Statins are Hydroxymethylglutaryl-coenzyme A reductase inhibitors, which are typically used to lower blood cholesterol. Additional beneficial effects, including improvement to pulmonary arterial hypertension (PAH), have also been confirmed. However, the mechanisms underlying this improvement have not yet been clarified. The present study was conducted to determine if fluvastatin was protective against experimental PAH development and to investigate the potential effects of fluvastatin on caveolin-1 (cav-1) expression. Rats were randomized to either receive a single subcutaneous injection of monocrotaline (MCT; 60 mg/kg; MCT group) or a single subcutaneous injection of MCT (60 mg/kg) followed by an oral gavage of fluvastatin (10 mg/kg) once daily until day 42 (M + F group). Rats in the MCT group received an equivalent volume of saline following the MCT injection. Six additional rats were given an equivalent volume of saline throughout as a control measure. PAH associated variables and cav-1 protein expression were measured in each group at various times during the experimental period. Hemodynamic and morphometric analysis revealed that M + F rats developed moderate, delayed PAH. Cav-1 western blot analysis demonstrated that cav-1 expression was not significantly different in fluvastatin treated rats; however, MCT injured rats given saline had markedly reduced cav-1 expression. It was concluded that fluvastatin may protect against PAH development and ameliorate MCT induced inhibition of cav-1 expression in rats.

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Introduction

Pulmonary arterial hypertension (PAH) is a condition of varying etiologies that results in an abnormally elevated mean pulmonary arterial pressure (PAP). PAH is characterized by multiple abnormalities, including endothelial cell proliferation, dysfunction of the peripheral pulmonary arterioles, thrombotic obliteration of the vascular lumen, abnormal vasomotor control and chronic remodeling of the vascular wall. In untreated cases, PAH can lead to severe right heart dysfunction and mortality (1). Although the availability of multiple therapeutic agents has improved the long-term survival for patients with PAH, the 5-year survival rate remains low (2).

Hydroxymethylglutaryl coenzyme A reductase inhibitors, or statins, are widely used for the treatment of hypercholesterolemia. Previous experiments and clinical trials have indicated that statins also exert a beneficial impact on PAH beyond their lipid-lowering effects, and may form the basis of potential future therapeutic strategies (3-5). The fungal-derived statin simvastatin can suppress the abnormal proliferation of pulmonary vascular smooth muscle cells, and induce the apoptosis of pathological vascular smooth muscle cells in a pneumonectomized and monocrotaline injured PAH model (6). Furthermore, it was reported that statins can exert protective effects against hypoxia-induced PAH (7). However, the mechanisms underlying the beneficial effects of statins have not yet been elucidated. It is well recognized that the intermolecular differences between statins can contribute to distinct additional pharmacological actions in PAH (8,9). Notably, a report by Satoh *et al* (10) indicated that the hydrophilic statin pravastatin can contribute to the development of pulmonary hypertension. The aim of the present study was to determine whether the synthetic statin fluvastatin could suppress the progression of experimental PAH. Caveolin-1 (cav-1) expression is depleted in the plexiform lesions and muscularized pulmonary arterioles that occur in patients with PAH (11), and may represent a potential upstream regulatory pathway of PAH development (12). Thus, potential associations between cav-1 expression and fluvastatin were also investigated in the PAH animal model.

Materials and methods

Animal and experimental design. A total of 136 male Wistar rats (Experimental Animal Center of Hubei Province, Wuhan, China) (250-300 g, 12 weeks old) were used in the present study. All experiment procedures were approved by the Animal Use Committee of Wuhan University (Wuhan, China) in accordance with the institutional guidelines that comply with national and international regulations.

Rats were randomly assigned to groups and received either a single subcutaneous injection of monocrotaline (MCT; 60 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; MCT group, n=66), or a single subcutaneous injection of MCT (60 mg/kg) followed by oral gavage of fluvastatin (MedChem Express, Monmouth Junction, NJ, USA) with 10 mg/kg once daily until day 42 (M + F group, n=42). MCT was prepared as previously described (12). Rats in the MCT group received an equivalent volume of saline following the MCT injection. An additional group of rats were given the equivalent volume of saline as a control measure (saline group, n=28). Rats were housed in a controlled environment under a 12-h light/dark cycle, with free access to standard rat chow and water at a controlled temperature of 20-22°C for 2 weeks prior to and throughout the experimental period. Morphometric changes, PAP, percent wall thickness (PWT), right ventricular hypertrophy index (RVHI) and cav-1 protein expression levels were measured at 0, 2, 4 and 6 week time intervals.

Hemodynamic measurement. At 0, 2, 4 and 6 week time intervals, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Once stable anesthesia was obtained, a skin incision was made on the neck to expose the trachea for cannulation to a rodent ventilator. Rats were ventilated with room air at a respiratory frequency of 70 breaths/min. Right ventricular systolic pressure (RVSP) was investigated using the tip of an intravenous trocar, which was pierced into the right ventricle. The opposite end of the trocar was connected to a pressure sensor to determine the RVSP (BL-420F; Chengdu Techman Software Co., Ltd., Chengdu, China). The left femoral artery was isolated and cannulated to a polyethylene catheter to record the systemic arterial pressure (SAP). Blood was sampled for hematocrit (HCT) measurements prior to the aforementioned recording.

Morphometric analysis. Following hemodynamic measurements, rats were euthanized by exsanguination, and the heart and lungs were removed *en bloc*. The heart was dissected and any excess blood was dried. The right ventricle (RV) free wall was separated from the left ventricle and septum (LV+S) to determine the wet weight of each section separately. RVHI was assessed by dividing the ratio of the RV by the sum of the septum plus LV weight (g). The right lower lobe of the lung was subsequently isolated, placed in liquid nitrogen and stored at -80°C until use in western blot analysis. The presence of pulmonary edema was evaluated by measuring the ratio of dry/wet lung weight as described previously (13).

Histological examination of the lung. A single lobe was excised from the lungs and fixed by perfusion with 4% (weight/volume) paraformaldehyde through a tracheal catheter at a transpulmonary pressure of 20 cm H_2O for 30 min. Following paraffin embedding, lungs were sectioned into 5 μm thick sections and subjected to staining using 0.2% hematoxylin for 5 min at room temperature and 0.5% eosin for 3 min at room temperature. Finally, 3 fields of view were analyzed and photographs were taken using a light microscope (Olympus Corporation, Tokyo, Japan; magnification, x20). Pulmonary arterial area (diameter, 100-150 μm) was measured. To assess the degree of pulmonary vascular remodeling, the PWT formula was applied (14): PWT (%)=[(external area-internal area)/external area] x100, in which the external and internal areas of the pulmonary arterioles are the areas bound by the external elastic lamina and lumen, respectively.

Cav-1 western blot analysis. Pulmonary expression of cav-1 was detected by western blot analysis. Lung samples from each group were homogenized in homogenization buffer (50 mM hydroxyethyl piperazine-ethanesulfonic acid, pH 7.55; 10% glycerol; 0.1% Triton X-100 and 1 mM dithiothreitol). Lysates were subsequently centrifuged at 7,000 x g for 10 min in order to remove the insoluble debris. Protein concentration in the supernatant was determined using a Bradford assay. Equal quantities of protein (20 μg) were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated at room temperature for 30 min in 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and subsequently washed with TBST (0.15 M NaCl; 0.05% Tween-20; 20 mM Tris-HCl, pH 8.0). They were then incubated with a rabbit polyclonal anti-cav-1 primary antibody (cat. no. ab2910; 1:1,000; Abcam, Cambridge, UK) and mouse monoclonal β-actin (cat. no. BM0627; 1:2,000; Wuhan Boster Biological Technology Ltd., Wuhan, China), at room temperature for 1 h. Following this, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. nos. BA1054 and BA1058; 1:2,000; Wuhan Boster Biological Technology Ltd.) for 1 h at room temperature and the antibody complexes were detected using enhanced chemiluminescence reagents (GE Healthcare, Chicago, IL, USA). An image analysis device (FAS-100; Toyobo Life Science, Osaka, Japan) was used to detect cav-1 immunoreactivity. The relative levels of proteins were then quantified using Image J 1.46 software (National Institutes of Health, Bethesda, MD, USA). The blots were stripped (cat. no. PS107; Epizyme, Inc., Cambridge, MA, USA) re-blotted for β-actin as a control and to ensure that equal amounts of proteins were loaded.

Reverse transcription-semi-quantitative polymerase chain reaction (sqPCR). Total RNA was extracted from lung tissue using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions and quantified using an ultraviolet spectrophotometer. RNA samples were reverse transcribed into cDNA using a reverse transcriptase kit (Takara Biotechnology Co., Ltd., Dalian, China). The cav-1 primer sequences were as follows: Forward, 5'-CAGTTGAGCGCCCACGCCAG-3' and reverse, 5'-GCGGCCTTCACCATCTTCTT-3'. The β-actin primer sequences were as follows: Forward, 5'-ATCCTGTTTCCG ACCTTCAACA-3' and reverse, 5'-CATCTCTTCCACGAA GAGCA-3' The reaction began with denaturation for 1 min at

94℃, followed by 30 cycles of replication (20 sec at 98℃ and 10 min at 68℃) and a final extension at 72℃ for 10 min using a GeneAmp PCR System 2400 (PerkinElmer, Inc., Waltham, MA, USA). The PCR products of cav-1 and β-actin mRNA were subsequently electrophoresed through a 2% agarose gel and stained with ethidium bromide (5 mg/l). The photograph of the ethidium bromide staining gel was taken using Gel-Doc 2000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The expression of cav-1 mRNA was represented by the relative yield compared with β-actin mRNA. Semi-quantitative analyses were quantified by using Image J software (National Institutes of Health).

Survival analysis. A total of 36 rats from the M + F (n=15), MCT (n=15) and saline (n=6) groups were followed for 6 weeks to assess survival rates. The day of MCT injection was defined as day 0.

Statistical analysis. Data are presented as mean ± standard error of mean. Student's t-test was used for comparison between 2 groups. For multiple group comparisons, analysis of variance was performed followed by the Fisher's least significant difference post hoc test. Survival analysis was performed using the Kaplan-Meier method with a log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of fluvastatin on the development of MCT-induced PAH. Rats had a significantly elevated RVSP (19.3±2.5 mmHg), RVHI (0.29±0.01) and PWT (48.7±2.31) following 2 weeks of MCT treatment in the MCT group. However, M + F rats did not have a significant increase in the aforementioned parameters when compared with those of saline treated rats following 2 weeks of treatment (RVSP, 16.3±2.5 mmHg; RVHI, 0.27±0.02 and PWT, 33.7±3.2%). PAH and right ventricular hypertrophy progressed further in the MCT group in the following 4 weeks. As presented in Fig. 1, MCT rats exhibited a further increase in RVSP (MCT, 30.0±5.0 mmHg; saline, 14.6±1.1; P<0.01), the RV/(LV+S) ratio (MCT, 0.34±0.03; saline, 0.27±0.01; P<0.01) and PWT (MCT, 68.0±7.0%; saline, 34.3±3.2%; P<0.001) in week 6. M + F rats had a significant elevation in RVSP and RVHI at week 6 when compared with the saline group. No differences in PWT were observed between M + F and the saline rats throughout the 6 week observation period (M + F, 37.7±3.2; saline, 32.3±2.3; P>0.05). Significantly lower values of RVSP (M + F, 22.3±3.5 mmHg; MCT, 30.0±5.0 mmHg; P<0.01) and PWT (M + F, 40.3±3.5% MCT, 68.0±7.0%; P<0.05) were observed in the M + F group compared with the MCT group at 6 weeks (Fig. 1).

Effects of fluvastatin on PAH associated variables. PAH associated variables were measured to observe the progression of MCT-induced PAH at 6 weeks (Table I). MCT rats developed a significantly reduced HCT percentage and an increased lung weight in comparison with the saline-treated rats (wet and dry). However, heart rate, SAP and dry/wet lung weight ratio were not significantly different between the groups. In

Table I. Effects of fluvastatin on pulmonary arterial hypertension associated variables in rats treated with saline, M + F and MCT.

Group	Saline	M + F	MCT
N number of rats	5	6	6
HR (beats/min)	271±5	269±9	275±4
SAP (mmHg)	82.3±5.8	84.2±6.7	85.3±9.2
HCT (%)	48±3	45±3	40±2[a]
DLW (g)	11.7±1.8	13.5±2.3[c]	17.2±2.6[a]
WLW (g)	55.6±3.6	64.4±4.7[a,c]	84.7±7.8[b]
DLW/WLW (%)	21.3±2.3	20.9±3.5	21.2±3.1

Data are presented as the mean ± standard deviation. [a]P<0.05, [b]P<0.01 vs. saline group; [c]P<0.01 vs. MCT group. HR, heart rate; SAP, systemic arterial pressure; HCT, hematocrit; DLW, dry lung weight; WLW, wet lung weight; MCT, monocrotaline; M + F, monocrotaline + fluvastatin.

comparison with saline treated rats, histological examination indicated marked pulmonary interstitial hyperplasia and inflammatory cell infiltration in the MCT group (Fig. 2), with a notably thickened alveolar duct and alveolar wall at 6 weeks following MCT injection (Fig. 2C). M + F rats also developed a moderate thickening of the alveolar septum (Fig. 2).

Effect of fluvastatin treatment on cav-1 mRNA and protein expression. The relative expression of cav-1 mRNA in saline treated rats at day 0 was 1.17±0.15 and no significant changes developed throughout the experimental period. MCT-injected rats exhibited significantly reduced cav-1 mRNA expression at the 2, 4 and 6 week time intervals compared with the saline group. In the M + F group, a significant downregulation of cav-1 mRNA expression was observed at 4 and 6 week time intervals when compared with the saline group (P<0.05; Fig. 3). Furthermore, the M + F group exhibited a significant increase in cav-1 expression at 4 and 6 week time intervals compared with MCT-injected rats (Fig. 3).

Cav-1 protein expression in the lung tissue was significantly reduced in the MCT group at the 2, 4 and 6 week time intervals (Fig. 4). By contrast, the M + F group did not exhibit a significant reduction in cav-1 protein expression within 6 weeks compared with the saline group; however, the M + F group exhibited significantly enhanced cav-1 protein expression at 4 and 6 week time intervals compared with MCT-injected rats (Fig. 4).

Survival analysis. Survival was measured from the date of MCT injection until mortality or 6 weeks following the MCT injection. During the 6 week period, there were 15 mortalities in the 36 rats studied, with 11 in the MCT group and 4 in the M + F group. The survival rate at 6 weeks was significantly higher in the M + F group compared with the MCT group (M + F, 66.7%; MCT, 26.7%; P<0.05; Fig. 5). There were no mortalities in the saline treated rats. All 15 mortalities were caused by progressive right heart failure with ascites, and pericardial and pleural effusions.
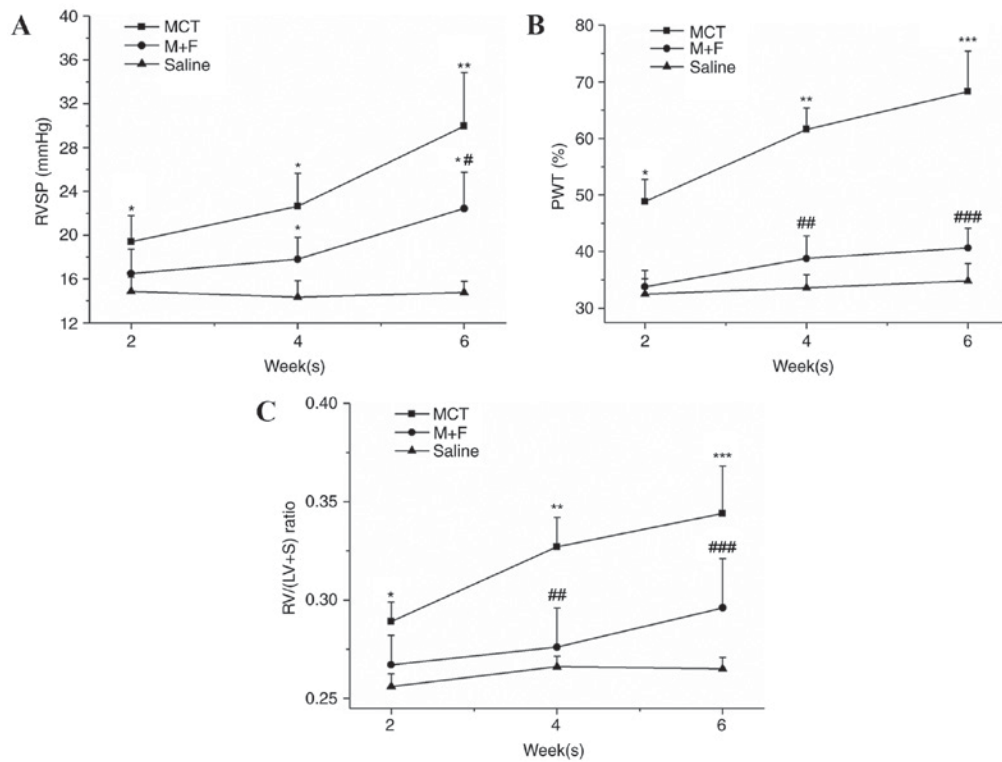
Figure 1. (A) RVSP, (B) PWT and (C) RV/(LV+S) weight ratio were examined every 2 weeks in the saline, M + F and MCT groups, respectively. Data are presented as the mean ± standard deviation of five measurements at various times. *P<0.05, **P<0.01 and ***P<0.001, vs. saline group; #P<0.05, ##P<0.01 and ###P<0.001 vs. MCT group. RVSP, right ventricular systolic pressure; PWT, percent wall thickness; RV/(LV+S), right ventricle (left ventricle + septum); MCT, monocrotaline; M + F, MCT + fluvastatin.
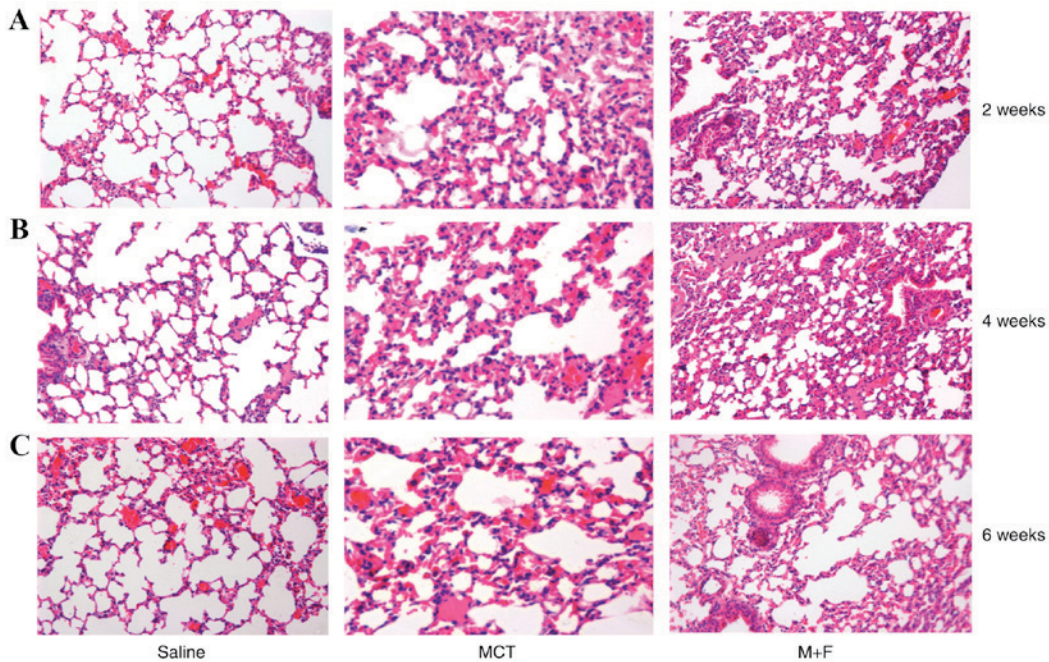

Figure 2. Histological examination of lung tissue in the saline-treated, MCT and fluvastatin treated rats at (A) 2 weeks (B) 4 weeks and (C) 6 weeks post-treatment. Magnification, x20. MCT, monocrotaline; M + F, monocrotaline + fluvastatin.

Discussion

Evidence from a variety of experimental and clinical studies has demonstrated that statins can lower serum cholesterol and also exert additional beneficial effects (15,16). Simvastatin has been reported to attenuate PAH through the regulation of the bone morphogenetic protein receptor type-2 (BMPR-2) signaling pathway in pulmonary artery endothelial cells (17,18). Simvastatin may also exert protective effects on endothelial nitric oxide synthase activity (7). Previous
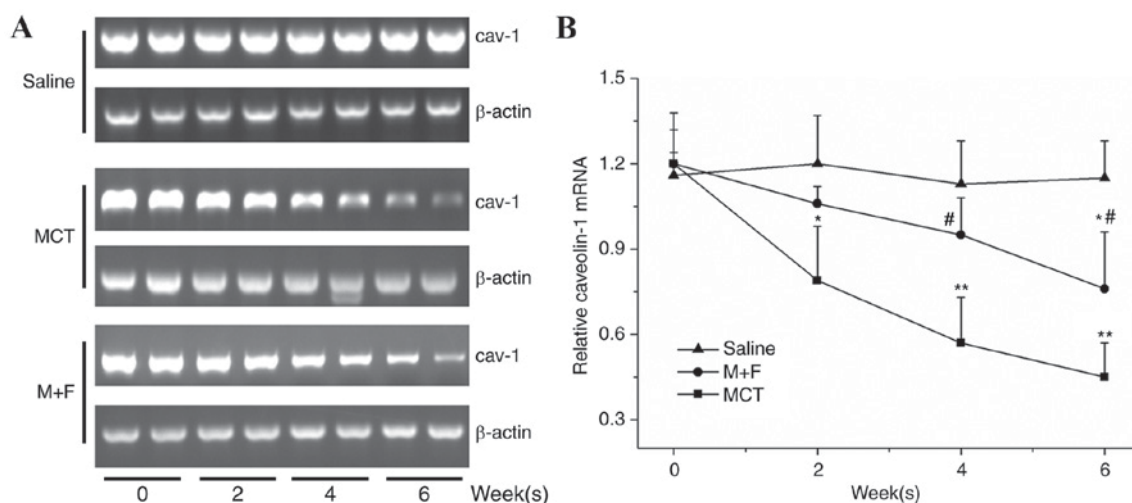
Figure 3. (A) Relative cav-1 mRNA expression as measured by semi-quantitative polymerase chain reaction in the saline, MCT and M + F groups. (B) Semi-quantification of cav-1 mRNA expression at week 0, 2, 4 and 6 week time intervals in the saline, MCT and M + F groups. Data are presented as the mean ± standard deviation of five independent experiments. *P<0.05 and **P<0.01 vs. saline group; #P<0.05 and ##P<0.01 vs. MCT group. cav-1, caveolin-1; MCT, monocrotaline; M + F, monocrotaline + fluvastatin.
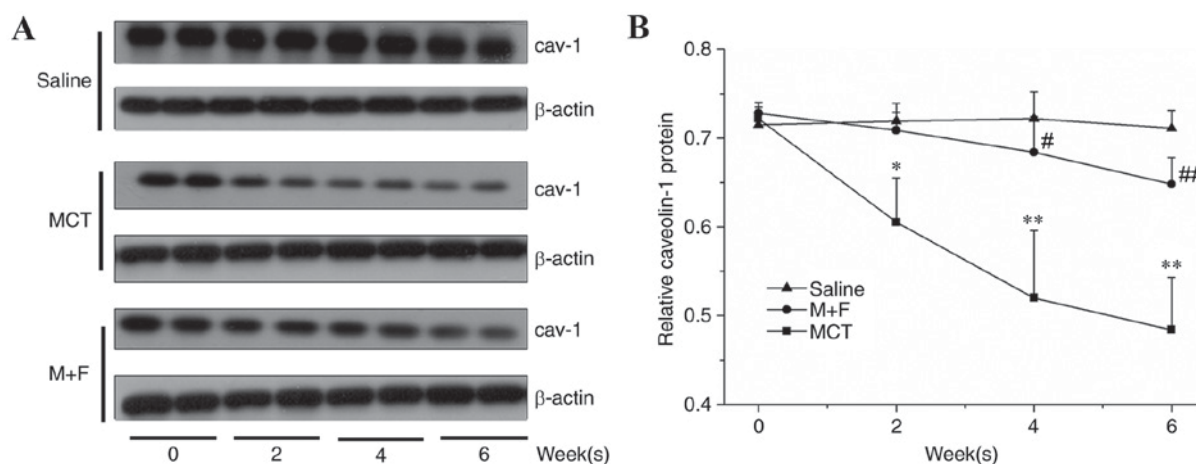

Figure 4. (A) Relative cav-1 protein expression as measured by western blotting analysis in the saline, MCT and M + F groups. β-actin was used as a control and to ensure equal protein loading. (B) Semi-quantification of cav-1 protein expression at 0, 2, 4 and 6 week time intervals in the saline, MCT and M + F groups. Data are presented as the mean ± standard deviation of five independent experiments. *P<0.05 and **P<0.01 vs. saline group; #P<0.05 and ##P<0.01 vs. MCT group. Cav-1, caveolin-1; MCT, monocrotaline; M + F, monocrotaline + fluvastatin.

studies have also indicated that statins exert beneficial effects in PAH (19,20) forming the basis for a potential future therapeutic strategy against PAH in humans. Previous *in vitro* and *in vivo* studies concerning statins and PAH have been largely focused on potential therapeutics against established lesions in PAH (10,21,22). The present study demonstrated that fluvastatin can reduce PAH development and ameliorate MCT-induced inhibition of cav-1 expression in rats.

MCT-induced PAH is an artificial model of pulmonary hypertension that is acknowledged to satisfactorily reproduce the processes occurring secondary to the dysfunction of the pulmonary arteries (23). In the present study, a daily gavage of 10 mg/kg fluvastatin was administered to M + F rats immediately following a single subcutaneous MCT injection, at which time PAH was not yet established. PAH evaluation was performed every 2 weeks during the experimental period to reduce the number of experimental animals required. Although it could not be concluded that
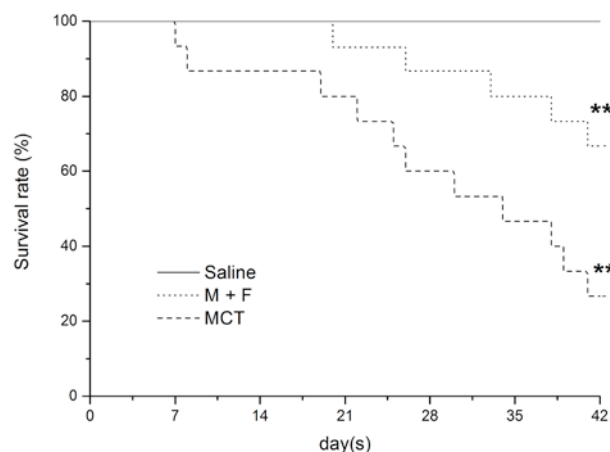

Figure 5. Kaplan-Meier survival analysis demonstrated that fluvastatin administration (M + F group) improved the rate of survival in comparison with the MCT group. **P<0.01 and ***P<0.001 vs. saline group; #P<0.05 vs. MCT group. MCT, monocrotaline; M + F, monocrotaline + fluvastatin.

M + F rats were completely protected from the development of PAH, development was delayed ≥2 weeks, as indicated by the results of hemodynamic and morphometric analysis. Pulmonary vascular remodeling is a hallmark pathological feature of PAH (24); PWT can be measured to evaluate the extent of this remodeling. In the present study, no significant changes in PWT were observed in M + F rats at 6 weeks, indicating that early fluvastatin administration may exert effective protection against pulmonary vascular remodeling; this may contribute to the reduced PAH development observed in M + F rats.

Cav-1 is a major component of the coat proteins that surround caveolae, which are flask-shaped plasma membrane invaginations that have important functions in vesicular transport and signal transduction (25). Knockout mice develop severe lung abnormalities characterized by hypercellularity, interstitial fibrosis and alveolar septa thickening, indicating that cav-1 may be involved in the development of normal lung vasculature (26). In the present study, pulmonary cav-1 protein expression was reduced in MCT rats, which is in accordance with other reported data (11,27). By contrast, significant differences in cav-1 protein expression in the lungs of M + F rats were not observed throughout the experimental period. The difference in cav-1 expression between the MCT and M + F groups was largely attributed to the 6 week fluvastatin intervention. However, a significant downregulation of cav-1 mRNA expression in the M + F group was observed at 6 weeks when compared with the saline group, indicating that fluvastatin confers only partial protection to cav-1 expression. A complete inhibition of cav-1 reduction in the M + F group within 6 weeks did not result in an expected complete inhibition of PAH development. RVSP and RVHI were moderately elevated, suggesting cav-1 was not the determining factor in this animal model. Survival analysis also revealed a significantly increased survival benefit in fluvastatin treated rats. Taken together, these results suggest that fluvastatin is effective in slowing PAH progression in an MCT-induced model, at least in part through the protection of cav-1 expression in rats.

Patients with congenital heart disease, BMPR-2 mutations or sickle cell anemia are particularly vulnerable to PAH development (28,29). Thus, the findings of the present study may be of clinical interest in respect to the development of therapeutic strategies aimed at the prevention of PAH in at-risk individuals. Furthermore, future research should explore the potential beneficial effects of other statins in models of PAH, as only fluvastatin was investigated in the present study.

There are several limitations to the findings of the present study. Interactions between fluvastatin and MCT were not investigated, which may potentially influence the successful establishment of the PAH animal model. Additionally, cav-1 expression cannot be concluded to be the main contributor to PAH development, as the expression of cav-2, cav-3 and other potentially involved proteins were not evaluated. Furthermore, as the established animal model may not accurately reproduce the pathological modifications present in various forms of PAH in humans, the findings of this pre-clinical study require confirmation in clinical trials.

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