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Therapeutic effect of sorafenib in monocrotaline-induced pulmonary arterial hypertension rats

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SUMMARY

Sorafenib is an inhibitor of multi-kinases including tyrosine and serine/threonine kinases. This study investigated the efficacy and safety of sorafenib, an inhibitor of multi-kinases, for the treatment of pulmonary arterial hypertension (PAH), in monocrotaline (MCT)-induced PAH rat models. PAH was induced by subcutaneous (sc) injection of 60 mg/kg monocrotaline (MCT) dissolved in 0.5 N HCl. Rats were assigned into the following groups: 1) control (C) group, 2) monocrotaline (M) group, and 3) sorafenib (S) group (MCT injection + sorafenib injection group). Rats in the treatment groups received a daily oral gavage of 2.5 mg/kg sorafenib for seven days. Our results showed that sorafenib treatment reduced systolic pulmonary artery pressure (SPAP) in PAH rats, and improved right ventricular (RV) hypertrophy, an indicator of RV pressure overload caused by elevated pulmonary artery pressure. In addition, the number of intra-acinar arteries and medial wall thickening in pulmonary arterioles (an index of vascular remodeling) were decreased. Our results revealed that sorafenib reduced ventricular hypertrophy and the expression of fibrosis. These results support the notion that sorafenib improves the functional properties of RV, thereby highlighting its potential benefits for heart and lung impairment.

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INTRODUCTION

Pulmonary arterial hypertension (PAH) is a severe pulmonary vascular disease characterized by sustained increase in pulmonary arterial pressure, and excessive thickening and remodeling of distal small pulmonary arteries (Badesch et al., 2007; Gong et al., 2017; Middleton et al., 2017). Disease progression of PAH is associated with an increase in mean pulmonary arterial pressure (MPAP), right ventricular (RV) enlargement, increased pulmonary vascular resistance, and smooth muscle hypertrophy in pulmonary arterioles (Ryan et al., 2015). Pulmonary hypertension (PH) is a worldwide public health challenge with an estimated population of affected people in resource-limited countries of 20 to 25 million in 2008 (Gidwani and Nair, 2014; Mocumbi et al., 2015). Severe PH can be life-threatening due to right heart failure (Rich et al., 1987). Despite advances with new therapeutics, PAH remains a devastating disease, since most of the approved therapies are expensive, fail to reverse vascular remodeling, and consequently offer only limited improvement in exercise capacity (McLaughlin et al., 2006).

A recently developed molecular-targeting agent, sorafenib is widely used for the treatment of cancer and carcinoma (Escudier et al., 2007; Llovet et al., 2008). Recent studies demonstrate that this multi-kinase inhibitor alleviates the disease in experimental models of pulmonary hypertension, and

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prevents right heart remodeling through improvement of endothelial dysfunction via inhibition of tyrosine kinase and serine/threonine kinase (Klein et al., 2008; Moreno-Vinasco et al., 2008). A dosing/cross-development study of sorafenib for the treatment of PAH has also been published (Seyahi et al., 2011). This study therefore aimed to evaluate the effect of sorafenib on hemodynamics, and the long-term outcomes of rats with MCT-induced PAH. To determine changes in pulmonary hemodynamics, right ventricular hypertrophy, pathology, and protein expressions, immunohistochemical staining and western blot analysis were performed to understand whether sorafenib treatment reduces the development of PAH in the rat model.

MATERIALS AND METHODS

Animals

Six-week-old male Sprague-Dawley rats were used. All rats were housed in climate- controlled conditions with a 12-hour light/12-hour dark cycle, and had free access to food and water. All animal experiments were approved by the appropriate Institutional Review Boards of the Seoul National University Bundang Hospital (BA1705-224/045-01) and conducted in accordance with National Institutes of Health Guide for the Care Use of Laboratory Animals (NIH publication No. 86-23, revised in 1996).

Pulmonary Arterial Hypertension (PAH) rat model

PAH was induced by subcutaneous (sc) injection of 60 mg/kg monocrotaline (MCT) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.5 N HCl. Rats were assigned into the following groups: 1) control (C) group (20 rats), vehicle injection with normal diet; 2) monocrotaline group (M) (20 rats), MCT injection with normal diet; and 3) sorafenib group (20 rats), MCT injection + sorafenib injection with normal diet. These animals were sacrificed at 7, 14, 21, and 28 days (each group, n = 5) after MCT administration. Tissues were removed and immediately frozen at -70°C for enzyme analysis.

Sorafenib delivery

Rats in the treatment groups received a daily oral gavage of 2.5 mg/kg sorafenib. Stock sorafenib solutions were prepared every 3 days, in EL-ethanol (50:50; Sigma Cremophor EL, 95% ethyl alcohol) at a final concentration of 4 mg/ml, and stored at room temperature protected from light exposure. Final dosing solutions were prepared on the day of use by diluting the stock solution to 1 mg/ml with water; the control groups received vehicle for injection through the same route.

Determination of the organ weights and right hypertrophy index

The animals were sacrificed at the scheduled time. The wet weights of excised right ventricle (RV), left ventricle (LV) plus interventricular septum (IVS) (LV+IVS) were measured. The RV to LV+IVS ratio [RV/(LV+IVS)] was used to determine the right hypertrophy index (RVI). The standard of right ventricular hypertrophy was defined as an RVI > 0.33 (Seyahi et al., 2011).

Pulmonary haemodynamics

Rats were anaesthetized with intraperitoneal injection of urethane (1.3 g/kg) in our study. While ketamine (90 mg/kg) and xylazine (10 mg/kg) were used at the recommended dose (Giroux et al., 2015). A combination of ketamine and xylazine was prepared as follows: 0.9 mL 10% ketamine solution +0.5 mL of 2% xylazine solution mixed with 0.6 mL of sterile saline. An 8-mm-long right internal jugular vein was isolated and ligated at the distal end. The vessel was cut at the proximal end of ligation. A catheter filled with heparin saline was rapidly inserted along the incision and slowly advanced for about 5 cm to enter the pulmonary artery. The standard of pulmonary hypertension was defined as systolic pulmonary artery pressure (SPAP) > 50 mmHg (Seyahi et al., 2011). Hemodynamic parameters were recorded at baseline and at 7, 14, 21 and 28 days.

Histologic findings of pulmonary arteries

Heart and Lung tissues were fixed with 10% buffered formalin and then embedded in paraffin. Sections were performed by four micron-thick hematoxylin-eosin stains to evaluate histopathologic changes of pulmonary blood vessels. The small pulmonary artery wall thickness was expressed as follows: % wall thickness.

Masson trichrome staining

Masson trichrome staining was carried out in accordance with well-characterized protocols. Briefly, heart and lung tissue sections were deparaffinized and hydrated in distilled water prior to a 1-hr treatment in Bouin's fixative (Richard-Allan Scientific; CA, USA; Catalog #NC9674780) at 56°C. Sections were washed in running distilled water until clear, and then stained in Weigert's iron hematoxylin (Richard-Allan Scientific; Catalog #NC9231529) for 10 minutes. Following a 10-minute wash in running water, sections were stained in Biebrich scarlet-acid fuchsin (Richard-Allan Scientific; Catalog #NC9424144) for 2 minutes. Sections were rinsed in distilled water followed by a 10-minute differentiation in phosphomolybdic-phosphotungstic acid (Richard-Allan Scientific; Catalog #NC9443038). Aniline blue (Richard-Allan Scientific; Catalog #NC9684104) was used as a counterstain for 10 minutes, and then sections were differentiated in 1% acetic acid for 3 minutes. Sections were dehydrated through a series of graded alcohols back to xylene, and then coverslipped and sealed using Cytoseal XYL (Richard-Allan Scientific).

Immunohistochemistry

Excised lung tissues were incubated overnight in 10% buffered formalin. Four- micrometer-thick sections were cut from paraffin embedded tissue blocks, deparaffinized in xylene, and rehydrated in graded alcohol solutions (70%-100%). Heat antigen retrieval was achieved by boiling the tissue sections in antigen retrieval solution in pH 6.0 or pH 9.0 (Dako, Carpinteria, CA, USA) for 10 minutes in a microwave prior to incubation at 4°C overnight with primary antibodies against endothelin-1 (ET-1; Abcam, Cambridge, MA, USA). After incubation with the appropriate biotinylated secondary antibodies for 30 minutes at 4°C and subsequently with streptavidin (Dako, Kyoto, Japan), color development was done using diaminobenzidine (DAB) as a chromogen and counterstained with hematoxylin.

Western blot analysis

The tissue was homogenized in 10 mM Tris HCl buffer, pH 7.4 containing 0.5 mM EDTA, pH 8.0, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_4VO_3 and a protease inhibitor cocktail (Roche-Boehringer-Mannheim, Mannheim, Germany). After centrifugation, the supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples equivalent to 25 μg of protein content were loaded and size-separated in 8-12% SDS-PAGE. The proteins on the acrylamide gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford MA, USA) at 400 mA in a transfer buffer containing 25 mM Tris and 192 mM glycine, pH 8.4. The nitrocellulose membranes was blocked in tris-buffered saline with 5% non-fat dry milk at room temperature for 1 h in 0.1% Tween 20 and incubated with the appropriated primary antibodies, including anti-Bcl-2 and anti-Survivin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), ERK, p38 MAPK, MEK1/2, VEGFR-2, p-ERK, p-VEGFR- 2, p-MEK1/2 and p-p38 (Abcam, Cambridge, Massachusetts) and anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), at 4°C overnight. The membranes were then incubated with horseradish peroxidase- conjugated secondary antibody (Cell Signaling Technology Inc., Danvers, MA, USA) for 1-h at room temperature. After washing, the membranes were visualized by a chemiluminescent ECL-detection kit from GE-Healthcare (Piscataway NJ, USA).

Statistical analyses

Results were expressed as the mean ± standard deviation. Differences between all other parameters for the three groups were evaluated by ANOVA followed by multiple-group comparisons. An unpaired two-tailed t-test and Mann-Whitney test were used, and a P value < 0.05 was considered statistically significant. SPSS 14.0 for windows (SPSS, Chicago IL, USA) was used for all statistical analyses.

RESULTS

Organ weight

The RV weight of the M group increased at 21 and 28 days after MCT administration. Weights of LV+IVS were not significantly different among the C, M, and S groups. RV/LV+IVS ratios were significantly increased at 21 and 28 days in the M group, as compared to those in the C group. However, RV/LV+IVS ratios were significantly decreased at

Table 1. Changes of SPAP and Organ Weights after Sorafenib treatment in PAH rats.

Day	Group	SASP (mmHg)	RV (g)	LV + IVS (g)	RV/(LV+IVS) %
	Control	23.1±1.1	0.145±0.02	0.551±0.03	0.263±0.17
7	M	23.9±2.4	0.146±0.03	0.563±0.03	0.259±0.25
	Sorafenib	24.3±1.7	0.146±0.02	0.562±0.02	0.259±0.19
	Control	24.4±1.3	0.157±0.02	0.662±0.03	0.237±0.18
14	M	33.9±2.9*	0.198±0.04	0.659±0.05	0.300±0.37*
	Sorafenib	29.7±1.3	0.179±0.03	0.653±0.04	0.274±0.22
	Control	24.2±1.6	0.165±0.03	0.712±0.04	0.231±0.21
21	M	50.5±4.6*	0.321±0.05*	0.656±0.06	0.489±0.57*
	Sorafenib	38.9±3.8#	0.212±0.04	0.702±0.04	0.301±0.31#
	Control	25.5±2.1	0.173±0.03	0.772±0.04	0.239±0.24
28	M	57.1±7.1*	0.366±0.09*	0.694±0.08	0.527±0.69*
	Sorafenib	40.2±4.6#	0.229±0.05#	0.734±0.05	0.311±0.33#

Values are presented as means ± standard deviation. Control, control group; M, monocrotaline group; Sorafenib, sorafenib group; SPAP, systolic pulmonary artery pressure; RV, right ventricle; LV, left ventricle; IVS, interventricular septum. *P < 0.05 compared with the C group, #P < 0.05 compared with the MCT group.

Table 2. Ratio of the medial thickening of pulmonary artery in each group (%).

Day	Control	M	Sorafenib
7	9.1±0.3	8.9±0.5	8.8±0.4
14	9.2±0.9	12.9±1.4	12.1±1.2
21	8.9±0.5	16.8±2.5*	16.3±2.6
28	8.9±0.6	22.1±2.9*	17.2±3.4#

*P < 0.05 vs. the corresponding value in the C group, #P < 0.05 vs. the corresponding value in the M group. Control: control group, MCT: monocrotaline group, Sorafenib: sorafenib group.

21 and 28 days in the M group, compared to those in the S group. Weights of LV+IVS were significantly lower in the M group and the S group, as compared to weights in the C group at 21 and 28 days. Heart weights showed significant increase in the M group, as compared to C group at 21 and 28 days. However, heart weights were significantly decreased in the S group as compared to the M group at 21 and 28 days (Table 1).

Changes in systolic pulmonary artery pressure (SPAP) after Sorafenib treatment in PAH rats

At 21 and 28 days, the mean SPAP values were significantly increased in the M group as compared to the C group. SPAP values were significantly decreased in the S group compared to those in the M group at 21 and 28 days (Table 1).

Medial wall thickness of the pulmonary artery

In the M group, the ratio of medial thickening to the external diameter of the pulmonary artery showed significant increase at 28 days, compared to the control group. However, the decrease was not significant in the S group when compared to the ratio of medial thickening of the pulmonary artery in the M group (Table 2).

Number of muscular pulmonary arterioles

The number of muscular pulmonary arterioles was significantly increased in the M group compared to that in the C group. At 21 and 28 days, the number of muscular pulmonary arterioles was significantly decreased in the S group compared to that in the M group (Table 3).

Table 3. Number of muscular pulmonary arterioles in each group.

Day	Control	M	Sorafenib
7	2.1±0.1	2.2±0.2	2.2±0.3
14	2.2±0.1	2.8±0.2	2.5±0.2
21	2.2±0.2	3.7±0.3*	3.2±0.2#
28	2.4±0.3	5.6±0.5*	4.1±0.3#

*P < 0.05 vs. the corresponding value in the C group, #P < 0.05 vs. the corresponding value in the M group. Control: control group, M: monocrotaline group, Sorafenib: sorafenib group.

Administration of sorafenib reduces RV and lung collagen deposition

To evaluate the efficacy of sorafenib on the development of cardiac fibrosis, lung fibrosis, and remodeling, histology patterns were determined in lung and RV samples collected at 28 days after administration of MCT. Results are shown in Fig. 1 and Fig. 2. After staining with Masson's Trichrome, lung and RV sections showed more pronounced collagen deposition in animals with PAH, compared to those in the control or S group (Figs. 1 and 2).

Immunohistochemistry analysis of lung samples

Immunohistochemistry (IHC) of lung tissues revealed that ET-1-positive cells were more prevalent in the S group, followed by those in the M group, in comparison with those in the C group at 28 days (Fig. 3A-F). Three weeks after sorafenib treatment, ET-1-positive cells were persistent in the lung area in the S group. The increased level of ET -1 immunoreactivity observed in the M group was statistically significant (p <0.05). The decreased level of ET-1-immunoreactivity was also significant in the S group when compared to the M group (Fig. 3G).

Western blot analysis

This experiment was designed to investigate alterations in the expression levels of Bcl-2 and survivin proteins in the lung after exposure to sorafenib. Changes in expression levels of Bcl-2 and survivin immunoreactivity in the lung were detected in the S group, which were significantly decreased compared to those in the M group (Figs. 4A, 4B). Actin bands indicated protein loading in the same sample (Fig. 4).

DISCUSSION

The present study showed that treatment with sorafenib reduces the mal-adaptive structural remodeling of the lung and heart, and prevents ventricular arrhythmias in MCT- induced PAH in rats. My results indicate that sorafenib reduces SPAP in PAH rats. In addition, there was a decrease in the number of intra-acinar arteries and medial wall thickening in pulmonary arterioles, an index of vascular remodeling.

Sorafenib is widely used as an anti-cancer agent for renal carcinoma (Escudier et al., 2007; Llovet et al., 2008). Previous studies have demonstrated that PAH and cancer show similar pathophysiologic features (Cool et al., 2003). Therefore, the possibility of sorafenib as an agent for the treatment of PAH has been explored. Reports indicate that sorafenib prevents pulmonary remodeling, improves the cardiac and pulmonary functions, and exerts in direct and myocardial anti-hypertrophic effects in rats with PAH induced by MCT (Klein et al., 2008).

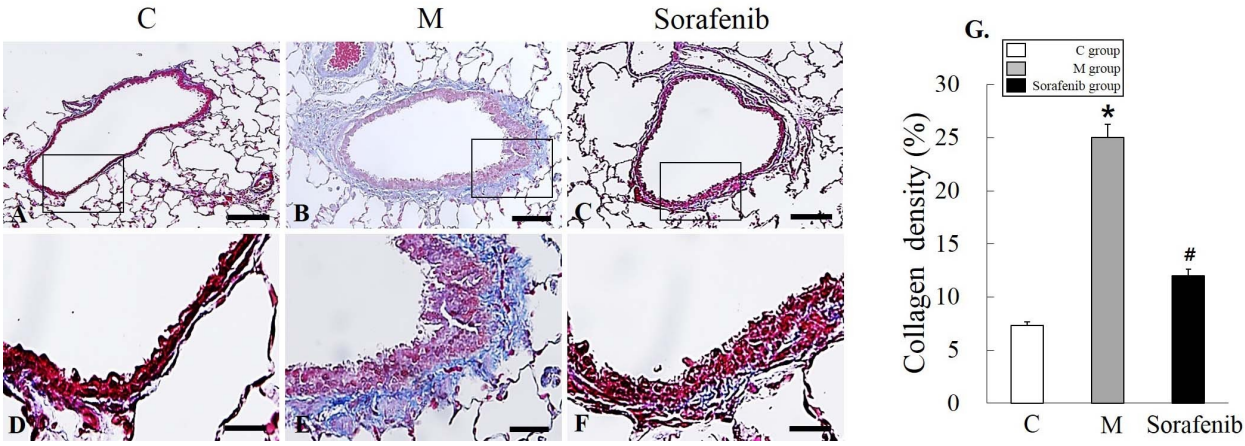

Fig 1. Representative images of lung stained with Masson Trichrome from C group (**A**), M group (**B**), and S group (**C**) at 28 days following the injection of monocrotaline. Panels **D**, **E**, and **F** are high power views of panels A, B, and C, respectively. **G**: Collagen content was greatly increased in the M group in comparison with the C group at 28 days. The S group showed a significant decrease in collagen content at 28 days. C group, control group; M group, monocrotaline group; S group, sorafenib group. **P* < 0.05 vs. C group, # *P* < 0.05 vs. M group. Scale bars = 50 μm (A, B, C), 20 μm (D, E, F).

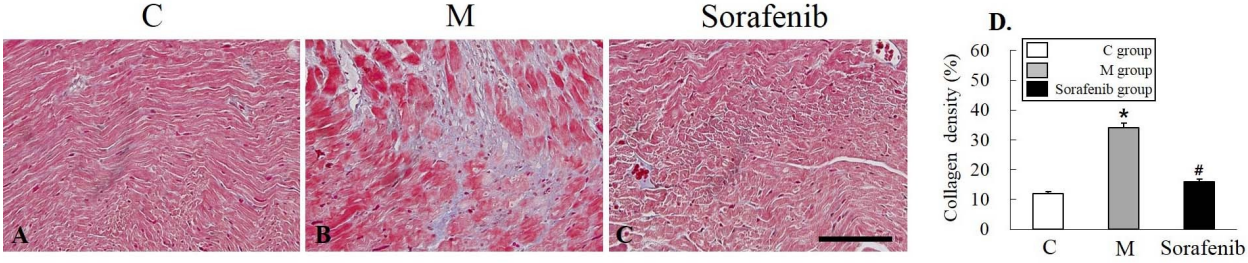

Fig 2. Representative images of right ventricle stained with Masson Trichrome from C group (**A**), M group (**B**), and S group (**C**) at 28 days following the injection of monocrotaline. **D:** Collagen content was greatly increased in the M group in comparison with the C group at 28 days. The S group showed a significant decrease in collagen content at 28 days. The S group showed a significant decrease in collagen content at 28 days. Fibrosis is colored blue. **P* <vs. C group, # *P* < 0.05 vs. M group. C group, control group; M group, monocrotaline group; S group, sorafenib group. Scale bars = 150 μm (A, B, C).

Furthermore, a phase Ib study of sorafenib in PAH patients has reported a tolerable dosing regimen for testing the therapeutic activity of “sorafenib”. Some improvement in the exercise capacity was also demonstrated, although there was no improvement in cardiac output (Gomberg-Maitland et al., 2010). The multikinase inhibitor sorafenib inhibits the tyrosine kinases PDGF receptor, VEGF receptors 2 and 3, c-kit, and Flt-3 in the nanomolar range (Moreno-Vinasco et al., 2008; Wilhelm et al., 2004). On the basis of its unique kinase inhibition profile targeting tyrosine as well as serine/threonine kinases, we hypothesized that sorafenib might prove useful for pulmonary hypertension (Wilhelm et al., 2004). Our data demonstrates a substantial effect on all major indices of cardiac and pulmonary function, including RV systolic pressure, cardiac output, and arterial pressure, in MCT-treated rats. In agreement with the functional benefits, morphometric indices of RV hypertrophy and pulmonary arterial muscularization were also significantly reduced. In addition, our results indicate that sorafenib favorably affects key features of pulmonary remodeling, such as proliferation and reduced apoptosis of pulmonary vascular endothelial cells. Endothelins (ET) are potent vasoconstricting peptides that have proliferative and pro-inflammatory properties. Isoform ET-1 is a well-established mediator of pulmonary vascular homeostasis (Bohm et al., 2007). Among other subtypes of PAH, higher levels of endothelin-1 (ET-1) are predictive of severity and mortality. We propose the following hypotheses regarding the mechanisms of how sorafenib decreases medial wall thickening. Sorafenib exerts vasodilatory (Abe et al., 2011), anti-inflammatory (Yang et al., 2017), anti-angiogenic (Sharma et al., 2011), and anti-proliferative (Cervello et al., 2013) effects through decreasing the expression of ET-1 in lung tissues (Fig. 3). The pulmonary vascular alterations in pulmonary arterial hypertension are characterized by proliferation of smooth muscle cells, migration of smooth muscle cells and myofibroblasts, and resistance of smooth muscle cells to apoptosis due to up-regulation of Bcl-2 and survivin (Geraci et al., 2001; Jones et al., 1997; McMurtry et al., 2005;

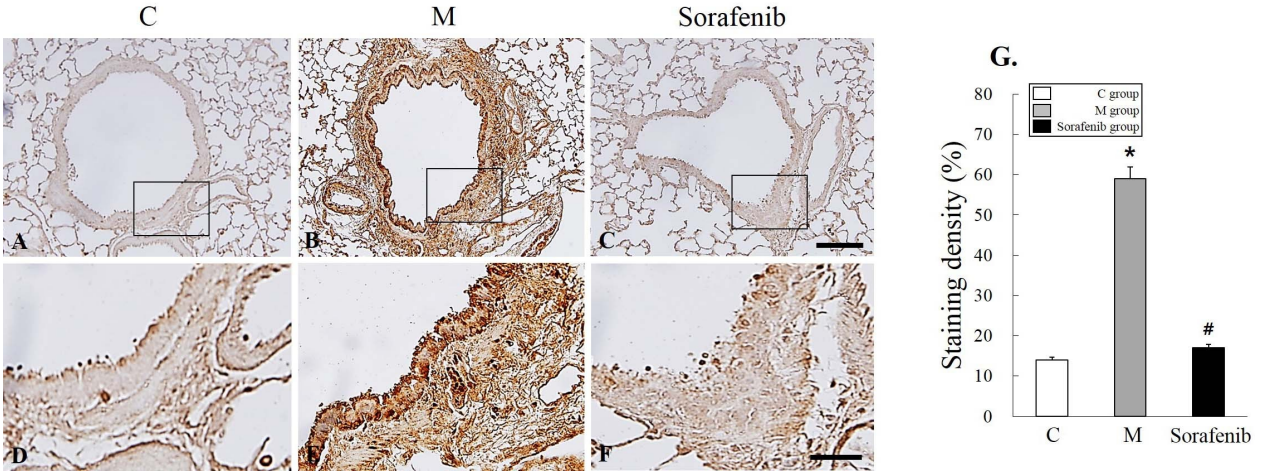

Fig 3. Localization of ET-1-immunoreactive cells in the lung tissues at 28 days (**A-F**). Immunohistochemical expression reveals that the positive cells of ET-1 are significantly greater in the M group than in the C group; however, they are comparatively lesser in the S group than in the M group (**G**). Panels D, E, and F are high power views of panels A, B, and C, respectively. ET-1, endothelin-1; C group, control group; M group, monocrotaline group; S group, sorafenib group. *P < 0.05 vs. C group, # P <0.05 vs. M group. Scale bars = 50 μm (A-C), 25 μm (D-F).

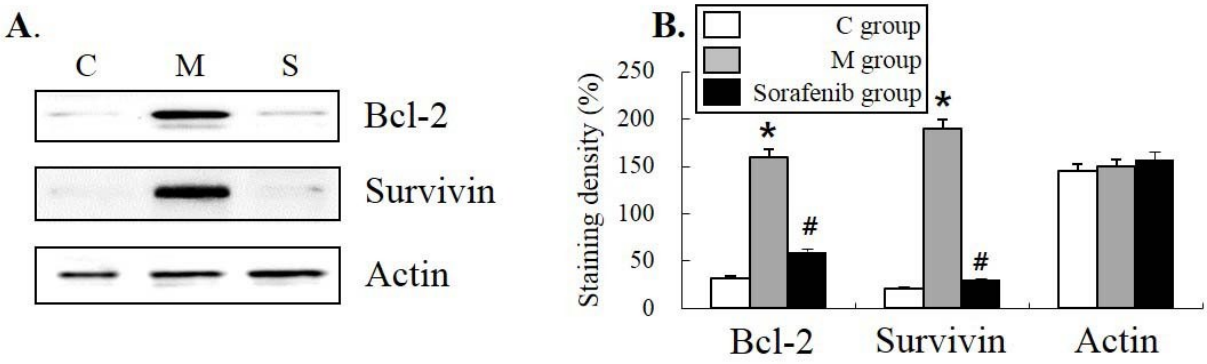

Fig 4. A: Western blot analysis of expression levels of Bcl-2, survivin and actin immunoreactivity in the lung. B: The semi-quantitative analysis confirms that S group exhibited decreased levels of immunoreactivity of Bcl-2 and survivin in the lung. C group, control group; M group, monocrotaline group; S group, sorafenib group (n = 4-5 per group). *P < 0.05 vs. C group, # P < 0.05 vs. M group.

Schermuly et al., 2005). Sorafenib has anti-apoptotic effects through decreasing the expression of Bcl-2 and survivin in lung tissues (Fig. 4). A significant increase in the pulmonary arterial pressure following MCT injection is accompanied by RV hypertrophy and increased lung and heart fibrosis when compared to the control group (Fig. 2 and Fig. 3). In the RV of rats with PAH, sorafenib treatment decreased the development of RV hypertrophy and normalized the expression of collagen content (Fig. 3). Additionally, in rats with PAH, SPAP of the heart was decreased by sorafenib treatment (Table 1). These results suggest that sorafenib probably decreases pulmonary vessel contraction and remodeling, leading to improvement in apoptosis and inhibition of fibrosis in PAH rats. These findings support our hypothesis that chronic sorafenib administration improves PAH. Thus, there is compelling evidence showing that sorafenib is a pulmonary-selective vasodilator that can be used as an alternative to current therapy for PAH.

In conclusion, sorafenib had favorable effects to improve symptoms and objective variables in rats with MCT-induced PAH. Further studies are required to determine the pathways involved which exert the efficacy of sorafenib in the improvement of PAH.

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