ORIGINAL ARTICLE

Rapamycin Improves Vascular Remodelling in a Controlled Rat Model of Monocrotaline-Induced Pulmonary Hypertension

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ABSTRACT

Background: *Pulmonary arterial hypertension (PAH) is a serious disease characterized by the progressive elevation of the pulmonary arterial resistance, leading to the right ventricular failure and death.*

Objective: *To evaluate the effect of rapamycin (RAPA), a potent cell-cycle inhibitor, on exercise capacity, right ventricular hypertrophy and pulmonary vascular remodelling on rats.*

Methods: *A total of 39 nine-week-old male Wistar rats (160–240 g) were divided into three groups: the control (n = 10), PAH control (n = 15) and PAH-RAPA (n = 14) groups. On the 1st day, 60 mg/kg monocrotaline was injected intraperitoneally to induce PAH in the PAH control group and PAH-RAPA groups. On the 21st day, 3 mg/kg/day RAPA was started orally, and the animals were followed for 35 days. On the 35th day, the exercise capacity of the rats was analysed through a modified forced swimming test. After measuring their right ventricular systolic pressure using an open-chest method, their hearts and lungs were excised and analysed histopathologically for right ventricular hypertrophy and pulmonary vascular remodelling.*

Results: *Rapamycin treatment provided limited and insignificant improvements in exercise capacity, right ventricular systolic pressure and right ventricular hypertrophy of the rats. However, there was significant recovery in the rats' pulmonary artery muscular layer thickness with the RAPA treatment (*$p < 0.049$*). On the 35th day, the mortality rate was 0% in the control group, 53.1% in the PAH control group and 42.9% in the PAH-RAPA group. No statistically significant decrease was observed in their mortality rates with the RAPA treatment (*$p > 0.16$*); however, a significant recovery was noted in terms of the rats' median life span (*$p < 0.006$*).*

Conclusion: *Pulmonary artificial hypertension is a progressive disease that is not curable with current therapies. Rapamycin may have the potential to reverse vascular remodelling and prolong life expectancy in cases of pulmonary hypertension.*

Keywords: Life expectancy, pulmonary hypertension, rapamycin, vascular remodelling

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a severe disease characterized by an increase in progressive pulmonary arterial resistance, which may cause right ventricle cardiac insufficiency and death (1). Although the reasons for PAH are complex, the vasoconstriction originating from vascular endothelial damage in the pulmonary arterioles, smooth muscle cell proliferation and hypertrophy and the constriction and the remodelling of the arterioles seem to be the primary factors (2).

The current treatments are aimed at restoring the balance between the vasoconstriction and the vasodilatation

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to prevent the proliferation of the endothelial and pulmonary artery's smooth muscle cells (3–5). However, despite these treatments, the disease is still not curable.

The inflammation in the vascular wall, the proliferation of the endothelial cells and the vascular smooth muscle, and vascular thrombosis are the histopathological hallmarks of PAH (6). Therefore, signalling the pathways involved in the proliferation should be targeted as a part of the novel treatment strategy.

Imatinib was found to have anti-proliferative properties and reversed the effect of PAH. A randomized, double-blind, placebo-controlled study showed that imatinib influenced the exercise capacity and the haemodynamics (7, 8). The positive effects of fasudil (a Rho kinase inhibitor) on altering the vasoconstriction and the pulmonary cell proliferation (9) afforded acute haemodynamic benefits in the patients with PAH (10). Rosiglitazone is an effective anti-proliferative agent and is a peroxisome proliferator-activated receptor agonist. It has been reported to improve the right ventricular hypertrophy and vascular remodelling in mice with hypoxia-induced PAH (11).

The mammalian target of rapamycin (mTOR) protein, which functions in the Akt pathway, is one of the molecular targets of anti-proliferative treatments. It also has an important role in controlling cell growth, proliferation and survival, and is regulated by mitogenic and nutrient signals (12, 13). Rapamycin (RAPA) is a bacterial macrolide isolated from *Streptomyces hygroscopicus* (14) and is an mTOR inhibitor. The blockage of mTOR by RAPA inhibits the proliferation in numerous cell lines, including cancer cells, coronary arterial smooth muscle cells, vascular progenitor cells and embryonic stem cells (15–18).

In this study, we aimed to evaluate the effects of RAPA on PAH induced by monocrotaline in terms of its effects on exercise capacity, right ventricular systolic pressure, right ventricular hypertrophy, pulmonary vascular remodelling, and mortality in rats.

MATERIALS AND METHODS

Study design and animals

A total of 39 nine-week-old male Wistar-Albino rats (160–240 g) were used. All the rats were provided by the Kocaeli University Animal Reproduction Centre and housed in the Animal Laboratory of Kocaeli University. The animals were caged in a controlled climate environment with 12-hour light/dark cycles. Standard rat feed and water were provided *ad libitum*. All the rats were allowed 2 weeks of acclimatization to this environment before the experiment began. The Kocaeli University Committee on the Use and Care of Animals approved the experiments, and all the investigations complied with the 1996 National Academy of Science Guide to the Care and Use of Laboratory Animals.

The rats were randomly assigned to one of the following groups: the untreated animals (control group, n = 10), the monocrotaline group only (PAH control, n = 15), and the monocrotaline plus RAPA group (PAH-RAPA, n = 14). In the PAH control group, monocrotaline (Sigma-Aldrich, St. Louis, MO, USA) 60 mg/kg was administered intraperitoneally on day 1 of the study period. In the PAH-RAPA group, monocrotaline 60 mg/kg was administered intraperitoneally on day 1 of the study period, and RAPA (Wyeth Pharmaceuticals Inc., Collegeville, PA, USA) 3 mg/kg was administered daily, orally, from day 21 until the end of this study period. The rats were monitored daily, and their mortality was recorded. After 5 weeks of monocrotaline application, all the animals were subjected to an exercise test. Then, their right ventricular pressure was measured under anaesthesia. Their hearts and lungs were excised for the histopathological investigations.

Exercise capacity

Their exercise capacity was assessed by the modified forced-swimming test (19). The animals were put in a cylinder tank (height, 50 cm; diameter, 30 cm) filled with water 25°C. The swimming time was defined as the total time from the immersion from which the floating time was subtracted.

Right ventricular pressure

All the rats were anaesthetized by the intraperitoneal application of 80 mg/kg ketamine hydrochloric acid (Ketalar, Eczacibasi Warner-Lambert Ilac Sanayi, Turkey) and 10 mg/kg xylazine hydrochloric acid (Rompun, Bayer, Turkey). Following the intubation of the trachea, the animals were ventilated using a rodent ventilator (model 7025 Ugo Basile, Comerio, Italy). Open-chest measurements were conducted. To measure their pulmonary arterial resistance, the chests of the rats were opened through a midline incision. An 18-gauge catheter filled with heparinized saline was inserted into the walls of their right ventricles and advanced into their pulmonary artery. The pressure recordings were performed using the MP 100A BIOPAC system (Santa Barbara, CA, USA).

Histology

The rat's histological examinations were done by histopathologists blinded to the study groups. After the measurement of the right heart resistance, the hearts and lungs of the decapitated animals were excised and fixed using neutral-buffered formalin (10%). The hearts, after the central transverse sectioning, were stored in paraffin and 2-µm-thick sections were made and stained with haematoxylin-eosin. Their right ventricular hypertrophy was expressed as the ratio of the right ventricular wall area (RV) to the left ventricular wall (area + interventricular septum) × 2 RV/[LV + S] × 2.

After sectioning into the 2-mm-thick samples, the lungs were placed in paraffin and 2-µm-thick sections were generated. The lung parenchyma incisions were dyed with haematoxylin-eosin, and immunohistochemically stained for α-smooth muscle actin (1:100, Dako, Glostrup, Denmark). The analyses of the pulmonary vascular remodelling of the rats were performed as follows: for each animal, 20 pulmonary arteries with an external diameter of 50–200 µm were selected randomly. The external diameter and medial muscular tissue thickness were measured, and the muscular wall thickness and external diameter were reported (19).

Statistical analyses

The statistical analyses were carried out using the MedCalc statistical software version, 12.7.7 (MedCalc Software Ltd., Ostend, Belgium). The Mann–Whitney *U* test was applied for the analyses of the two groups that were not independent and not normally distributed. The median, minimum and maximum values were used. Log-rank tests were conducted to compare the survival rates between the groups. The statistical significance level was set at $p < 0.05$.

RESULTS

Exercise capacity

The median exercise capacity significantly decreased in the PAH group compared to the control group (3.75 *vs* 6.43 minutes, respectively, $p < 0.009$). This decrease was reversed by RAPA, but the difference between the PAH control and PAH-RAPA groups was not significant (3.75 *vs* 6.85 minutes, respectively, $p > 0.51$) (Fig. 1).

Right ventricular systolic pressure

The right ventricular systolic pressure of the PAH control group (median, 26 mmHg; range, 16–28 mmHg) was significantly higher than that of the control group (median, 12 mmHg; range, 8–16 mmHg) ($p < 0.05$). However, there was no significant difference between the right ventricular systolic pressures of the PAH control and PAH-RAPA groups (median, 23.75 mmHg; range, 16–31.4 mmHg) ($p > 0.89$) (Fig. 2).

Right ventricular hypertrophy

The right ventricular hypertrophy rates were 0.38 (range, 0.33–0.46) in the control group, 0.54 (range, 0.44–0.95) in the PAH control group, and 0.46 (range, 0.39–0.65) in the PAH-RAPA group. A statistically significant difference was observed between the control and the PAH control groups ($p < 0.01$), but not between the PAH-RAPA and the PAH control groups ($p > 0.12$) (Fig. 3).

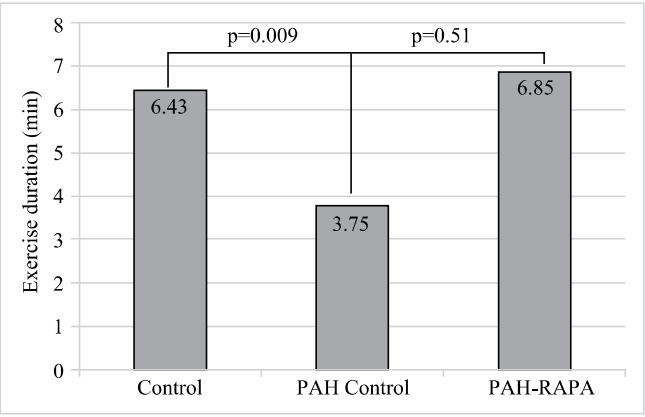

Figure 1: Exercise duration (min) in the study groups. Although the exercise duration was lower in the PAH control group than in the control and PAH-RAPA groups, the difference was significant for only between the control and PAH control groups (n = 6–10).
PAH = pulmonary arterial hypertension; RAPA = rapamycin.

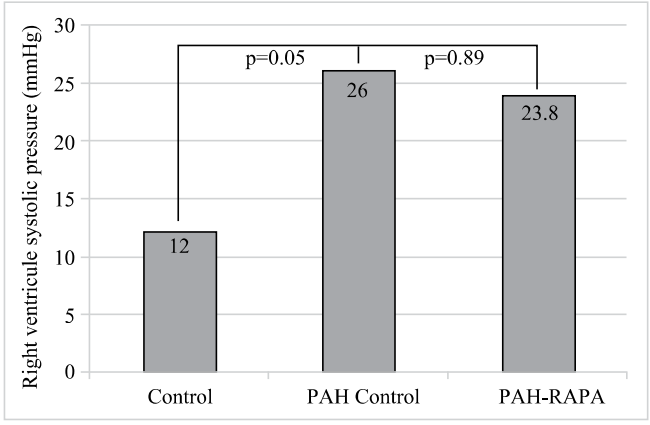

Figure 2: Right ventricular systolic pressure (mmHg) was significantly higher in the PAH control group than in the control group, but not significantly different between the PAH control and the PAH-RAPA groups (n = 3–6).
PAH = pulmonary arterial hypertension; RAPA = rapamycin.

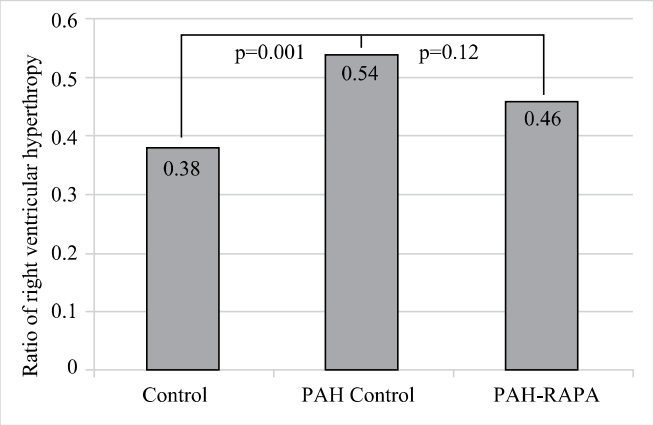

Figure 3: Right ventricular hypertrophy ratio ([RV/LV + S] × 2) was significantly higher in PAH control group than in the control group, but not significantly different between the PAH control and PAH-RAPA groups (n = 5–7).
LV = left ventricular wall area; PAH = pulmonary arterial hypertension; RAPA = rapamycin; RV = right ventricular wall area; S = interventricular septum.

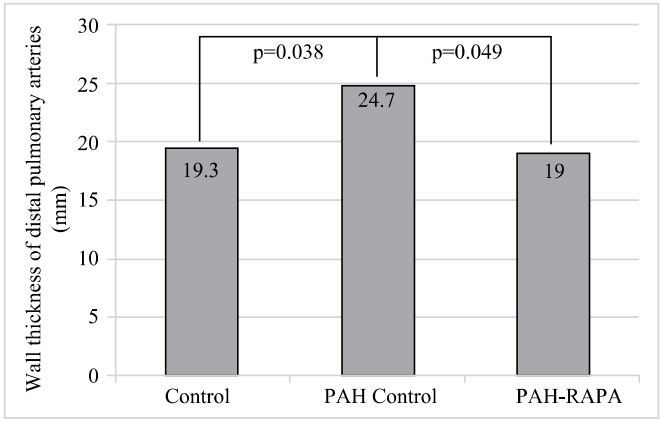

Figure 4: Pulmonary arterial hypertension was associated with increased wall thickness in the distal pulmonary arteries (corrected for vessel size), which was reduced with rapamycin therapy (n = 4–8).
PAH = pulmonary arterial hypertension; RAPA = rapamycin.

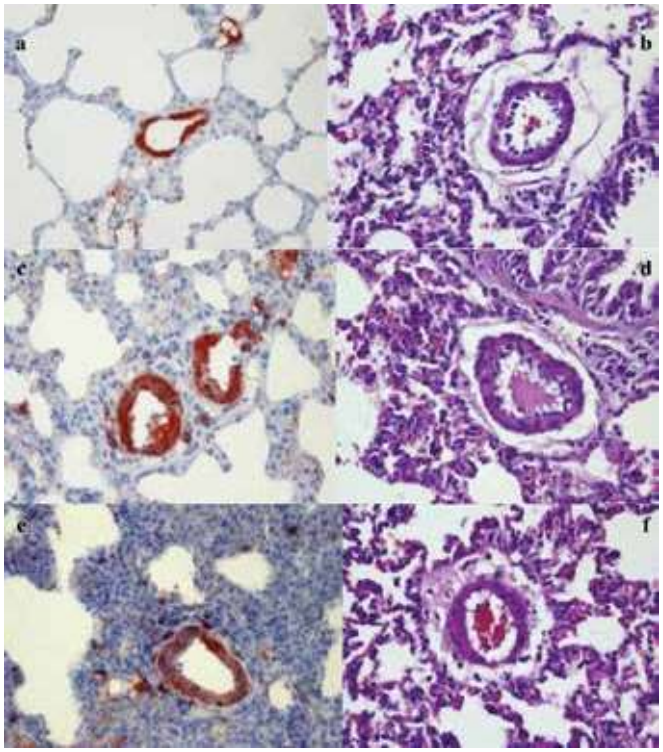

Figure 5: Histological images of distal pulmonary arteries stained with α-smooth muscle actin (left side) and haematoxylin-eosin (right side) for control (a, b), PAH control (c, d), and PAH + RAPA (e, f) groups with ×400 magnification.
PAH = pulmonary arterial hypertension; RAPA = rapamycin.

Distal pulmonary artery wall muscular thickness

The distal pulmonary artery wall muscular thickness increased significantly in the PAH control group (median, 24.7; range, 18.35–27) compared with the control group (median, 19.03; range, 17.6–21.65), this increase was significantly reversed to the control level by the RAPA treatment (median, 18.98; range, 15.6–23.8) (Figs. 4 and 5).

Survival

Eight rats (53.1%) in the PAH control group, and 6 (42.9%) in the PAH-RAPA group, and none in the control group died during the 35 days of the follow-up (Fig. 6). The median lifetime durations were 32 and 35 days in the PAH control and PAH-RAPA groups, respectively (log-rank test, $p < 0.006$). The mortality rate differed significantly between the control and the PAH control groups ($p > 0.006$); however, there was no significant difference between the PAH control and PAH-RAPA groups ($p > 0.71$) (Fig. 7).

DISCUSSION

The PAH is a progressive disease characterized by abnormal high pressure in pulmonary arteries as a result of the functional and the structural fluctuations in the pulmonary vascular bed. The increases in the pulmonary artery pressure and pulmonary vascular resistance occur as a result of the vasoconstriction and the structural changes in the small pulmonary arteries. Although notable progress has been made in the treatment of PAH, the prognosis remains bad. Three classes of vasodilatory agents are used in PAH treatment: prostanoids, endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors (20). Although PAH originates from abnormal pulmonary vasoconstriction, the disease is thought to proceed from pulmonary vascular remodelling. Eventually, these changes result in the irreversible

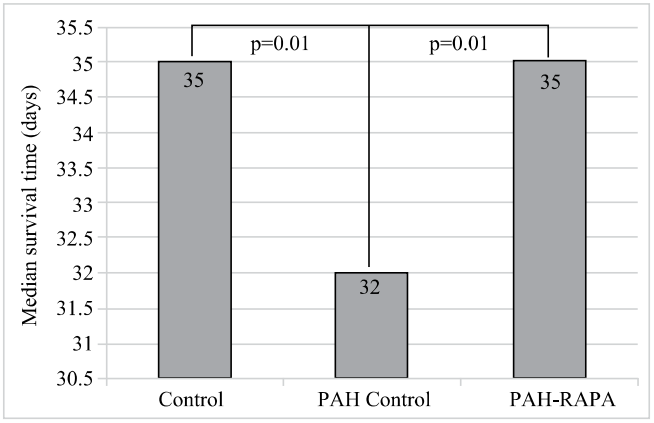

Figure 6: Kaplan–Meier survival curves of the three groups. Mortality rates were 0% in control group, 53.1% in the PAH control group and 42.9% in the PAH-RAPA group. There was a significant difference between the control and the PAH control groups, but not between the PAH control and the PAH-RAPA groups ($p < 0.006$ and $p > 0.71$, respectively).
PAH = pulmonary arterial hypertension; RAPA = rapamycin.

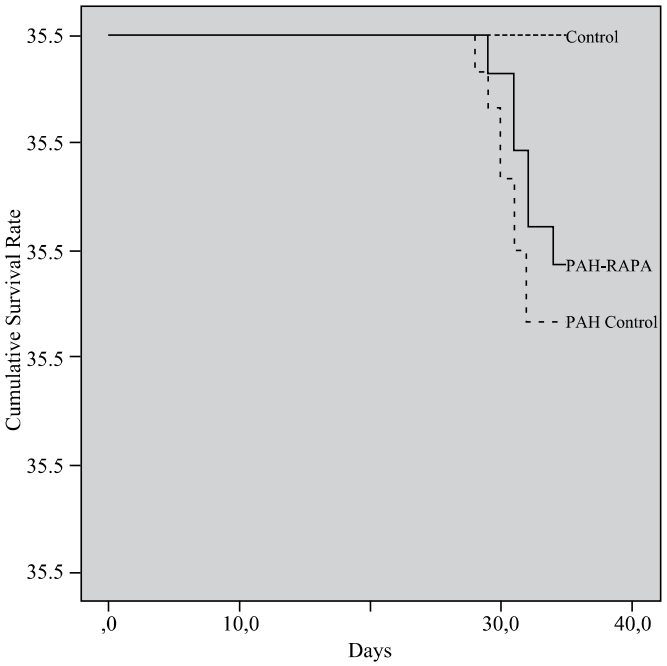

Figure 7: The median survival time in 35 days was significantly lower in the PAH control group than in the control and the PAH-RAPA groups (n = 10–15).
PAH = pulmonary arterial hypertension; RAPA = rapamycin.

increases in the pulmonary vascular resistance and right cardiac failure. Therefore, developing treatments that inhibit or reverse vascular remodelling is critical for the long-term management of PAH.

Rapamycin is an immunosuppressant used in transplant patients to prevent rejection (21). It has recently also been used for cancer treatment, at the restenosis of the coronary artery stents (22, 23). Moreover, RAPA has been found to be effective against liver, kidney, pulmonary fibrogenesis (24–26), probably by preventing proliferation and inhibiting extracellular matrix production (27).

In our study, although, RAPA had no significant effect on exercise capacity, right ventricular hypertrophy, right ventricular systolic pressure, and 35-day mortality, it had positive effects on pulmonary artery muscularization and 35-day life expectancy. The preventative effect of RAPA on monocrotaline-induced PAH in rats was first reported by Nishimura. However, RAPA was unable to reverse PAH in this study (28). Another study indicated that 2 mg/kg oral RAPA had a protective effect, which was linked to haeme oxygenase-1 (29). Paddenberg *et al* (30) evaluated the curative and preventative effects of 3 mg/kg/day intraperitoneal RAPA application on PAH induced by hypoxia. A decrease in proliferative activity, and positive effects on pulmonary vascular remodelling, and right ventricular hypertrophy, were detected. No effects on right ventricular hypertrophy were reported, similar to our study; however, positive effects on the muscularization of the intrapulmonary arteries were detected. Houssaini *et al* found that 5 mg/kg/day RAPA in monocrotaline-induced rats improved the pulmonary arterial pressure, right ventricular systolic pressure, and the number of muscularized pulmonary veins (31).

According to Ogawa *et al*, RAPA had antiproliferative effects on the pulmonary artery's smooth muscle cells and inhibited store-operated calcium entry in the patients with chronic thromboembolic PAH (32). McMurtry *et al* examined the effects of RAPA and statin on the monocrotaline-induced PAH (33). The treatment was initiated on the 12th day after monocrotaline application, and 2.5 mg/kg/day RAPA was administered orally for 12 days. However, no positive effects of the drugs on the pulmonary arterial pressure levels, vascular modelling and right ventricular hypertrophy were detected. Everolimus, a RAPA analogue, was well tolerated by 10 patients with progressive PAH despite associated vasodilator treatment. A significant improvement in the pulmonary vascular resistance and statistically non-significant improvement in the exercise capacity in an open-label pilot human study with everolimus were noted (34).

Pulmonary arterial hypertension develops after 3 weeks in the monocrotaline-induced PAH model (35). To analyse the reversal effects of RAPA, the treatment was initiated on the 21st day and was of 14-day duration.

We preferred not to use RAPA as a prevention strategy because PAH was diagnosed relatively late during the course of the disease. It had been reported that RAPA had a preventative effect on PAH when administered prior to its development. Numerous studies had shown that RAPA had reversal effects on PAH, and particularly vascular remodelling. The controversial results regarding the reversal effects of RAPA might be related to the dose used. More positive effects on PAH were noted when ≥ 3 mg/kg/day was administered. Besides, the human study of Seyfarth *et al* in which partial improvement at progressive PAH patients in spite of other treatments was detected and they observed its protective effect more distinctively in animal studies made us think that RAPA may be a more efficient treatment at the early stage of PAH (34).

The analysis of exercise capacity in this current study is an important parameter because it denotes the functional significance of PAH and is used as an end-point in clinical studies. Thus, the inclusion of this parameter contributes to the interpretations of the results of the experimental studies from the clinical perspective.

CONCLUSION

In conclusion, the positive effects of RAPA on pulmonary arterial muscularization were detected in our study. Despite the lack of statistical significance, improvements in exercise capacity, right ventricular systolic pressure, and right ventricular hypertrophy were detected. The use of RAPA derivatives has been approved for immune suppression after organ transplantation, for the prevention of vessel restenosis after angioplasty, and for the treatment of some malignancies. Therefore, RAPA may have the potential as a further option for the treatment of PAH.

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AUTHORS' NOTE

AS drafted the manuscript and performed the chemical application and right ventricle systolic pressure (RVSP) measurements; AS performed the RVSP measurements; CV and BYB performed the pathological examinations; CO performed the chemical application; and AT performed the exercise testing. All the authors read and approved the final manuscript.

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