ABSTRACT

Objective: Pulmonary arterial hypertension (PAH) is a serious disease characterized by progressive elevation of pulmonary arterial resistance, leading to right ventricular failure and death. We aimed to evaluate the effect of rapamycin (RAPA), a potent cell-cycle inhibitor, on exercise capacity, right ventricular hypertrophy, and pulmonary vascular remodeling.

Methods: Thirty-nine 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 first day, 60 mg/kg monocrotaline was injected intraperitoneally to induce PAH in the PAH control 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 analyzed through a modified forced swimming test. After measuring right ventricular systolic pressure using an open-chest method, the heart and lungs were excised and analyzed histopathologically for right ventricular hypertrophy and pulmonary vascular remodeling.

Results: RAPA treatment provided limited, but not significant, improvements in exercise capacity, right ventricular systolic pressure, and right ventricular hypertrophy. However, there was significant recovery in pulmonary artery muscular layer thickness with 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 mortality rates with RAPA treatment (p = 0.16); however, a significant recovery was noted in terms of median life span (p = 0.006).

Conclusions: PAH is a progressive disease that is not curable with current therapies. RAPA may have the potential reverse vascular remodeling and prolong life expectancy in cases of pulmonary hypertension.

Keywords: Life expectancy, pulmonary hypertension, rapamycin, vascular remodeling
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, vasoconstriction originating from vascular endothelial damage in pulmonary arterioles, smooth muscle cell proliferation and hypertrophy, and constriction and remodeling of arterioles seem to be primary factors (2).

Current treatments aim at restoring the balance between vasoconstriction and vasodilatation to prevent proliferation of endothelial and pulmonary artery smooth muscle cells (3-5). However, despite these treatments, the disease is still not curable.

Inflammation in the vascular wall, proliferation of endothelial cells and vascular smooth muscle, and vascular thrombosis are histopathological hallmarks of PAH (6). Therefore, signaling pathways involved in proliferation should be targeted as part of a 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 that imatinib influenced exercise capacity and hemodynamics (7, 8). The positive effects of fasudil (a Rho kinase inhibitor) on altering vasoconstriction and pulmonary cell proliferation (9) afforded acute hemodynamic benefits in 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 right ventricular hypertrophy and vascular remodeling 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, 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. Blockage of mTOR by RAPA inhibits proliferation in numerous cell lines, including cancer cells, coronary arterial smooth muscle cells, vascular progenitor cells, and embryonic stem cells (15-18).

We aimed to evaluate the effects of RAPA on PAH induced by monocrotalin in terms of its effects on exercise capacity, right ventricular systolic pressure, right ventricular hypertrophy, pulmonary vascular remodeling, and mortality.

**METHODS**

**Study design and animals**

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

Rats were randomly assigned to one of the following groups: untreated animals (controls, n=10), monocrotaline group only (PAH control, n=15), and monocrotaline plus RAPA group (PAH-RAPA, n=14). In PAH control group, monocrotaline [Sigma-Aldrich, USA] 60 mg/kg was administered intraperitoneally on day 1 of the study period. In PAH-RAPA group, monocrotaline 60 mg/kg was administered intraperitoneally on day 1 of the study period, and
RAPA (Wyeth Pharmaceuticals Inc., USA) 3 mg/kg was administered daily by orally from day 21 until the end of the study period. Rats were monitored daily and mortality was recorded. After five weeks of monocrotaline application, all animals were subjected to an exercise test. Then, right ventricular pressure was measured under anesthesia. Heart and lungs were excised for histopathological investigation.

**Exercise capacity**

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 immersion, from which floating time was subtracted.

**Right ventricular pressure**

All rats were anesthetized by 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 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 pulmonary arterial resistance, the chest of the rat was opened through a midline incision. An 18-gauge catheter filled with heparinized saline was inserted into the wall of the right ventricle and advanced into the pulmonary artery. Pressure recordings were performed using the MP 100A BIOPAC system (Santa Barbara, California).
**Histology**

Histological examination was performed by histopathologists blinded to study groups. After measurement of the right heart resistance, the heart and lungs of the decapitated animals were excised and fixed using neutral-buffered formalin (10%).

The heart, after central transverse sectioning, was stored in paraffin and 2-μm-thick sections were made and stained with hematoxylin-eosin. Right ventricular hypertrophy was expressed as the ratio of right ventricular wall area to (left ventricular wall area+interventricular septum)×2 (RV/[LV+S]×2).

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

**Statistical analysis**

Statistical analysis was carried out using the MedCalc statistical software version, 12.7.7. Mann Whitney U test was applied for analyses of two groups that were not independent and not normally distributed. Median, minimum and maximum values were used. Log-rank tests were conducted to compare survival rates between groups. The statistical significance level was set at p<0.05.
RESULTS

Exercise capacity

Median exercise capacity significantly decreased with PAH application compared to control group (3.75 vs 6.43 min, 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 min, respectively, p=0.51) (Figure 1).

![Figure 1. Exercise duration (min) in study groups.](image)

Although exercise duration was lower in PAH control group than control and PAH-RAPA groups, the difference was significant for only between control and PAH control groups (n=6–10).

Right ventricular systolic pressure

The right ventricular systolic pressure of PAH control group (median, 26 mmHg; range, 16-28 mmHg) was significantly higher than that of control group (median, 12 mmHg; range, 8-16 mmHg) (p=0.05). But there was no significant difference between the right ventricular systolic pressures of PAH control and PAH-RAPA group (median, 23.75 mmHg; range, 16-31.4 mmHg) (p=0.89) (Figure 2).
Figure 2. Right ventricle systolic pressure (mmHg) was significantly higher in PAH control group than control group, but not significantly different between PAH control and PAH-RAPA groups (n=3–6).

Right ventricular hypertrophy

Right ventricular hypertrophy rates were 0.38 (range, 0.33-0.46) in the control group, 0.54 (range, 0.44-0.95) in 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 PAH control groups (p=0.01), but not between the PAH-RAPA and PAH control groups (p=0.12) (Figure 3).

Figure 3. Right ventricle hypertrophy ratio [(RV/LV+S)×2] was significantly higher in PAH control group than control group, but not significantly different between PAH control and PAH-RAPA groups (n=5–7).
Distal pulmonary artery wall muscular thickness

Distal pulmonary artery wall muscular thickness increases significantly in PAH control group (median, 24.7; range, 18.35-27) compared to control group (median, 19.03; range, 17.6-21.65), this increase was significantly reversed to control level by RAPA treatment (median, 18.98; range, 15.6-23.8) (Figures 4 and 5).

**Figure 4.** PAH was associated with increased wall thickness in distal pulmonary arteries (corrected for vessel size), which was reduced with RAPA therapy (n=4–8).

**Figure 5.** Histological images of distal pulmonary arteries stained with α-smooth muscle actin (left side) and hematoxylin-eosin (right side) for control (a, b), PAH control (c, d) and PAH+RAPA (e, f) groups with x400 magnification.
Survival

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

Figure 6. Kaplan-Meier survival curves of the three groups. Mortality rates were 0% in control group, 53.1% in PAH control group and 42.9% in PAH-RAPA group. There was a significant difference between control and PAH control groups, but not between PAH control and PAH-RAPA groups (p=0.006 and p=0.71, respectively).
**DISCUSSION**

PAH is a progressive disease characterized by abnormal high pressure in pulmonary arteries as a result of functional and structural fluctuations in the pulmonary vascular bed. Increases in pulmonary artery pressure and pulmonary vascular resistance occur as a result of vasoconstriction and structural changes in 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 remodeling. Eventually, these changes result in irreversible increases in pulmonary vascular resistance and right cardiac failure. Therefore, developing treatments that inhibit or reverse vascular remodeling is critical for the long-term management of PAH.
RAPA is an immunosuppressant used in transplant patients to prevent rejection (21). It has recently also been used for cancer treatment, at restenosis of coronary artery stents (22,23). Moreover, RAPA has been found to be effective against liver, kidney, pulmonary fibrogenesis (24–26), likely 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 of 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 heme 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 remodeling, right ventricular hypertrophy were detected. No effects on right ventricular hypertrophy were reported, similar to our study, but positive effects on muscularization of intrapulmonary arteries were detected. Houssaini et al. found that 5 mg/kg/day RAPA in monocrotaline-induced rats improved pulmonary arterial pressure, right ventricular systolic pressure, and the number of muscularized pulmonary veins (31).

According to Ogava et al., RAPA had antiproliferative effects on pulmonary artery smooth muscle cells and inhibited store-operated calcium entry in patients with chronic thromboembolic PAH (32).

McMurty et al. examined the effects of RAPA and statin on 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. No positive effects of the drugs on pulmonary arterial pressure levels, vascular modeling 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 pulmonary vascular resistance and statistically nonsignificant improvement in exercise capacity in an open-label pilot human study with everolimus (34).

PAH develops after 3 weeks in the monocrotaline-induced PAH model (35). To analyze 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 since PAH was diagnosed relatively late during the course of the disease. It has been reported that RAPA had a preventative effect on PAH when administered prior to its development. Several studies have reported that RAPA has reversal effects on PAH, and particularly vascular remodeling. Controversial results regarding the reversal effects of RAPA may be related to the dose used. More positive effects on PAH were noted when ≥3 mg/kg/day was administered. Besides, at human study of Seyfarth et al. detecting partial improvement at progressive PAH patients in spite of other treatments and observing protective effect more distinctively at animal studies made us think that RAPA may be more efficient treatment at early stage of PAH (34).

The analysis of exercise capacity in the 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 interpretation of the results of experimental studies from the clinical perspective.
CONCLUSION

In conclusion, 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. Use of RAPA derivatives has been approved for immune suppression after organ transplantation, for prevention of vessel restenosis after angioplasty, and for treatment of some malignancies. Therefore, RAPA may have potential as a further option for treatment of PAH.

Abbreviations

PAH: Pulmonary arterial hypertension; RAPA: Rapamycin; mTOR: Mammalian target of rapamycin; RV: Right ventricular wall area; LV: Left ventricular wall area; S: Interventricular septum.

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Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

AS drafted the manuscript, performed the chemical application and RVSP measurements; SV performed RVSP measurements, CV and BYB performed pathological examination; CO performed chemical application, AT performed exercise testing. All authors read and approved the final manuscript.
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