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Original Article

Physiological and pathological effects of diminazene on pulmonary hypertension: a controlled rat model study

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Abstract: Objectives: To evaluate the effects of diminazene (DMZ) on monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH). Methods: In total, 32 nine-week-old male Wistar-Albino rats (170-240 g) were divided into three groups: control (n=10), PAH (n=15), and PAH+DMZ groups (n=7). On the first day, 60 mg/kg MCT was injected intraperitoneally in the PAH and PAH+DMZ groups. On the 21st day, 15 mg/kg/day DMZ was injected, and the animals were followed for 35 days. On the 35th day, the exercise capacity of rats was analyzed through a modified forced swimming test. After measuring right ventricular systolic pressure using an open-chest method, right ventricle hypertrophy and pulmonary vascular remodeling were evaluated histopathologically. Results: On the 35th day, the mortality rate was zero in the control group, 53.1% in the PAH group, and 14.3% in the PAH+DMZ group. A significant decrease was observed in mortality rates with DMZ, and significant recovery was noted in median life spans (P=0.16 and P=0.01, respectively). DMZ had no significant effect on exercise capacity, right ventricle hypertrophy, or right ventricle systolic pressure, whereas there was significant recovery in pulmonary artery muscular layer thickness (P=0.046). Conclusions: DMZ prolonged life expectancy in PAH and decreased pulmonary arterial muscularization. Thus, it may be a new candidate treatment for PAH.

Keywords: Diminazene, pulmonary hypertension, mortality, ventricle hypertrophy

Introduction

Pulmonary arterial hypertension (PAH) is characterized by progressive pulmonary vascular resistance (PVR) and is a disease that has a poor prognosis, causing right ventricle (RV) hypertrophy, right ventricular failure, and ultimately, death [1, 2]. The primary factors of this disease include abnormal vasoconstriction due to vascular endothelial cell dysfunction in the pulmonary arterioles, smooth muscle cell proliferation and hypertrophy, contraction of the arterioles, and remodeling. However, the actual causes of the disorder remain obscure. Treatment options include calcium channel blockers, endothelin receptor antagonists, prostanoids, and phosphodiesterase 5 blockers that have vasodilatory effects [3]. Although there have been new developments in treatment, the mortality rate for PAH remains high, with survival at two years from diagnosis being about 85% [4, 5]. Despite improvements for the treatment of the disease, clinical deterioration may be postponed, but not stopped. Thus, there is a continuing need for new treatments for PAH.

The renin-angiotensin system (RAS) plays an important role in endothelial function and vascular remodeling. While the angiotensin-converting enzyme (ACE)-angiotensin II (Ang II)-Ang II receptor type 1 (AT1R) axis supports vasoconstriction, proliferation, and fibrosis, the ACE2-Ang-(1-7)-Mas axis has vasoprotective effects [6, 7]. Diminazene (DMZ) is an ACE2 activator [8]. It is an agent used for the treatment of sleeping sickness [9].

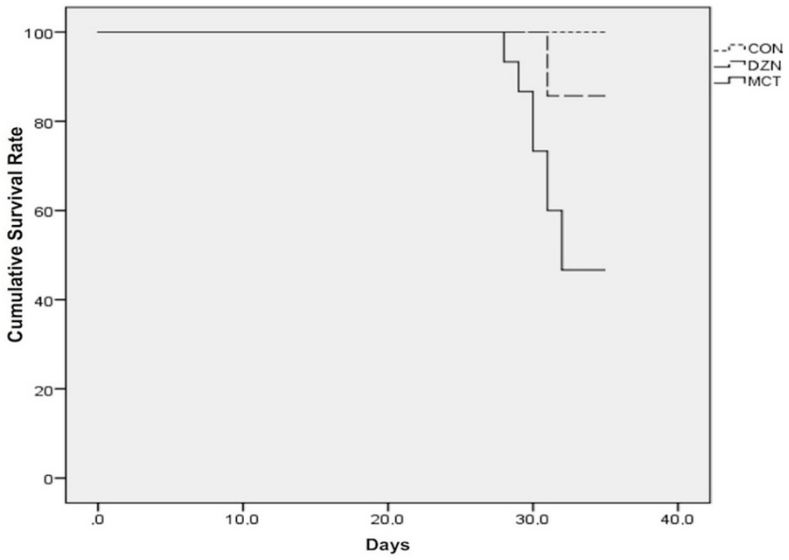

Figure 1. Kaplan-Meier survival curves of the three groups. Mortality rates were 0% in control group, 53.1% in PAH group and 14.3% in PAH+DMZ group. There was significant difference between control and PAH groups, but not between PAH and PAH+DMZ groups (P=0.006 and P=0.16, respectively).

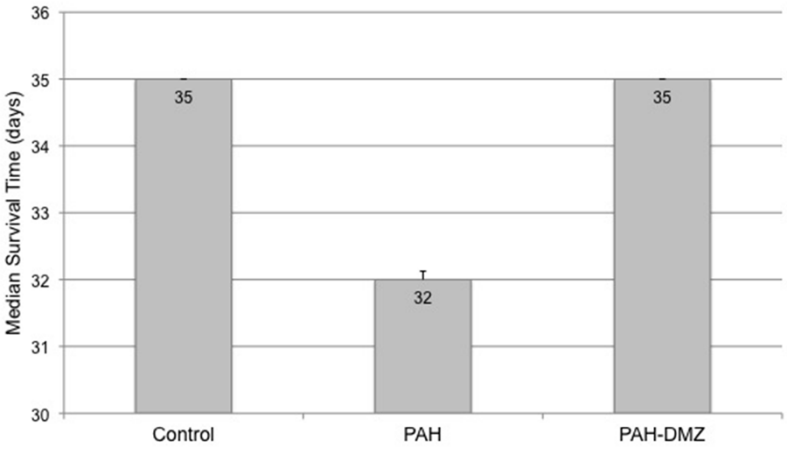

Figure 2. Median survival time (days) during 35 days of follow-up. Median survival time was significantly lower in PAH group than control and PAH+DMZ groups (P=0.01 for both). Error bars represent standard error of the mean.

In this study, we aimed to evaluate the effects of DMZ in PAH induced with monocrotaline on survival, exercise capacity, RV systolic pressure, RV hypertrophy, and pulmonary artery remodeling.

Methods

Study design and animals

In total, 32 nine-week-old male Wistar-Albino rats (170-240 g) were used. All of the rats were provided by the Kocaeli University Animal Reproduction Center, and were kept in the Experimental Animal Laboratory at Kocaeli University. They were kept in cages under controlled temperature and humidity with a 12/12 h light/dark cycle. Rats were fed *ad libitum*. Before the experiment, all of the rats were acclimated to the environment for 2 weeks. The Kocaeli University Committee on the Use and Care of Animals approved the experiments. All of the investigations conformed to the 1996 National Academy of Science's Guide for the Care and Use of Laboratory Animals.

Rats were divided into the following groups: untreated animals (control group, n=10), monocrotaline only (PAH group, monocrotaline [Sigma-Aldrich, USA], 60 mg/kg applied intraperitoneally (i.p.) on day 1 of the study period, n=15), and monocrotaline plus DMZ (PAH-DMZ group, monocrotaline, 60 mg/kg applied i.p. on day 1 of the study period, plus DMZ [diminazene aceturate, Sigma-Aldrich], 15 mg/kg, applied daily intraperitoneally from the 21st day until the end of the study period, n=7). At the beginning of the study, there were seven rats per group. However, some rats had to be added to control and PAH groups afterwards due to increased mortality rate in PAH group and failure of RV systolic pressure measurement in the PAH and control groups, which resulted in a lower number of rats in PAH-DMZ group than control and PAH groups.

Rats were monitored daily, and mortality was recorded. After five weeks of monocrotaline application, exercise tests were conducted for all animals. Then, after anesthesia, RV systolic

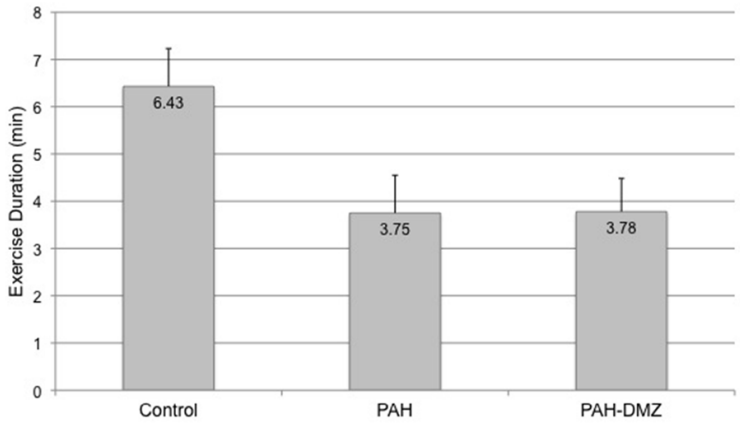

Figure 3. Exercise duration (min) was lower in PAH rats than control group (P=0.009), but similar between PAH and PAH+DMZ groups (P=1.0). Error bars represent standard error of the mean.

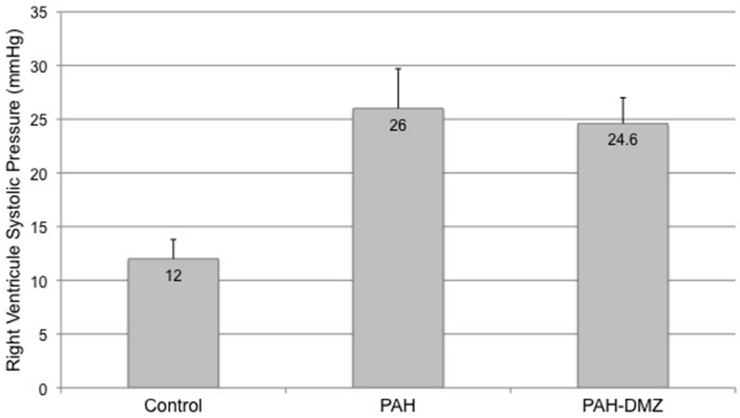

Figure 4. The right ventricule systolic pressure (mmHg) was significantly higher in PAH group than control group (P=0.05). But, there was no significant difference in the right ventricule systolic pressure between PAH and PAH+DMZ groups (P=0.79). Error bars represent standard error of the mean.

pressure was measured. The heart and lungs were excised for histopathological investigations.

Exercise capacity

Exercise capacity was measured using a modified forced swimming test. Rats were placed in a tank filled with water, 50 cm long and 30 cm wide, at 25°C. Swimming duration was calculated subtracting the motionless period from the swimming time of the rats [10].

Right ventricle systolic pressure

All rats were anesthetized with i.p. application of 80 mg/kg ketamine hydrochloride (Ketalar; Eczacibasi Warner-Lambert Ilac Sanayi, Turkey) and 10 mg/kg xylazine hydrochloride (Rompun, Bayer, Turkey). Following intubation of the trachea, the animals were ventilated with a rodent ventilator (model 7025 Ugo Basile, Comerio, Italy). Open-chest measurements were conducted as previously described [11]. To measure RV systolic pressure, the chest of the rat was opened through a midline incision. An 18-gauge catheter filled with heparinized saline was inserted into the RV. Pressure recordings were made using a MP 100A BIOPAC system (Santa Barbara, CA, USA).

Histology

The histology was assessed by a histopathologist blinded to the animal groups. After measurement of right heart resistance, the heart and lungs of decapitated animals were excised and fixed using neutral-buffered formalin (10%).

Right ventricle hypertrophy

The heart after central transversal section was processed for histology in paraffin wax. Then, 2 μm sections were stained with hematoxylin and eosin. RV hypertrophy was expressed as follows: right ventricular (RV) wall area/(LV wall area + interventricular septum area)/2 [2×RV/LV+S].

Pulmonary vascular remodeling

After cutting the lungs into 2 mm slices, they were embedded in paraffin wax and 2 μm sections were prepared. Sections were stained with hematoxylin and eosin and also for α-smooth muscle actin (1:100, Dako, Glostrup, Denmark). Analysis of pulmonary vascular remodeling was performed as previously described. For each animal, 20 pulmonary artery sections with an external diameter of 50-200 μm were picked randomly. External diameter and medial muscular tissue thickness

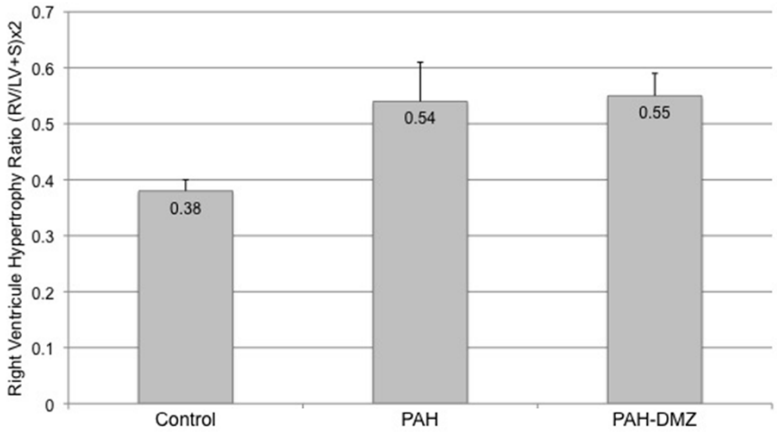

Figure 5. Right ventricule hypertrophy ratio was significantly different between PAH and control groups (P=0.001), but not between PAH and PAH+DMZ groups (P=0.87). Error bars represent standard error of the mean.

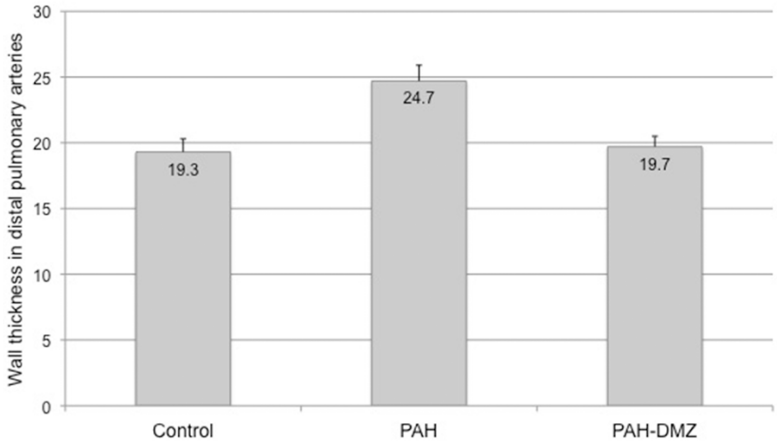

Figure 6. PAH was associated with increased medial wall thickness (corrected for vessel size) (P=0.038), which was significantly reduced with DMZ therapy (P=0.046). Error bars represent standard error of the mean.

were measured and muscular wall thickness/external diameter rate was recorded [10].

Statistical analysis

Statistical analyses were carried out using the MedCalc software (ver. 12.7.7). Normality of all parameters was evaluated by Shapiro Wilks Test. The Mann-Whitney U-test was used for two-group analyses (Control vs PAH, Control vs DMZ, PAH vs DMZ at all continuous variables) that were not independent or normally distributed. Median, minimum, and maximum values are reported. For comparing survival rates between Control, PAH, DMZ groups, the log-rank test was used. Statistical significance was set at 0.05.

Results

When mortality in all of the groups was analyzed, there were no (0%) deaths in the control group, eight (53.1%) in the PAH group, and one (14.3%) in the PAH-DMZ group. Between the control and PAH groups, the difference was statistically significant (P=0.006); however, the difference between the PAH and PAH-DMZ groups was not significant (P=0.16). When the 35-day survey was analyzed, the median lifetime was 35 days, the median in the PAH group was 32 days and the median in the PHD-DMZ group was 35 days; there was a significant difference between the groups (P=0.01; **Figures 1** and **2**).

When median swimming time values were analyzed, exercise capacity was 6.43 (4.06-12) min in the control group, 3.75 (1.21-6.65) min in the PAH group, and 3.78 (2.3-6.3) min in the PAH-DMZ group. A statistically significant difference was seen between the control and PAH groups (P=0.009). There were no significant differences between the PAH and PAH-DMZ groups (**Figure 3**).

When RV systolic pressure was analyzed, the median value in the control group was 12 (8-16). The RV systolic pressure median value of the PAH group was 26 (16-28), and compared to the control group, the difference was at the limit of statistical significance (P=0.05). The PAH-DMZ median value was 24.65 (20.3-34.3), and there was no significant improvement versus the PAH group (P=0.79; **Figure 4**).

RV hypertrophy rate median values were 0.38 (0.33-0.46) in the control group, 0.54 (0.44-0.95) in the PAH group, and 0.57 (0.43-0.67) in the PAH-DMZ group. A statistically significant

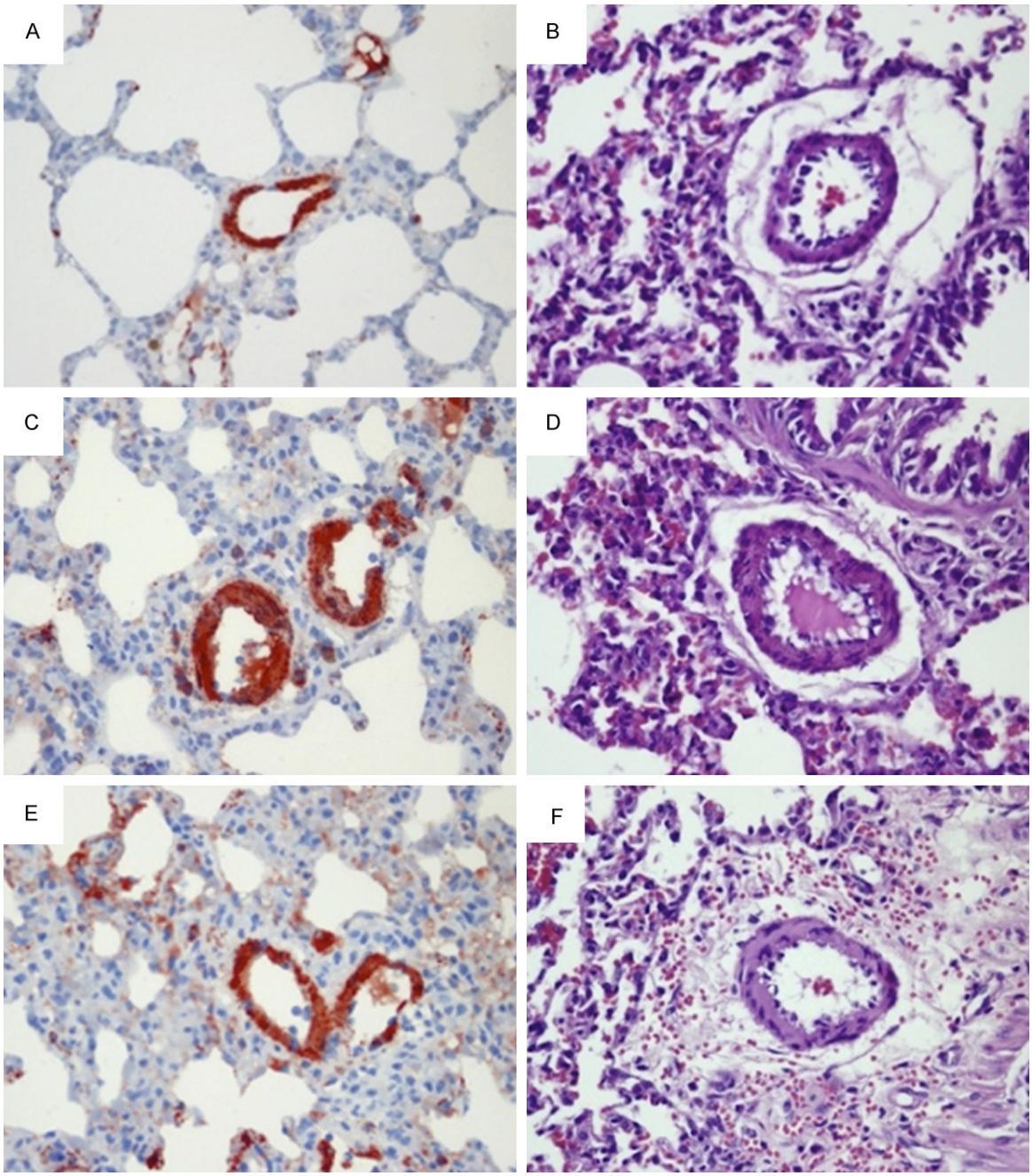

Figure 7. Histological images of distal pulmonary arteries stained with α-smooth muscle actin (left side) and hematoxylin-eosin (right side) with ×400 magnification for control (A, B), PAH (C, D) and PAH+DMZ (E, F) groups.

difference was observed between the control and PAH groups (P=0.01). Comparing the PAH-DMZ and PAH groups, the difference was not statistically significant (P=0.87; **Figure 5**).

The distal pulmonary artery wall muscular thickness was 19.03 (17.6-21.65) in the control group, 24.7 (18.35-27) in the PAH group, and 19.68 (17.51-2.25) in the PAH-DMZ group. A statistically significant difference was observed between control and PAH groups (P=0.038). Significant improvement was detected between the PAH and PAH-DMZ groups (P=0.046; **Figures 6** and **7**).

Discussion

PAH is a serious disease and the long-term prognosis is still poor. Although PAH has various causes, the cardiac and pulmonary vascu-

lar histopathological findings are similar. PAH is primarily a disease of the small pulmonary arteries. It is characterized by a progressive increase in vascular proliferation, remodeling, and PVR. The increase in PVR causes endothelial dysfunction, resulting in vasoconstriction, pulmonary vascular wall remodeling, and thrombosis [12]. Although hemodynamic changes represent one dimension of the illness, it is thought that the most important point in the pathogenesis is the increased proliferation of endothelial cells and decreased apoptosis [13].

One factor causing endothelial dysfunction and vascular impairment is the renin angiotensin system (RAS) [14]. Activation of the classical ACE-AngII-AT1R axis of RAS, which includes ACE, is related to the pulmonary hemodynamics that cause PAH [15]. ACE2, discovered more recently as a homolog of ACE, is relevant to many physiological and pathophysiological processes. ACE2 is a monocarbopeptidase. ACE2 is expressed in many tissues, such as the lungs, in Clara cells, type I and II alveolar epithelial cells, macrophages, endothelium, smooth muscle cells of blood vessels, and bronchial epithelia [16]. Widespread oscillation of RAS components in lungs suggest that pulmonary ACE2 activation may be effective for lung injury pathogenesis, and that it may be a new treatment target in PAH [17].

ACE2 has an important role in lung pathophysiology. It has been detected in various animal studies that ACE2 protected the lung from acute injury [18] and respiratory distress syndrome [19], relieved fibrosis induced with bleomycin [20], improved hypoxemia in an acute respiratory distress syndrome piglet model [21] prevented the development of lung failure [22], and that decreased ACE2 expression is related to lung fibrosis [23] and can cause lung failure [24]. It was observed in a pressure overload mouse model that with recombinant human ACE2 treatment, RV hypertrophy was decreased and RV systolic and diastolic function improved without affecting LV function [25]. There have been various studies regarding the importance of ACE2 in PAH. It has been shown in some studies that angiotensin signaling contributed to pulmonary artery smooth muscle cell proliferation and vasoconstriction, with upregulation of the AT1 receptor, and downregulation of ACE2 and angiotensin (1-7) [26-29]. The importance of neprilysin, which is a source of Ang-(1-7), has been reported in another mice study regarding the RAS-ACE2 pathway in neprilysin null mice. After chronic hypoxia exposure in these mice, increased pulmonary resistance and distal arterial muscularization were noted [30].

There are also reports of positive effects of ACE 2 activation or overexpression treatment in PAH. In the study of Yamazato et al [31], ACE2 overexpression relieved PAH, which was induced with monocrotalin. It was found that XNT, which is a pharmacological activator of ACE2, provided increased interleukin-10 levels, which is an anti-inflammatory cytokine and prevents right ventricular hypertrophy, pulmonary vascular wall thickness changes, interstitial fibrosis, and RV systolic pressure increases in PAH [6]. In a rat study, it was found that ACE2 activation, achieved through continuous injection of resorcinolnaphthalein, prevented development of high pulmonary arterial pressure, RV hypertrophy, and neointimal formation [32]. In the study of Shenoy et al [20], decreased RV systolic pressure was detected with ACE2 overexpression in pulmonary fibrosis induced with bleomycin. In addition, significant improvements in RV systolic pressure, RV wall thickness, and pulmonary artery wall thickness with Ang-(1-7) overexpression were noted in PAH, induced with monocrotaline [20].

In the study by Kulemina et al [8], it was found that DMZ, which is an anti-protozoan chemotherapeutic used to treat trypanosomiasis in domestic livestock, provided off-target ACE2 activity [8]. We concluded in our study that DMZ, which is an ACE2 activator, contributed to increasing the lifetime and improving pulmonary arterial muscularization. We did not find any difference in RV systolic pressure, RV hypertrophy, or exercise capacity with DMZ treatment. It is known that after 3 weeks, pulmonary hypertension develops in the induced PAH model [33]. To analyze the reversal effects of DMZ, treatment began on the 21st day and lasted for 14 days. Because analyses were made at the end of the fifth week, when post-monocrotaline deaths often occur, it would be expected that deaths would occur in the non-treatment PAH group, and that abnormal hemodynamics, pathology, and exercise capacity findings would be encountered more often.

Vascular remodeling is a hallmark of PAH. It has been proposed in many reports that the effect of ACE2 on hyperplasia in vascular components is significant. It has been shown in *in vitro* and *in vivo* studies of ACE2 that hyperproliferation, migration of pulmonary smooth cells, and muscularization of small pulmonary arteries are involved [20, 34]. We also detected improvements in pulmonary arterial muscularization in rats treated with DMZ.

Some studies have analyzed the effects of DMZ in PAH models. In the study by Rigatto et al [35], where the effects of DMZ on autonomic modulation were observed in PAH rats induced with monocrotaline, it was found that monocrotaline increased sympathetic modulation and decreased heart rate variability and that DMZ improved these effects. Shenoy et al [36]. analyzed the protective and reversing effects of DMZ in a PAH rat model and reported a significant protective effect of DMZ on RV systolic pressure, RV hypertrophy, and pulmonary artery muscularization, in addition to positive reversing effects on RV systolic pressure and RV hypertrophy. Our findings were not as remarkable as those of Shenoy et al [36]. It should be noted that the monocrotaline dose in the study of Shenoy et al was lower than that applied in our study. For this reason mortality rate in PAH groups may be lower than our study. In our study, the reason for insignificant difference between DMZ group and PAH group in terms of RV systolic pressure, RV hypertrophy, and exercise capacity may be high mortality rate at PAH group. In the previous studies, the effects of DMZ were not analyzed in terms of mortality or exercise capacity. The analysis of exercise capacity in the present study is an important parameter because it denotes the functional significance of PAH and is used as an end-point in clinical studies.

Publications regarding the side effects of DMZ have mentioned that toxicity is encountered occasionally, even at standard therapeutic doses used in farm animals [37]. However, in a study by Abaru [9], who analyzed 99 African trypanosomiasis patients retrospectively, it was found that side effects were minor and transient.

In conclusion, DMZ treatment prolonged life in PAH rats and decreased pulmonary arteriolar muscularization. Although with DMZ treatment, RV systolic pressure and exercise capacity improved, there was no significant change. However, it is considered that as a result of the high death rate in the PAH group, animals with more negative values were likely not included in the analysis. DMZ, an ACE2 activator, may offer a new treatment approach in PAH therapy. There is a need for further studies to analyze long-term side effects and for clinical trials.

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Disclosure of conflict of interest

None.

Authors' contribution

AS drafted the manuscript, carried out experiments; SA carried out experiments, CV and BYB performed pathological examination; CO carried out experiments, AT performed exercise testing, NDK drafted the manuscript, UAA performed statistical analysis. All authors read and approved the final manuscript.

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