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ORIGINAL ARTICLE

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Alamandin and especially melatonin attenuate pulmonary arterial hypertension induced by monocrotalin

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Abstract

Background: Despite the available treatments, pulmonary arterial hypertension (PAH) prognosis is poor.

Objectives: We aimed to investigate the effects of the alamandine (ALA), melatonin (MEL), and ALA + MEL in PAH.

Methods: The rats were randomly divided into Control ($n = 10$), monocrotaline (MCT) ($n = 12$), ALA ($n = 12$), MEL ($n = 12$), and ALA + MEL ($n = 12$) groups. PAH was induced by MCT. The ALA, MEL, and ALA + MEL groups received 50 μg/kg/day ALA, 10 mg/kg/day MEL, and ALA + MEL, respectively, for 35 days. Echocardiographic and hemodynamic measurements and tissue analyses (morphometric, histopathological, ELISA, and western blot) were performed.

Results: Monotherapies, especially MEL, reduced the right ventricular (RV) systolic pressure. Only MEL increased the pulmonary artery acceleration time. MCT increased the RV/left ventricle (LV) + interventricular septum (IVS) ratio. While ALA and ALA + MEL slightly decreased the RV/(LV + IVS), MEL significantly restored it. MCT increased the tunica intima-media (TIM) thickness, PCNA and α-SMA of pulmonary arterioles, histopathological score (HS) (inflammatory infiltration etc.) of the lung, and RV. All treatments reduced the TIM thickness (especially MEL), PCNA, and α-SMA. All treatments significantly decreased the HS of the lung; however, MEL and ALA + MEL produced greater benefits. All treatments attenuated the HS of RV. MCT caused a significant increase in lung lysyl oxidase (LOX) activity. All treatments restored the LOX; however, MEL and ALA + MEL provided greater improvement. While lung Nrf-2 was increased in MCT-treated rats, MEL reduced it.

Conclusion: ALA, MEL, and ALA + MEL attenuate PAH and protect RV via antiproliferative, anti-remodeling, antihypertrophic, anti-inflammatory, and free radical scavenging (only MEL) capabilities. Overall, MEL produced the best outcomes.

KEYWORDS
alamandine, melatonin, monocrotaline, pulmonary arterial hypertension, right ventricular systolic pressure

1 | INTRODUCTION

Pulmonary arterial hypertension (PAH) is a deadly illness. In PAH, right ventricular systolic pressure (RVSP), which reflects mPAP, causes RV hypertrophy (RVH), dysfunction, and failure. Current treatments have limited efficacy for PAH.[1] Thus, drug research for PAH treatment has increased.[2]

Increased vasoconstrictor/proliferative signals in PAH led to remodeling in the pulmonary arterioles. This increases the mPAP.[2] Also, oxidant damage and inflammation play important roles in PAH. Increased reactive oxygen species (ROS) generation induces pulmonary vascular endothelial damage and increases apoptosis resistance and proliferation in PASMCs. Subsequently, oxidant damage accelerates pulmonary vascular remodeling.[3]

Alamandin (ALA) is a new endogenous peptide that is included in the beneficial part of the renin-angiotensin system (RAS). It works via the membrane Mas-related G protein-coupled receptor member D (MrgD) receptor. This receptor is present in smooth muscle cells in arteries, endothelial cells, and cardiomyocytes.[4] Studies have shown the antihypertensive and anti-remodeling effects of ALA. Lautner et al reported that perorally administrated an inclusion compound of alamandine/β-hydroxypropyl cyclodextrin provided a long-term antihypertensive effect in spontaneously hypertensive rats and antifibrotic effects including a decrease in cardiac collagen I, III, and fibronectin accumulation in isoproterenol-treated rats via binding MrgD receptor.[5]

Recently, our research team showed that ALA reduces the systolic arterial pressure in rat model of Ang II-induced hypertension.[6] de Souza-Neto et al reported that ALA attenuates transverse aortic constriction (TAC)-induced arterial remodeling in ascending aorta of mice.[7] Additionally, ALA has been shown to have MrgD-dependent antiproliferative activity on human pancreatic carcinoma cells by inhibition of PI3K/AKT/mTOR and BRAF/MKK/ERK1/2 pathways, activation of transcriptional factor FoxO1, and the downregulation of actin cytoskeleton proteins.[8] Although proliferation and remodeling play an important role in PAH pathogenesis, very limited research has been published investigating the effects of ALA-MrgD receptor signaling in PAH. Recently Soltani Hekmat et al reported protective effects of ALA in a rat PAH model. They showed that ALA improves hemodynamic parameters, oxidative stress markers, inflammatory factors, and electrocardiographic data, as monotherapy.[9] Unlike them, in our study, we investigated some biochemical parameters assessing different signaling pathways, and also performed detailed histopathological evaluation for small pulmonary arteries, and made echocardiographic examinations. We also examined the effect of ALA both alone and in combination with MEL. Because of aforementioned reasons, in this rat PAH model, we investigated whether ALA reduces pulmonary arterial pressure, attenuates pulmonary arterial and cardiac remodeling, and ultimately improves PAH via its antiproliferative effect.

Melatonin, a powerful antioxidant, is mainly produced and released by the epiphysis.[10] MEL has three receptors, namely, MT1, MT2 and MT3 receptors. MT1 and MT2 receptors are G protein-coupled receptors. M3 receptor is a quinone reductase enzyme whose function is to inhibit the electron transfer of quinones, thus protecting against oxidative stress. The cardiovascular system effects of MEL are mediated by MT1 and MT2 receptors. These receptors are found in cerebral arteries, coronary arteries, systemic arteries, aorta, and cardiac ventricular wall. MEL has both receptor-mediated and receptor-independent effects.[11,12] MEL shows free radical scavenging, antioxidant and anti-inflammatory effects.[3] MEL reduces ROS (superoxide anion radical ($O2-$), and hydroxyl radical (OH)) and reactive nitrogen species (RNS) (NO) by donating electrons. Thus, MEL acts as a free radical scavenger with a receptor-independent effect.[13,14] MEL also increases the levels of antioxidant enzymes such as superoxide dismutase and glutathione reductase by binding MT1 and MT2 receptors.[15] As a conclusion, MEL protects cellular compounds against oxidation by reactive oxygen species.[16] By neutralizing harmful nitrogen molecules like nitric oxide and peroxynitrite anion, which cause nitrosative damage, melatonin performs another important antioxidant role. Additionally, nitric oxide synthase, an enzyme that raises oxidative stress, is suppressed by melatonin.[13] Melatonin has anti-inflammatory properties that include the ability to induce the release of anti-inflammatory mediators (IL-10) while suppressing the release of proinflammatory cytokines including TNF-α, IL-6, and IL-1, which cause tissue damage.[17] Given these characteristics, we hypothesized that MEL, which possesses antioxidant and anti-inflammatory actions, might be helpful in PAH, in which oxidative damage and inflammation play a significant part in the pathogenesis. Another reason for investigating the effect of MEL in this study is that the effects of MEL, a cheap and well-tolerated drug,[18] on PH are not well known. We also were inspired to investigate the MEL because of the studies that showed it to be beneficial in cardiovascular pathologies including systemic arterial hypertension, atherosclerosis, myocardial ischemia/reperfusion, and PH.[18]

In summary, while ALA may act mainly through inhibition of proliferative signals, MEL may block especially oxidative stress pathways. Thus, ALA and MEL have therapeutic potential by affecting different parts of the pathophysiology of PAH. We thought that if ALA and MEL are used in combination, they can treat PAH more effectively than monotherapies as they affect different parts of PAH pathogenesis. Therefore, in this study, we examined the potentially beneficial effects of ALA or MEL alone or ALA + MEL combination in the PAH

model hemodynamically, histopathologically, and biochemically to elucidate the underlying mechanisms.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Ethical approval (2020/5-4) was provided by the Local Ethics Committee of Animal Experiments at Inonu University. Animals were provided by the Laboratory Animal Production and Research Center at Inonu University. The housing of the rats was carried out in polycarbonate cages under the following conditions: a 12-h light/dark cycle, a temperature of 20–25°C, a humidity of 60%, ad libitum feeding with fresh tap water, and a standard rat cow.

By using 58 male Sprague Dawley rats (220–380 g, 10–11 weeks), we formed five groups at random (Figure 1):

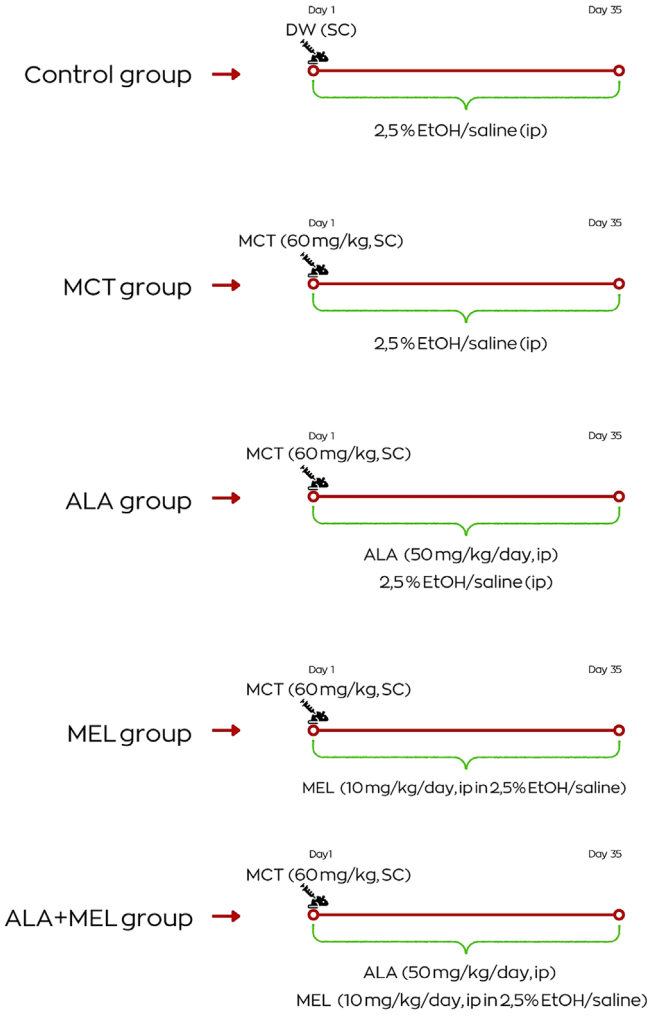

FIGURE 1 Schematic picture of the rats' group allocation and intervention.

1. Control group ($n = 10$): On the first day, subcutaneous (SC) distilled water was applied once. On days 1–35, a 2.5% solution of ethanol in saline was administered intraperitoneally (ip).
2. MCT group ($n = 12$): On the first day, 60 mg/kg single-dose SC MCT was applied once. On days 1–35, a 2.5% solution of ethanol in saline was administered ip.
3. MEL group ($n = 12$): On the first day, 60 mg/kg single-dose SC MCT was applied once. On days 1–35, 10 mg/kg/day ip MEL dissolved in a 2.5% solution of ethanol in saline was administered.
4. ALA group ($n = 12$): On the first day, 60 mg/kg single-dose SC MCT was applied once. On days 1–35, 50 μg/kg/day of ip ALA and an ip 2.5% solution of ethanol in saline were administered.
5. ALA + MEL group ($n = 12$): On the first day, 60 mg/kg single-dose SC MCT was applied once. On days 1–35, ALA and MEL were applied as in the other groups.

The doses of MCT (Sigma-Aldrich USA; CAS number: 315-22-0), MEL (Sigma-Aldrich USA; CAS number: 73-31-4), and ALA (Phoenix Pharmaceuticals, Inc. USA; CAS number: 1176306-10-7) were determined based on earlier reports.[19–21]

2.2 | Echocardiography

Echocardiographic measurements were performed by a single, blinded investigator. Animals were anesthetized by ethyl carbamate (1.2 mg/kg, ip) (Acros Organics, New Jersey, USA; CAS number: 51-79-6). Anesthetized rats were examined by transthoracic echocardiography via a 10S sector probe on a Vivid S6 device (GE Healthcare, United States) using previously described methods.[22] Firstly, the chests of the anesthetized rats were shaved. 2D-MMode and Doppler measurements were performed in the left lateral decubitus position. Aortic velocity, peak velocity of early diastolic transmitral flow (E), LA diameter, end-systolic and end-diastolic interventricular septal thickness (IVSs and IVSd), end-systolic and end-diastolic left ventricular diameter (LVIDs and LVIDd), end-systolic and end-diastolic left ventricular posterior wall thickness (LVPWs and LVPWd), left ventricular ejection fraction (LVEF), HR, pulmonary artery acceleration time (PAAT), tricuspid annular plane systolic excursion (TAPSE), RV diameter, and RV/LV ratio were measured. Parasternal long axis and apical four/five-chamber images were taken into account for 2D and color flow imaging and spectral Doppler evaluation of the mitral valve and/or aortic outflow tract. Measurements of the LA, LV, and aorta were made using the parasternal long axis. PAAT measurements were taken over the parasternal short axis. Recordings of Doppler spectra were taken during five to

10 heart cycles at a scanning rate of 100 mm/s. Color Doppler was studied at the preset limit of 0.44 m/s Nyquist. Estimated systolic PAP measurement on echocardiography was not performed because it would not yield practical and accurate results.

2.3 | Hemodynamic measurements

After echocardiographic measurements, invasive hemodynamic measurements (SAP, DAP, HR, and RVSP) were performed by a single, blinded investigator in anesthetized rats. SAP, DAP, and HR were recorded through the cannula placed in the left carotid artery (Biopac MP-100 Data Recording System). The calculation of mean arterial pressure (MAP) was made as follows: (2 × DAP + SAP)/3.

RVSP recording was taken using a slightly curved cannula placed in the right jugular vein (Biopac MP-100 Data Recording System).

The anesthetized rats were euthanized by removing the heart. The left lung was fixed in 10% formalin for histopathological examinations. The right lung was reserved for ELISA and Western blot and kept at −80°C until it was analyzed. Hearts were fixed in 10% formalin and then cut transversely, and the atria were separated from the ventricles. Then the RV-free wall was removed. The Fulton index was calculated as follows: the weight of the RV-free wall/the weight of the LV + IVS.

2.4 | Histopathology

Leica DFC-280 research microscope and Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK) were used for analyses. These analyzes were performed by a single, blinded investigator.

2.4.1 | Histochemistry

Lung and RV sections were prepared and stained with hematoxylin–eosin (H-E) staining following previously described procedures[23] and then evaluated with light microscopy.

Lung sections were examined for inflammatory infiltration, alveolar macrophage density, alveolar septal thickness, and interstitial edema. Randomly selected 10 areas were examined and scored as 0: no change, 1: mild, 2: moderate, and 3: severe change according to the degree of histopathological changes.[24] The tunica intima-media (TIM) thickness of randomly selected 20 pulmonary arteries was measured.[25]

RV sections were evaluated for interstitial edema, inflammatory infiltration, and granulation. Randomly selected 10 areas were examined and scored as 0: no change, 1: mild, 2: moderate, and 3: severe change according to the degree of histopathological changes.[26] In addition, the diameter of the randomly selected 20 cardiomyocytes at the nuclear level was measured.[27]

2.4.2 | Immunohistochemistry

Lung sections were prepared and stained with PCNA and α-SMA immunostaining following previously described procedures[23] and then evaluated with light microscopy. PCNA and α-SMA immunoreactivity assessments were performed on 20 randomly selected pulmonary arteries from each section at 40× magnification. Staining was scored semiquantitatively based on the extent of immunoreactivity (0: no staining, 1: 1%–25%, 2: 26%–50%, 3: 51%–75%, 4: 76%–100%) and severity (0: absent, +1: mild, +2: moderate, +3: severe). The total staining score was determined as follows: prevalence x severity.[23]

2.5 | Enzyme-linked immunosorbent assay (ELISA)

Lung samples weighing 0.1 g each were homogenized under ice isolation in a PBS (pH 7.4) solution, including 1 mL of protease inhibitor. The homogenates were centrifugated at +4°C for 15 min at 14 000 rpm with a cooled centrifuge. Finally, the obtained supernatants were stored at +4°C until the measurements. The total concentration of the proteins was detected via the Bradford method.[28]

In the lung homogenate, activities of LOX (Catalog no. ab112139; Abcam), ACE2 (Catalog no. ab273297; Abcam), and MPO (Catalog no. ab105136; Abcam) and levels of NOX4 (Catalog no. SEB924Ra, USCN) were detected by utilizing commercial ELISA kits following the manufacturer's instructions.

2.6 | Western blot

The western blot procedure was conducted as described earlier.[29] Concisely, total proteins extracted from lung tissues were size-fractionated with 4%–20% Mini-PROTEAN TGX (Bio-Rad, California) gel electrophoresis, transferred to a polyvinylidene difluoride (PVDF) membrane via the Trans-Blot Turbo Transfer System (Bio-Rad, California), and incubated overnight with primary antibodies against CypA (Cell Signaling, Massachusetts), RhoA (Cell Signaling, Massachusetts), ROCK-1 (Cell Signaling, Massachusetts), ROCK-2 (Cell Signaling, Massachusetts), MMP-2 (Santacruz Biotechnology, Texas), MMP-9 (Santacruz Biotechnology, Texas), and Nrf-2 (Santacruz Biotechnology, Texas).

The next day, the membrane was incubated with horseradish peroxidase (HRP)-linked secondary antibody for 1 h at room temperature and then labeled with chemiluminescence (ECL) (Bio-Rad, California). Each blot was performed in at least three replicates. Protein loading was checked by monoclonal antibodies against β-actin (Cell Signaling, Massachusetts). Blots were produced utilizing the Clarity Western ECL Substrate Kit (Bio-Rad, California) and visualized using the ChemiDoc MP System (Bio-Rad, California). Blot intensities were determined densitometrically using ImageJ (National Institute of Health, Bethesda, Maryland). The levels of proteins were normalized to the β-actin levels and presented as a percentage of the vehicle-treated rat.

2.7 | Statistical analysis

Statistical analysis was made by a web application developed by Arslan et al.[30] In the comparison between groups, the Kruskal–Wallis H test was utilized for non-normally distributed data while ANOVA (Tamhane or Tukey) test was utilized for normally distributed data. $p < 0.05$ was determined as the significance threshold. Data were given as median (minimum-maximum) (Med [Min–Max]) or arithmetic mean ± standard deviation (AM ± SD), depending on the distribution.

3 | RESULTS

3.1 | Biometric findings

Biometric findings are detailed in Table 1. Briefly, all groups had similar pretest rat weights ($p > 0.05$). The Fulton index (RV/LV + IVS) was higher in the MCT group versus the Control group ($p < 0.001$). A decrease was observed in all treatment groups (especially in the MEL group) compared to the MCT group.

3.2 | Hemodynamic findings

Hemodynamic findings are detailed in Table 2. Briefly, a significant elevation in RVSP was seen in the MCT group compared to the Control group ($p < 0.05$). A reduction was observed in all treatment groups (especially in the MEL group) compared to the MCT group. A significant decrease in SAP, DAP, and MAP was observed in the MCT group versus the Control group ($p < 0.05$). An elevation was observed in SAP, DAP, and MAP in the MEL and ALA + MEL groups compared to the MCT group. While there was an increase in SAP and MAP in the ALA group compared to the MCT group, a decrease was observed in terms of DAP.

3.3 | Echocardiography

Our results are detailed in Table 2. Briefly, PAAT was lower in the MCT group versus the Control group. PAAT was higher in the MEL group compared to the MCT group. A significant elevation in RV/LV was determined in the MCT group compared to the Control group ($p < 0.001$). RV/LV values were significantly lower in the ALA and ALA + MEL groups than in the MCT and MEL groups. LVEF was significantly lower in the MCT group versus the Control group ($p < 0.001$). LVEF was higher in the ALA, MEL, and ALA + MEL groups compared to the MCT group ($p < 0.001$). LVEF was higher in the ALA and ALA + MEL groups compared to the MEL group.

TABLE 1 The impact of the ALA, MEL, and ALA + MEL treatments on biometric parameters.

Parameters	Groups					*p* value
	Control	MCT	ALA	MEL	ALA + MEL	
Pre-test BW (g)	272 (224–340)	302 (273–334)	274.5 (245–343)	309 (217–382)	277 (239–334)	0.054
Post-test BW (g)	340 (295–414)	363 (313–407)	259[a,b,c] (215–373)	317[b] (256–400)	255[a,b,c] (193–333)	<0.001
Lung weight (g)	1.74 (1.17–2.05)	2.9[d] (2.40–3.54)	2.41[b,d,e] (1.94–3.27)	1.93[b,d] (1.5–2.92)	2.36[b,d,e] (2.11–3.18)	<0.001
Heart weight (g)	1.24 (0.97–1.67)	1.56[d] (1.15–1.85)	1.21[b] (0.84–1.46)	1.15[b] (0.9–1.72)	1.2[b] (0.83–1.59)	0.016
RV free wall (g)	0.15 (0.13–0.2)	0.21[d] (0.15–0.3)	0.17 (0.13–0.25)	0.12[b] (0.1–0.25)	0.18[e] (0.15–0.25)	0.042
LV + IVS (g)	0.7 (0.57–0.73)	0.59[a] (0.50–0.73)	0.58[a] (0.4–0.73)	0.63 (0.51–0.73)	0.53[a] (0.44–0.66)	0.019
Fulton index (RV/LV + IVS)	0.22 (0.20–0.27)	0.34[d] (0.26–0.41)	0.3[d,e] (0.26–0.41)	0.24[b] (0.16–0.37)	0.33[d,e] (0.27–0.47)	<0.001

Note: The variables are summarized as "median (min–max)."
Abbreviations: IVS, interventricular septum; LV, left ventricle; RV, right ventricle.
[a]Significant decrease versus the Control group ($p < 0.05$).
[b]Significant decrease versus the MCT group ($p < 0.05$).
[c]Significant decrease versus the MEL group ($p < 0.05$).
[d]Significant increase versus the Control group ($p < 0.05$).
[e]Significant increase versus the MEL group ($p < 0.05$).

TABLE 2 The effects of the ALA, MEL, and ALA + MEL treatments on hemodynamic and echocardiographic parameters.

	Groups					
Parameters	**Control**	**MCT**	**ALA**	**MEL**	**ALA + MEL**	***p* value**
RVSP (mmHg)	53 (40–100)	103[a] (63–122)	87 (51–111)	78[b] (45–131)	110[a] (63–156)	0.008
SAP (mmHg)	133 (77–154)	80[c] (72–113)	94[c] (78–116)	93[c] (81–116)	97[c] (49–136)	0.015
DAP (mmHg)	80 (65–102)	54[c] (47–91)	50[b,c] (33–85)	61[c] (33–87)	71 (26–116)	0.019
MAP (mmHg)	98 (69–110)	63[c] (55–98)	66[c] (53–93)	71[c] (49–97)	77 (34–122)	0.01
HR (beats/min)	262 (246–395)	262 (205–310)	319 (247–570)	249 (159–303)	307 (205–486)	0.067
IVSd (mm)	2 (1–3)	2 (2–3)	2 (1–2)	2 (1–3)	2 (2–3)	0.51
LVIDd (mm)	5,5 (3–6)	4 (3–5)	4 (3–6)	5 (4–6)	4 (3–5)	0.067
LVIDs (mm)	2 (1–2)	2 (1–3)	1[d,e] (1–2)	2 (1–3)	1[c,d,e] (1–1)	0.002
LVPWd (mm)	2,5 (2–3)	2 (1–3)	2 (2–3)	2 (2–3)	2 (2–3)	0.65
LVPWs (mm)	3 (1–4)	3 (2–4)	3 (3–4)	3 (3–3)	3 (3–4)	0.074
LVEF (%)	97 (93–99)	93[c] (79–96)	98[f,g] (96–100)	96[f] (89–98)	98[a,f,g] (96–100)	<0.001
PAAT (ms)	30 (22–41)	22 (18–37)	22[c,e] (15–30)	33 (11–41)	22[c,e] (15–22)	0.015
RV diameter (mm)	4.12 (3.85–5.04)	4.56 (3.6–5.1)	4.03[d,e] (3.52–4.52)	4.82[a,f] (4.65–5.53)	4.12[d,e] (3.79–4.61)	<0.001
RV/LV (mm/mm)	0.87 (0.84–1.03)	1.16[a] (0.76–1.29)	0.87[d,e] (0.78–1.01)	1.1[a] (0.93–1.25)	0.92[d,e] (0.84–1.08)	<0.001

Note: The variables are summarized as "median (min–max)." Regarding RVSP; MEL tended to decrease compared to MCT ($p = 0.062$), ALA tended to elevate compared to the Control ($p = 0.065$), ALA tended to decrease compared to ALA + MEL ($p = 0.054$). PAAT tended to decrease in MCT versus the control group ($p = 0.067$); it tended to decrease in ALA + MEL versus the MCT ($p = 0.086$).

Abbreviations: DAP, diastolic arterial pressure; HR, Heart rate; IVSd, end-diastolic interventricular septal thickness; LV, left ventricle; LVEF, left ventricular ejection fraction; LVIDd, left ventricular end-diastolic diameter; LVIDs, left ventricular end-systolic diameter; LVPWd, left ventricular end-diastolic posterior wall thickness; LVPWs, left ventricular end-systolic posterior wall thickness; MAP, Mean arterial pressure; PAAT, pulmonary artery acceleration time; RV, right ventricle; RVSP, Right ventricular systolic pressure; SAP, Systolic arterial pressure.

[a]Significant increase compared to the Control group ($p < 0.05$).

[b]Significant decrease compared to ALA + MEL group ($p < 0.05$).

[c]Significant decrease compared to the Control group ($p < 0.05$).

[d]Significant decrease versus the MCT group ($p < 0.05$).

[e]Significant decrease versus the MEL group ($p < 0.05$).

[f]Significant increase versus the MCT group ($p < 0.05$).

[g]Significant increase versus the MEL group ($p < 0.05$).

3.4 | Histopathology

3.4.1 | Lung

In the Control group, bronchi, bronchioles, alveoli, and pulmonary interstitium had normal histology, except for mild inflammatory infiltration (Figure 2(1)).

Significant changes were observed in the MCT group in comparison with the Control group ($p < 0.0001$) (Figure 2(1)). The inflammatory infiltration significantly increased in the MCT group. The infiltration was diffusely observed throughout the pulmonary interstitium, especially in the perivascular area. Another finding was an increase in alveolar macrophage density. In addition to being observed intensively on the alveolar wall, alveolar macrophages were also observed in groups within the alveolar lumen. Other findings were the thickening of the alveolar septum and interstitial edema. A significant increase in the TIM thickness of the pulmonary arterioles (PAs) was determined in this group in comparison with the Control group ($p < 0.0001$) (Figure 2(2)).

Histopathological changes were milder in all treatment groups compared to the MCT group ($p < 0.0001$) (Figure 2(1)). Besides, the alterations in the MEL and ALA + MEL groups were milder than in the ALA group ($p < 0.0001$). The results of the histopathological evaluation and mean TIM thickness are given in Table 3. TIM thickening was milder in all treatment groups compared to the MCT group ($p < 0.0001$). Besides, TIM thickness was similar in all treatment groups (Figure 2(2)).

PCNA-positive cell nuclei were stained as brown due to immunohistochemical staining (Figure 2(3)). The extent and intensity of PCNA immunoreactivity were mild in the Control group. Otherwise, the intensity of immunoreactivity was markedly elevated in the MCT group ($p < 0.0001$). A marked decline was seen in the intensity of immunoreactivity in all treatment groups compared to the MCT group ($p < 0.05$); however, they were similar (Table 3).

α-SMA positive cell cytoplasm stained brown due to immunohistochemical staining (Figure 2(4)). α-SMA immunoreactivity was markedly elevated in the MCT group contrasted to the Control group ($p < 0.0001$). A marked decline was seen in the intensity of immunoreactivity in all treatment groups against the MCT group ($p < 0.0001$). The reduction in the ALA + MEL group was more pronounced than ALA and MEL groups ($p < 0.005$) (Table 3).

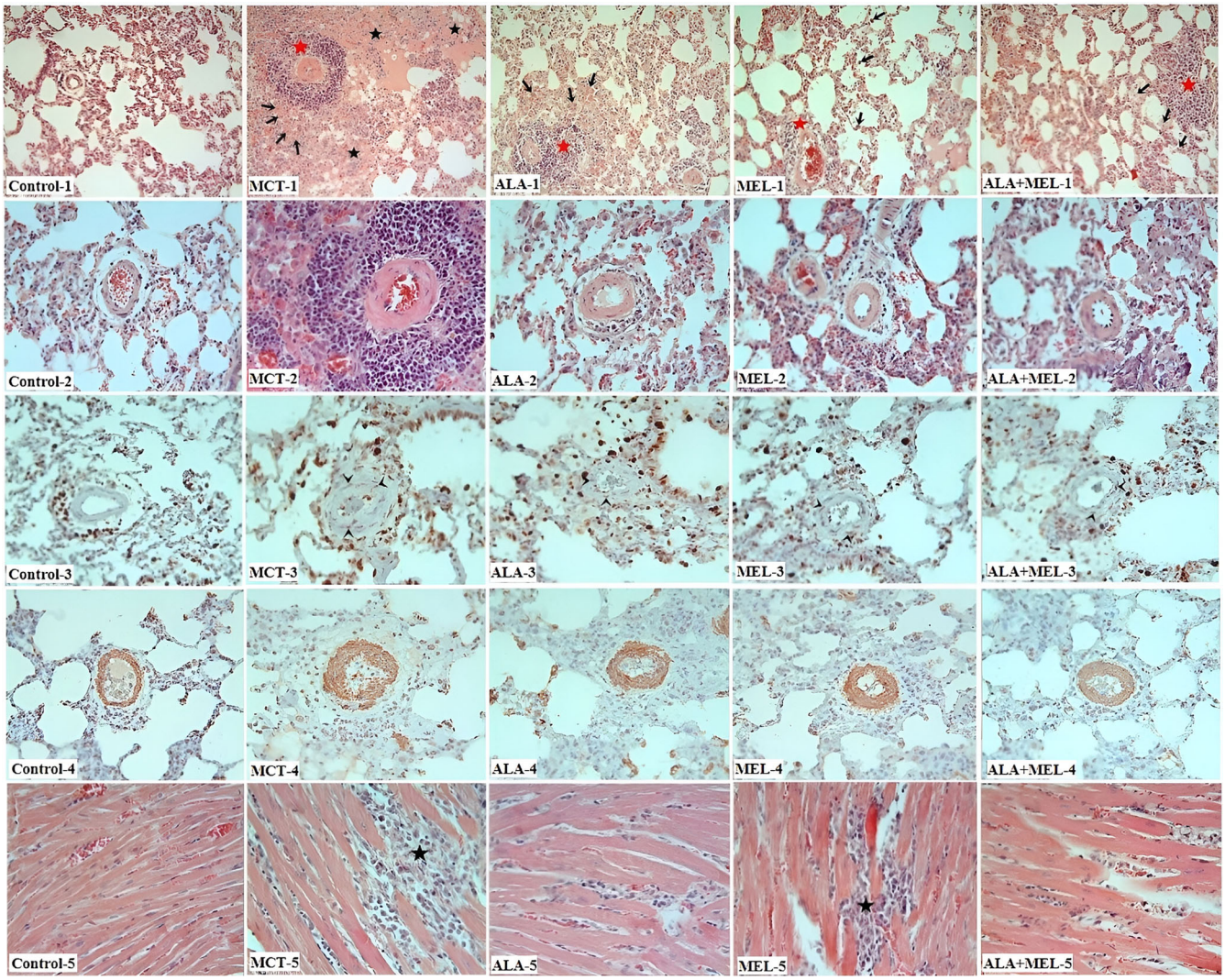

FIGURE 2 Histopathological effects of ALA, MEL, and ALA + MEL treatments on lung, pulmonary arteriole and right ventricle in PAH. (1) lung: in the Control group, the lung has a normal histology. In the MCT group, perivascular infiltration (red star), alveolar macrophages groups in the alveolar lumen (arrow), interstitial edema (black star) draw attention. Significantly milder histopathological changes are observed in ALA, MEL, and ALA + MEL groups compared to the MCT group. H-E; ×200. (2) pulmonary arteriole: significant thickening of the arteriolar wall in the MCT group is remarkable contrary to the Control group; a significant decrease in arteriolar wall thickness in the ALA, MEL, and ALA + MEL groups is remarkable in contrast to the MCT group. H-E; ×400. (3) PCNA immunoreactivity in pulmonary arteriole: PCNA immunoreactivity (arrowhead) in vascular smooth muscle cells (VSMCs) in all groups. PCNA immunohistochemical staining ×400. (4) α-SMA immunoreactivity in pulmonary arteriole: α-SMA immunoreactivity in VSMCs in all groups. α-SMA immunohistochemical staining ×400. (5) right ventricle: Control group; the myocardium has a normal histology. MCT group; large granulation tissue (star) among cardiomyocytes draw attention. ALA and ALA + MEL groups; histopathological changes appear to be markedly reduced. MEL group; marked granulation tissue is observed. H-E; ×400.

3.4.2 | Heart

Myocardial tissue with normal histology was seen in the Control group (Figure 2(5)).

As distinct from the Control group, myofibril loss in cardiomyocytes, local edema, inflammatory infiltration, and granulation tissue were noted in the myocardium of the MCT group ($p < 0.0001$) (Figure 2(5)). Cardiomyocyte diameter (CD) was higher in the MCT group versus the Control group ($p < 0.0001$).

Histopathological changes, especially the granulation tissue, were remarkably decreased in the ALA and ALA + MEL groups against the MCT group ($p < 0.005$) (Figure 2(5)). On the other hand, the changes seen in the MEL group were milder than in the MCT group (Figure 2(5)). All treatment groups were similar to the MCT group in terms of CDs. The results are presented in Table 3.

3.5 | ELISA and Western blot findings

Our ELISA results are detailed in Table 4. Briefly, the LOX was higher in the MCT group in contrast to

TABLE 3 The effects of the ALA, MEL, and ALA + MEL treatments on histopathological parameters.

Groups	HS (lung) Med (min–max)	TIM thickness (PAs) Mean ± SD	PCNA (PAs) Med (min–max)	α-SMA (PAs) Med (min–max)	HS (heart) Med (min–max)	CD Mean ± SD
Control	1 (0–2)	12.6 ± 3.5	0 (0–2)	8 (8–12)	0 (0–0)	14.2 ± 2.7
MCT	3 (2–3)[a]	23.4 ± 4.9[a]	1 (0–9)[a]	12 (8–12)[a]	1 (0–3)[a]	19.3 ± 3.9[a]
ALA	3 (0–3)[b]	18.8 ± 4.5[b]	1 (0–9)[c]	8 (8–12)[b]	0 (0–3)[d]	18.4 ± 2.9
MEL	2 (0–3)[b,e]	17.5 ± 4.1[b]	0 (0–9)[c]	8 (8–12)[b]	0 (0–3)	18.0 ± 3.9
ALA + MEL	2 (0–3)[b,e]	18.5 ± 4.2[b]	0 (0–9)[c]	8 (8–12)[b,f]	0 (0–3)[d]	18.3 ± 3.5

Abbreviations: CD, cardiomyocyte diameter; HS, histopathological score.
[a]Higher versus Control group ($p < 0.0001$).
[b]Lower versus MCT group ($p < 0.0001$).
[c]Lower versus MCT group ($p < 0.05$).
[d]Significant decrease versus MCT group ($p < 0.005$).
[e]Lower versus ALA group ($p < 0.0001$).
[f]Lower versus ALA and MEL groups ($p < 0.005$).

TABLE 4 Comparison of biochemical findings LOX, ACE2, MPO between the groups.

Parameters	Groups					*p* value
	Control	MCT	ALA	MEL	ALA + MEL	
LOX (μg/mg protein)	5.58 (5.09–11.14)	8.62[a] (8.11–11.37)	6.05[b] (4.09–7.97)	4.99[b] (3.96–7.26)	4.98[b] (4.70–6.31)	**<0.001**
ACE2 (pmol/min/mg protein)	15.28 (9.66–15.75)	13.15 (12.35–14.71)	13.72 (10.74–15.94)	10.59 (7.47–13.65)	11.63 (6.00–13.03)	0.053
MPO (nmol/min/mg protein)	8.02 (5.84–8.25)	6.09 (5.49–11.29)	5.31[c] (4.68–8.53)	6.70 (5.46–8.93)	5.11[b,c,d] (4.42–6.34)	**0.019**
NOX4 (ng/mg protein)	0.2 (0.16–0.26)	0.08[c] (0.04–0.12)	0.09[c] (0.08–0.15)	0.08[c] (0.07–0.14)	0.09[c] (0.07–0.13)	**0.004**

Note: The variables are summarized as "median (min–max)." Regarding LOX, the MEL group tends to decrease versus the ALA group ($p = 0.088$); the MEL group tends to decrease versus the Control group ($p = 0.06$).
Abbreviations: ACE2, Angiotensin-converting enzyme 2; LOX, Lysyl oxidase; MPO, myeloperoxidase; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4.
[a]Marked elevation versus Control group ($p < 0.05$).
[b]Marked decrease versus MCT group ($p < 0.05$).
[c]Marked decrease versus Control group ($p < 0.05$).
[d]Marked decrease versus MEL group ($p < 0.05$).

the Control group ($p < 0.001$). LOX was significantly lower in all treatment groups contrary to the MCT group ($p < 0.001$). LOX value was lower in the MEL and ALA + MEL groups compared to the ALA group.

Western blot outcomes were demonstrated in Figure 3. In summary, the Nrf2 was significantly higher in the MCT group than in the Control group ($p < 0.001$). The Nrf2 was markedly lower in the MEL group compared to the MCT group.

4 | DISCUSSION

In this study, MCT led to a marked elevation in RVSP, as previously reported.[31] Monotherapies, especially MEL treatment, slightly reduced the RVSP. This indicates that all treatments especially MEL improve PAH hemodynamics. Echocardiographically, in favor of PAH, MCT reduced the PAAT. Only the MEL treatment caused an elevation in PAAT. MCT significantly increased the RV/LV ratio as previously shown.[32] All treatments provided amelioration in the RV/LV ratio. Also, MCT increased the RV/(LV + IVS) ratio reflecting RVH, as previously reported.[31] While ALA and ALA + MEL treatments slightly decreased the RV/(LV + IVS) ratio, MEL significantly reduced it. This suggests that all treatments, especially MEL, exert an anti-hypertrophic effect on the RV.

Additionally, while MCT significantly decreased the LVEF, all treatments led to a significant elevation in LVEF. The improvement provided by ALA and combined treatment was more pronounced than MEL treatment. Hemodynamically, while MCT significantly reduced the SAP, DAP, and MAP, MEL and ALA + MEL treatments caused an improvement in arterial blood pressure. ALA led to improvements in SAP and

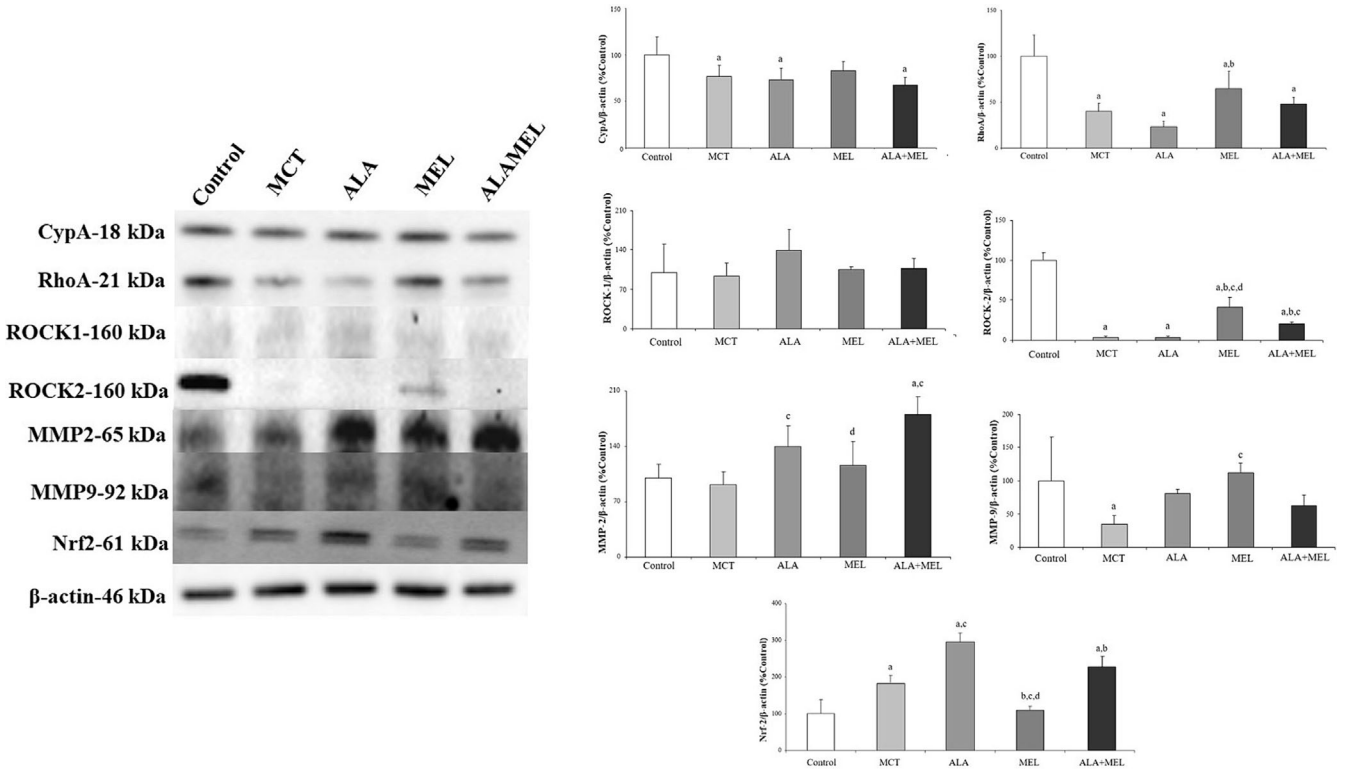

FIGURE 3 Comparison of western blot findings between the groups. (a) Significantly different from Control group ($p < 0.05$). (b) Significantly different from ALA group ($p < 0.05$). (c) Significantly different from MCT group ($p < 0.05$). (d) Significantly different from ALA + MEL group ($p < 0.05$). Regarding RhoA, the MEL group tended to increase versus the MCT group ($p = 0.068$); the ALA + MEL group tended to increase versus the ALA group ($p = 0.059$). Regarding MMP-2, ALA tended to increase versus the control ($p = 0.062$); ALA + MEL tended to increase versus the ALA ($p = 0.057$) group.

MAP; however, it reduced the DAP. Also, all treatments alleviate left ventricular function and systemic hemodynamics except that ALA lowers DAP.

In our study, MCT increased the TIM thickness of PA. Also, MCT caused an increase in the histopathological score (HS) including inflammatory cell infiltration and edema in the pulmonary interstitium, alveolar macrophage density, and an increase in alveolar septum thickness, especially in the perivascular area. MCT also led to a marked increase in PCNA and α-SMA immunoreactivity in the PA. These changes were similar to those shown by Wu et al.[31] All treatments, especially MEL, caused a significant reduction in TIM thickness, indicating PA wall thickness. In addition, all treatments reduced the PCNA, indicating PASMC proliferation. Also, all treatments significantly reduced α-SMA, reflecting the proliferation and/or hypertrophy of PASMCs. These findings indicate that all treatments-especially MEL-reduced the PA wall thickness via their antiproliferative and antihypertrophic effects. Additionally, all treatments significantly decreased the HS; however, MEL and ALAMEL yielded higher benefits than ALA. This suggests that MEL exhibited potent anti-inflammatory effects as previously reported,[33] and MEL was stronger than ALA to suppress lung inflammation, and combination treatment yielded no further help. As a result, it can be said that all treatments, especially MEL, ameliorate PAH via antiproliferative, antihypertrophic, and anti-inflammatory effects.

In our study, MCT caused a significant increase in the HS, including myofibril loss in cardiomyocytes, interstitial edema, inflammatory infiltration, and granulation in RV myocardium, as previously shown.[34] All treatments reduced the HS of RV. This suggests that all treatments exert anti-inflammatory effects in the RV myocardium in addition to the lung; therefore, these treatments protect the RV against the detrimental effects of PAH. Also, CD was elevated by MCT; however, it was slightly reduced by all treatments, indicating the antihypertrophic effects of the treatments.

According to the histopathological results of the PA, lung, and RV, it can be said that combined treatment provides no added advantage to the MEL treatment in terms of PAH treatment and protection of the RV against PAH.

High LOX activity seems to be critical in ECM remodeling by leading to excessive cross-linking in collagen and elastin fibers, thus playing a crucial role in PAH pathogenesis.[35] Nave et al reported high LOX expression in the lungs and PASMCs of rats with PAH induced by MCT.[35] Consistently, we found that MCT caused a significant increase in lung LOX activity. All

treatments restored the LOX activity; however, MEL and ALA + MEL treatments provided the most obvious improvement. These indicate that all treatments, especially MEL, reduce ECM remodeling in PA by inhibiting LOX, thus preventing PAH.

Nrf-2, the main controller of the antioxidant response including the expression of antioxidant enzymes, is considered to be critical in PAH pathogenesis.[36] In our study, lung Nrf-2 levels were increased in MCT-treated rats. It can be attributed to the compensation against oxidative stress. Literature data also support our result. Veit et al reported high Nrf-2 expression in PASMCs of MCT-induced PAH rats.[37] Martin et al showed high Nrf-2 levels in Sugen/hypoxia PAH rats.[38] In addition, Lopez-Bernardo et al reported that 4-hydroxynonenal, a lipid peroxidation product, increased Nrf-2 expression in mouse cardiomyocytes.[39] In our study, lung Nrf-2 levels were found to be lower in MEL-treated rats compared to the MCT-treated rats. This finding can be explained by the fact that MEL reduces the need for lung Nrf-2 elevation by scavenging free radicals. On the other hand, lung Nrf-2 levels were found to be higher in ALA-treated rats compared to the MCT-treated rats. Also, lung Nrf-2 level in the ALA + MEL group was not significantly different from the MCT group. A possible explanation is that ALA suppresses MEL-induced lung Nrf-2 lowering effect when combined with MEL. In this regard, Romero et al reported that Ang (1–7), Mas receptor agonist,[40] augmented the cellular levels of Nrf2 in human umbilical vein endothelial cells in a Mas-dependent manner.[41] As known, the molecular structures of ALA and Ang (1–7) are quite alike. A heptapeptide, alamandine with the amino acid sequence Ala–Arg–Val–Tyr–Ile–His–Pro differs from Ang (1–7) solely in that its N-terminal alanine residue is substituted for the aspartate residue. It was proposed that alamandine is produced either directly from Ang (1–7) by decarboxylating its aspartate residue or catalytically from Ang A by the action of ACE2.[42] This similarity between ALA and Ang (1–7) may explain why ALA increased Nrf2 and thus neutralized the some effects of MEL in our study. For instance ALA + MEL was not as effective as MEL alone on ameliorating RVSP, PAAT, and Fulton index. There are not enough data for clear explanation; however, future studies will shed light on this circumstance.

According to the available literature, there is very limited data regarding the effect of ALA in PAH. Recently Soltani Hekmat et al reported some protective effects of ALA on monocrotaline-induced PAH in rats. They showed that while MCT increases RVSP and Fulton index, elevates lung MDA and reduces SOD and CAT, elevates inflammatory markers (TNF-α, NF-κB, IL-6, IL-1β, iNOS) in lung; ALA reversed these changes. They also demonstrated histopathologically that ALA reduced MCT-induced lung inflammation.[9] The results of this study coincide with our results. Also, there is some additional data to support our findings. Vasodilator, antihypertensive, cardioprotective, antifibrotic, and anti-inflammatory effects of ALA have been demonstrated.[4,7] de Souza-Neto et al reported that ALA prevents the thickening in the media layer and inhibits the increase in cell density in the adventitia layer in ascending aorta of mice that underwent TAC surgery. Thus, ALA attenuated TAC-induced arterial remodeling.[7]

In the current work, MEL treatment ameliorated PAH and protected RV from the detrimental effects of PAH via its antiproliferative, anti-remodeling, antihypertrophic, anti-inflammatory, and free radical scavenging properties. A few studies determined the efficacy of MEL against PH. The findings of these studies are in line with the results of our study. Zhang et al showed low MEL serum levels in PAH patients. Additionally, they reported MEL's beneficial effects including a reduction in RVSP, RVH, and remodeling in PAH models. They suggested that MEL is effective against PAH through inhibition of inflammasome in macrophages.[20] Also, Maarman et al reported that MEL reduces RVH, oxidative stress, and cardiac fibrosis and heals RV function in MCT-induced PAH rats. They showed antioxidant, antifibrotic, and cardioprotective effects of MEL in PAH. However, the effects of MEL on in vivo PAH hemodynamics (in vivo PAP or RVSP measurement or echocardiography) or lung histopathology were not evaluated.[43]

5 | STUDY LIMITATIONS

One of the limitations of the study is that the mPAP value could not be measured due to the lack of laboratory facilities. Therefore, we measured the RVSP value reflecting mPAP.

In addition, vascular contraction and relaxation responses of small pulmonary arteries could be measured to determine whether the possible vasodilatory effects of treatments play a role in the decrease in pulmonary artery pressure.

Also, to explain the antiproliferative mechanism of ALA and MEL, proliferative signaling pathways such as Cyclin-D–CDK4/6, cyclin-E–CDK2, Ras–Raf–MEK–ERK, or PI3K/Akt/mTOR could be examined.

Additionally, to reveal the anti-inflammatory mechanism of ALA and MEL, inflammation pathways such as NF-κB, MAPK, or JAK–STAT could be investigated.

6 | CONCLUSION

ALA, MEL, and ALA + MEL treatments attenuate PAH and protect RV via their antiproliferative, anti-remodeling, antihypertrophic, anti-inflammatory, and free radical scavenging (only MEL) properties. In total, the best results were obtained with the MEL treatment.

New well-designed studies are needed to validate the outcomes we achieved.

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CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The current research was practiced under the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.[44] The ethical approval was provided by Local Ethics Committee for Animal Experiments at Inonu University (protocol number: 2020/5-4).

AUTHORSHIP STATEMENT

All authors, especially Seyhan Ayik, Hakan Parlakpinar, and Haci Ahmet Acet, participate in the hypothesis setting and planning/programming of the experiments. Management of the research, material preparation, and data collection were performed by Seyhan Ayik and Hakan Parlakpinar. Experiments were executed by Seyhan Ayik, Mehmet Gunata, Onural Ozhan, and Seyfullah Taha Inan. Histopathological evaluations were performed by Azibe Yildiz and Nigar Vardi. Western blot was performed by Nilay Ates and Ertugrul Kilic. ELISA was performed by Samir Abbas Ali Noma and Ahmet Ulu. Seyhan Ayik conducted the statistical analyses. All authors, especially Seyhan Ayik, Hakan Parlakpinar, and Haci Ahmet Acet, provided contributions to the commentary on the outcomes. Seyhan Ayik produced the manuscript's preliminary version, and all authors participated in the interpretation of the early forms of the manuscripts. Reading and approval of the paper's complete version were carried out by all authors.

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