# Matrix metalloproteinases are possible targets in monocrotaline-induced pulmonary hypertension: investigation of anti-remodeling effects of alagebrium and everolimus

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## Abstract

**Objective:** In our study, sildenafil alone and everolimus or alagebrium in combination with sildenafil were investigated in terms of their additional therapeutic and anti-remodeling activity in monocrotaline-induced pulmonary hypertension (PH) model in rats. In particular, the inter-relationships between PH and matrix metalloproteinases (MMPs) were investigated.
**Methods:** The pulmonary artery responses of male Sprague Dawley rats were recorded using myography, and the quantities and activities of MMPs were analyzed in homogenates of the pulmonary arteries and lungs by enzyme-linked immunosorbent assays, activity assays, and gelatin zymography techniques.
**Results:** Our results indicated that the therapeutic effects of sildenafil were accompanied by its suppressor effects on MMP activity. It was also shown that everolimus or alagebrium in combination with sildenafil showed additional regulatory effects on MMPs as well as functional responses on pulmonary artery pressure. Therefore, the enzymes in the MMP superfamily are likely to be target molecules for the treatment of PH.
**Conclusion:** In conclusion, MMPs were involved in the pathogenesis of PH, and our results suggested that the addition of everolimus or alagebrium to sildenafil therapy may be beneficial in PH. Our results indicated that agents that limit pulmonary vascular hypertrophy and inflammation via their anti-remodeling effects significantly ameliorate mortality and morbidity in PH. *(Anatol J Cardiol 2017; 17: 8-17)*
**Keywords:** Pulmonary hypertension, vascular remodeling, monocrotaline, everolimus, alagebrium, matrix metalloproteinases

## Introduction

Pulmonary hypertension (PH) is a progressive disease caused by vascular structural remodeling and increased vascular resistance, which lead to the increase in intrapulmonary pressure, right ventricle failure and death (1, 2). Vascular remodeling occurs in all layers of pulmonary artery and is characterized by medial hypertrophy, alterations of intimal proliferation, and adventitial thickening (3, 4). Inflammation, proliferation, and alterations of extracellular matrix (ECM) turnover are mechanisms, which are believed to play a role during this process (2). The matrix metalloproteinase (MMP) enzyme superfamily and their tissue inhibitors (TIMPs) are responsible for ECM integrity (5–7). These enzymes play important roles in matrix turnover, tissue remodeling (8), angiogenesis, and morphogenesis. It has been shown that the activity of MMPs is altered in cardiovascular pathologies (hypertension, atherosclerosis, and aneurysm) (6, 7). In addition, increased expression and activity of MMP2 have been described in idiopathic PH (9). Therefore, the activity of MMPs following the increase in the pressure of pulmonary bed might be a new predictor for PH.

Vasodilatory agents, such as prostacyclin analogues, endothelin antagonists, and phosphodiesterase type 5 enzyme (PDE-5) inhibitors, are widely used in the clinical management of PH (10). However, the current treatment strategies retard but do not stop the progression of the disease (11). Recently, new strategies targeting irreversible pulmonary vascular remodeling have been revealed (12, 13). In previous studies targeting abnormal inflammatory and immune responses, which contribute to remodeling process, patients with PH were shown to respond to immunosuppressive therapy alone or in combination with vasodilators (14). Everolimus, which inhibits the growth factor-mediated cell proliferation controlled by mTOR in hematopoietic cells and non-haematopoietic endothelial cells (15), smooth muscle cells, and fibroblasts, is an immunosuppressant with fewer cardiovascular adverse effects (16). In addition, the formation of

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glucose-amino acid cross-links has a role in triggering cardiovascular disorders. Alagebrium, which is the only agent capable of breaking these cross-links, has been shown to reduce endothelial dysfunction and vascular remodeling (17).

Monocrotaline (MCT), is an alkaloid from Crotolaria species, causes experimental PH in rats. Because of the strong homology of the MCT model with clinical PH, this model allows the investigation of the possible anti-remodeling effects of anti-proliferative and anti-inflammatory agents combined with vasodilator agents (18). In our study, sildenafil, everolimus, alagebrium, and combinations of everolimus or alagebrium with sildenafil were investigated for their efficacy on the reversal of MCT-induced PH in rats. Because of the insufficiency of classical vasodilator therapy in the management of PH, sildenafil monotherapy was compared with sildenafil+everolimus and sildenafil+alagebrium combination therapies to investigate the value of vascular remodeling as a promising target. The effects of these treatments on the extent of the improvement in the functional and structural parameters, which reflect the remodeling in pulmonary arteries in PH, were evaluated. In addition, we aimed to determine the activities of MMP enzymes and their TIMPs to illuminate their role in the pathogenesis of PH and the likelihood of any relationship with our treatments. Therefore, MMP-1, MMP-2, MMP-3, and MMP-9, which are thought to be the important members of the superfamily associated with vascular abnormalities (19), and their TIMPs, TIMP-1, and TIMP-2, were evaluated in our PH model.

## Methods

### Animals

Adult male albino rats of the Sprague Dawley strain weighing 250–300 mg were housed in polypropylene cages in a room kept at a standard temperature of 22°C±3°C and under a 12-h light/12-h dark cycle in our laboratory animal facility. The rats were provided with water and a standard diet ad libitum. The experimental protocol was approved by the Ethical Committee on Animal Experimentation (No: 153-2010). After acclimatising for 7 days, the animals were randomly divided into seven groups (with eight rats per group): (1) Control (C) (n=8) rats received an intraperitoneal (i.p.) injection of vehicle. After 3 weeks, rats received saline orally (p.o.) via gavage for 21 days. (2) MCT (n=8) rats received MCT (i.p.), and after 3 weeks, they received saline via gavage for 21 days. (3) SLD (n=8) rats received MCT (i.p.), and after 3 weeks, they received sildenafil (50 mg $kg^{-1}$) via gavage for 21 days. (4) EVE (n=8) rats received MCT (i.p.), and after 3 weeks, they received everolimus (3 mg $kg^{-1}$) for 21 days. (5) ALE (n=8) rats received MCT (i.p.), and after 3 weeks, they received alagebrium (10 mg $kg^{-1}$) via gavage for 21 days. (6) SLD+EVE (n=8) rats received MCT (i.p.), and after 3 weeks, they received sildenafil (p.o.; 50 mg $kg^{-1}$) and everolimus (p.o.; 3 mg $kg^{-1}$) via gavage for 21 days. (7) SLD+ALE (n=8) rats received MCT (i.p.), and after 3 weeks, they received sildenafil (50 mg $kg^{-1}$) and alagebrium (10 mg $kg^{-1}$) via gavage for 21 days. The doses were selected on the basis of previous studies (20, 21).

### Experimental protocol

### Drugs

Sildenafil was kindly provided by Deva (Istanbul, Turkey) and everolimus was kindly provided by Novartis Pharma AG (Basel, Switzerland). Alagebrium was purchased from Unispec Chemicals Co. (Nanjing, China).

### Induction of PH by MCT

MCT was purchased from Sigma, USA. It was dissolved in 1N HCl and buffered to pH 7.0 with 1N NaOH. Rats received MCT (60 mg $kg^{-1}$) or its vehicle by a single i.p. injection. 21 days after the injection, PH had developed (1, 22, 23).

### Hemodynamic studies

When active treatment was stopped after 24 h, rats were anaesthetised with ketamine (Pfizer, Turkey) (60 mg $kg^{-1}$) and xylazine (Bayer, Turkey) (5 mg $kg^{-1}$). In brief, a 23-gauge needle placed on the tip of a polyethylene-50 (PE-50) catheter was inserted into the right ventricle (RV) and direct RV pressure (RVSP) was recorded by MP150 Data Acquisition System (Biopac, USA).

### Wire-myography

Following RVSP measurement, the heart and lungs were removed and transferred to cold Krebs–Henseleit solution (in g: NaCl 6.9, KCl 0.35, $CaCl^2$ 0.29, $MgCl^2$ 0.24, KH2PO4 0.16, NaHCO3 2.1, and D-glucose 2; dissolved in 1 L double-distilled water). Extralobar pulmonary artery (left branch) was dissected and mounted in a myograph (DMT Model610, Aarhus, Denmark). Vessels were equilibrated and pretensioned as previously described (21). To assess viability, a contraction with Krebs–Henseleit solution containing 60 mM KCl was performed. After a wash-out of the bath and resting period of 15 min, which is a routine between constriction and relaxation periods, pulmonary arteries were contracted by serotonin (5-HT) (Sigma-Aldrich, St. Louis, USA; at concentrations between $1x10^{-9}$ and $1x10^{-4}$ M). Vessel relaxations toward sodium nitroprusside (SNP) (Nipruss, Schwarz Pharma AG, Germany; at concentrations between $1x10^{-9}$ and $1x10^{-4}$ M) and acetylcholine (ACh) (Sigma-Aldrich, St. Louis, USA; at concentrations between $1x10^{-9}$ and $1x10^{-3}$ M) were tested after the constriction induced by 5-HT.

### Measurement of right ventricular hypertrophy

Right ventricular hypertrophy (RVH) was determined by measuring the RV wall separating from left ventricle and the septum. The ratio of RV to heart weight (RV/HW) was calculated to assess RVH (24).

### Measurement of MMPs and TIMPs

The lungs and pulmonary arteries were washed with a phosphate-buffered saline (PBS) solution, pH 7.4. They were suspended 1:20 (w:v) in cold buffer (50 mM Tris-base, 0.15 mM NaCl, 10 mM $CaCl^2$, 0.05% Brij35) and homogenized. The homogenates were centrifuged at 10,000 xg for 10 min at 4°C. The supernatants

were removed and used for determination of MMPs and TIMPs. Therefore, ELISAs, activity assay kits and the gelatin zymography technique (SDS-PAGE) were performed.

### Gelatin zymography

Both active- and pro-forms of MMP-2 and MMP-9 were measured using gelatin zymography technique described in previous studies (19, 25). The gels, containing 7.5% polyacrylamide, 0.1% type I gelatin and 10% SDS, were copolymerized to measure MMP activities. MMP-2 and MMP-9 standards (Enzo Life Sci., USA) containing both pro- and active-forms of MMP-2 and MMP-9 were also applied in separate wells so that 5 ng enzyme was present in each. The electrophoresis was performed for 3 h, at 4°C with a constant voltage of 80 V. After electrophoresis, the gels were rinsed twice with 2.5% Triton X-100 for 30 min and incubated in 1 M Tris-HCl (pH 7.6), 50 mM NaCl and 13 mM $CaCl^2$ overnight at 37°C. The following day, the gels were stained for 2 h with staining solution (0.1% Coomassie brilliant blue, 40% methanol and 10% acetic acid) and subsequently incubated in destaining solution (40% methanol and 10% acetic acid) for 0.5–2 h. A special software (Totallab 1D, UK) was used to determine the relative enzymatic activity in Arbitrary Units per ng protein (AU).

### ELISAs and activity assays

The total amounts of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, and TIMP-2 in pulmonary tissue homogenates were measured using ELISA techniques according to the manufacturer's instructions (Cusabio, China). The active-forms of MMP-2 and MMP-9 were determined by activity assay kits according to the manufacturer's instructions (Amersham Biotrak, GE Healthcare Life Sci., PA, USA).

### Light microscopic analysis

Pulmonary artery tissue samples were fixed in a 10% buffered formalin solution for 48 h and embedded in paraffin, cut into 5-µm sections and stained with haematoxylin and eosin. An Olympus BH-2 (Olympus Corp., Japan) microscope was used to examine all tissue sections.

### Statistical analysis

In vitro vessel responses were analyzed by two-way ANOVA followed by Tukey's multiple comparison tests using GraphPad Prism version 5.0 software (GraphPad Inc., USA). The results of the gelatin zymography were evaluated with the Kruskal–Wallis test using the SigmaStat 3.5 programme (Systat Software, USA). Other statistical analyses were performed with one-way ANOVA followed by Tukey's HSD test with the SPSS 15.0 software package (IBM SPSS Statistics, USA). RVSP and RV/HW ratios are expressed as the mean±standard deviation. In vitro vessel responses are represented as contraction % or relaxation % and standard error. A p value of <0.05 was considered statistically significant.

## Results

### Assessment of PH

RVSP was increased in the MCT group compared with the controls (p<0.05). EVE or ALE therapy alone failed to attenuate MCT-induced increase in RVSP. SLD and SLD+ALE therapy attenuated PH, and a further reduction in RVSP was observed in SLD+EVE treatment group (Fig. 1).

RV weight was increased in MCT group when compared with control group (p<0.05). Similarly, increased RV weight was obtained in ALE and EVE groups whereas SLD, SLD+ALE and SLD+EVE therapy decreased RV hypertrophy when compared with MCT group (p<0.05). A further reduction was observed in RV hypertrophy in SLD+ALE and SLD+EVE groups with respect to SLD group (p<0.05) (Fig. 2).

### Results of wire-myography

### Contractile responses to 5-HT

Contractile responses were significantly increased in the MCT group (Fig. 2). At the $10^{-4}$ M concentration of 5-HT, increased constriction responses were observed in SLD, ALE and

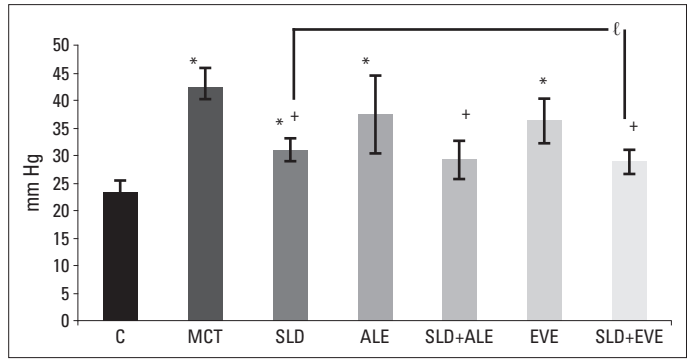


**Figure 1.** Assessment of right ventricular pressure in anaesthetised rats (n:8). *Significant difference between C group (*P*<0.05); +Significant difference between MCT group (*P*<0.05); ℓSignificant difference between SLD group (*P*<0.05). One-way ANOVA posthoc Tukey test

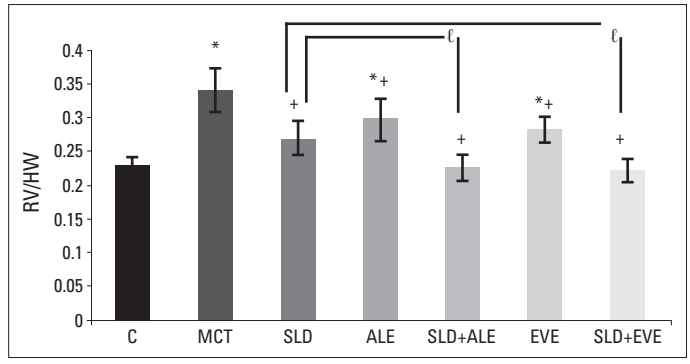


**Figure 2.** Assessment of right ventricular hypertrophy in anaesthetised rats (n:8). RV/HW - Right ventricle/Heart weight ratio. *Significant difference between C group (*P*<0.05); +Significant difference between MCT group (*P*<0.05); ℓSignificant difference between SLD group (*P*<0.05). One-way ANOVA posthoc Tukey test

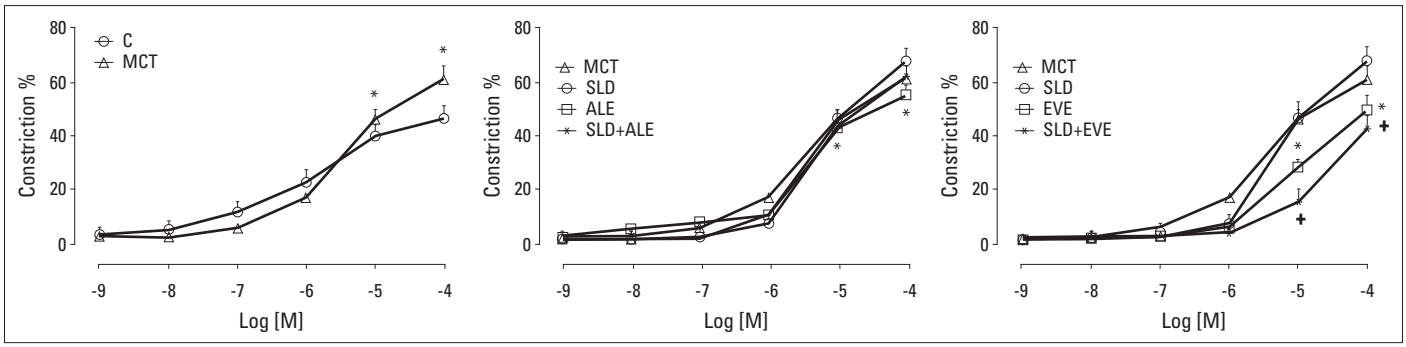


**Figure 3.** Assessment of 5-HT contraction responses in pulmonary arteries isolated from rats (n:8). *Significant difference between C group (*P*<0.05); +Significant difference between MCT group (*P*<0.05). Two-way ANOVA posthoc Tukey test

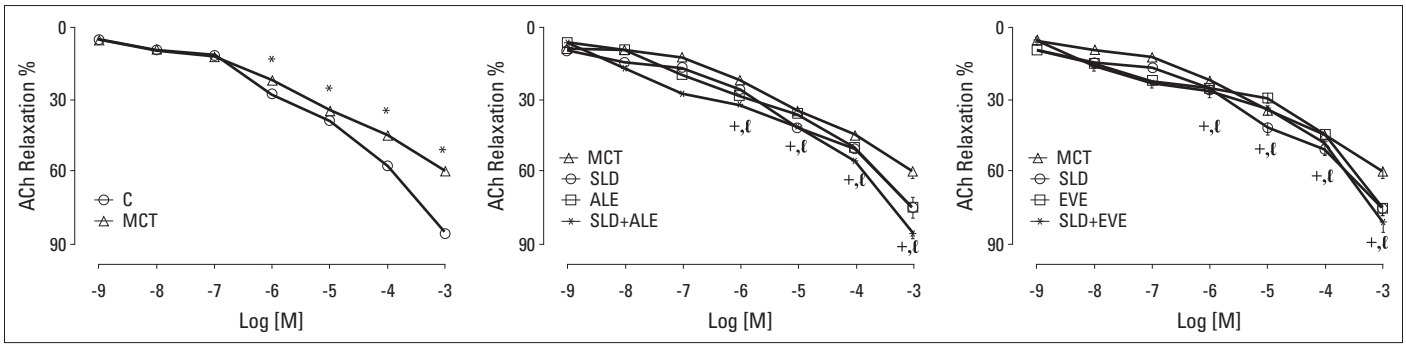


**Figure 4.** Assessment of endothelium-dependent (AcH) relaxation responses against 5-HT contractions in pulmonary arteries isolated from rats (n:8). *Significant difference between C group (*P*<0.05); +Significant difference between MCT group (*P*<0.05); ℓSignificant difference between SLD group (*P*<0.05). Two-way ANOVA posthoc Tukey test

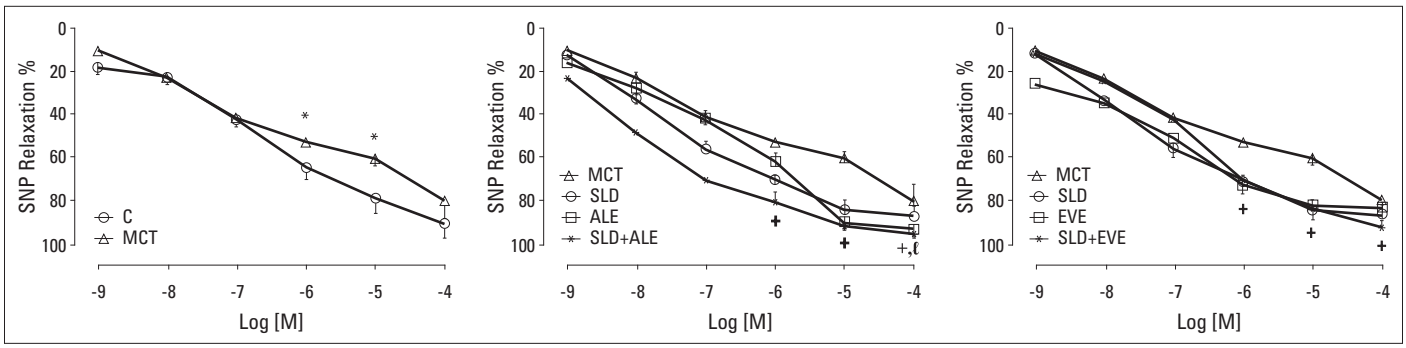


**Figure 5.** Assessment of endothelium-independent (SNP) relaxation responses against 5-HT contractions in pulmonary arteries isolated from rats (n:8). *Significant difference between C group (*P*<0.05); +Significant difference between MCT group (*P*<0.05); ℓSignificant difference between SLD group (*P*<0.05). Two-way ANOVA posthoc Tukey test

EVE groups compared with the control group (p<0.05). However, the constriction responses to $10^{-5}$ and $10^{-4}$ M concentrations of 5-HT for the EVE and SLD+EVE groups were significantly decreased relative to the MCT group (Fig. 3).

### Endothelium-dependent relaxations against 5-HT constriction

For the highest concentrations of ACh ($10^{-6}$ to $10^{-3}$ M), significantly decreased relaxation responses were observed in the MCT group compared with those in the control group. For the similar ACh concentrations in the treatment groups, significantly increased relaxation responses were recorded. In particular, in the SLD+EVE and SLD+ALE groups, the relaxation responses were not different from that of the control group (Fig. 4).

### Endothelium-independent relaxations against 5-HT constriction

In the MCT group, significantly decreased relaxation responses were obtained at high concentrations ($10^{-6}$ to $10^{-4}$ M) of SNP compared with those in the control group. In the treatment groups, increased relaxation responses were observed compared with the MCT group at high SNP concentrations (p<0.05). In particular, at the $10^{-4}$ M SNP concentration, increased relaxation responses were observed in the SLD+ALE group (p<0.05) (Fig. 5).

**Table 1. Distribution of MMP and TIMP related data among groups (ng mL$^{-1}$)**

| MMP, ng mL$^{-1}$ | C | MCT | SLD | ALE | SLD+ALE | EVE | SLD+EVE |
|---|---|---|---|---|---|---|---|
| MMP-1 | 15.50±0.30 | 14.90±1.10 | 13.80±2.10 | 13.70±2.10 | 12.50±2.70 | 14.30±2.10 | 13.90±2.20 |
| MMP-2, total | 5.40±0.80 | 20.20±2.80* | 6.10±0.60 | 9.50±0.70*,+ | 4.80±0.80+,ℓ | 10.80±2.40*,+ | 4.76±0.78+,ℓ |
| MMP-3 | 76.70±13.60 | 41.10±9.90* | 59.70±11.40 | 47.90±7.30* | 93.30±17.50+,ℓ | 63.40±12.20 | 129.90±26.20*,+,ℓ |
| MMP-9, total | 3.6±0.7 | 5.90±0.90* | 3.80±0.90+ | 5.10±0.90* | 3.30±0.70+ | 5.10±0.90* | 3.01±0.60+,ℓ |
| TIMP-1 | 8.60±1.10 | 12.60±0.20* | 10.30±1.60+ | 11.10±1.70* | 9.50±1.10+ | 11.30±1.40* | 9.30±1.20+ |
| TIMP-2 | 0.15±0.04 | 0.39±0.09* | 0.2±0.03+ | 0.23±0.06*,+ | 0.19±0.01+ | 0.25±0.02*,+ | 0.19±0.05+ |
| Active MMP-2 | 1.36±0.21 | 4.68±0.79* | 1.28±0.12+ | 1.85±0.17+,ℓ | 1.00±0.08+ | 2.06±0.40*,+,ℓ | 0.77±0.12+,ℓ |
| Active MMP-9 | 1.26±0.2 | 3.32±0.76* | 2.09±0.3+ | 2.99±0.69* | 1.74±0.33+ | 2.43±0.52*,+ | 1.33±0.31+,ℓ |
| MMP9/TIMP1 | 0.38±0.05 | 0.47±0.05* | 0.39±0.05+ | 0.48±0.11* | 0.33±0.04+,ℓ | 0.47±0.11* | 0.31±0.07*,+,ℓ |
| MMP2/TIMP2 | 37.72±7.82 | 54.35±12.49* | 31.43±4.83+ | 38.34±5.95+ | 25.12±3.93+,ℓ | 43.17±7.43 | 25.23±6.22+,ℓ |

ALE - alagebrium administered group; C - control group; EVE - everolimus administered group; MCT - Group received monocrotaline-injection i.p.; MMP - matrix metalloproteinase; SLD - sildenafil administered group; SLD+ALE - sildenafil and alagebrium combination administered group; SLD+EVE - sildenafil and everolimus combination administered group; TIMP - tissue inhibitors of matrix metalloproteinases. One-way ANOVA posthoc Tukey test. *Significant difference between C group ($P$<0.05); +Significant difference between MCT group ($P$<0.05); ℓSignificant difference between SLD group ($P$<0.05)

**Table 2. Gelatin zymography results, AU/50 ng protein**

| Groups | MMP levels | | | |
|---|---|---|---|---|
| | Pro MMP-9 | Active MMP-9 | Pro MMP-2 | Active MMP-2 |
| C | 199.81±34.0 | 251.82±19.60 | 292.83±34.77 | 218.36±28.70 |
| MCT | 491.22±69.04* | 817.02±27.05* | 1040.09±60.78* | 1519.29±366.10* |
| SLD | 291.50±34.73+ | 365.51±31.27+ | 547.50±48.84+ | 439.45±57.97+ |
| ALE | 362.98±114.50*,+ | 558.10±49.27*,+ | 564.37±95.70*,+ | 552.80±158.30*,+ |
| SLD+ALE | 263.34±70.40+ | 318.38±24.10+ | 431.17±70.60+,ℓ | 333.77±65.30+,ℓ |
| EVE | 384.63±53.80*,+ | 544.26±21.20*,+ | 532.73±68.20*,+ | 594.06±120.10*,+ |
| SLD+EVE | 242.90±12.98+,ℓ | 283.18±27.10+,ℓ | 425.83±30.20+,ℓ | 313.17±49.60+,ℓ |

AU - arbitary units; ALE - alagebrium administered group; C - control group; EVE - everolimus administered group; MCT - group received monocrotaline-injection i.p.; MMP - matrix metalloproteinase; SLD - sildenafil administered group; SLD+ALE - sildenafil and alagebrium combination administered group; SLD+EVE - sildenafil and everolimus combination administered group; TIMP - tissue inhibitors of matrix metalloproteinases. Kruskal-Wallis posthoc Dunn's test. *Significant difference between C group ($P$<0.05); +Significant difference between MCT group ($P$<0.05); ℓSignificant difference between SLD group ($P$<0.05)

### MMP levels in tissue homogenates

#### *MMP-1*

The MMP-1 levels in pulmonary tissue homogenates did not show any significant difference among groups (Table 1).

#### *MMP-2*

The total MMP-2 levels (pro- and active- forms) in the pulmonary tissues of the MCT group were significantly increased. Furthermore, the MMP-2 levels in all treatment groups were decreased compared with the MCT group (p<0.05) (Table 1). Similarly, the gelatin zymography results (Fig. 6) indicated that the pro-MMP-2 levels were significantly increased in MCT-induced pulmonary hypertensive rats and were decreased in the treatment groups (Table 2). Remarkably, the MMP-2 activity was significantly decreased in the SLD+ALE and SLD+EVE groups compared with the SLD group and also these results were similar to gelatin zymography results (Table 1, 2).

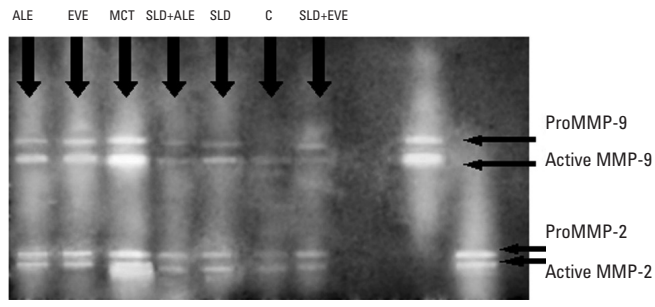


**Figure 6.** Gelatin zymogram showing MMPs

#### *MMP-3*

The MMP-3 levels in the pulmonary tissue were decreased in the MCT group compared with the control group (p<0.05). In the SLD+ALE and SLD+EVE groups, higher levels were observed than in the MCT group (p<0.05) (Table 1).

***MMP-9***

The total MMP-9 levels in the tissues were increased in the MCT group compared with the control group ($p<0.05$). In the SLD+EVE group, the total MMP-9 amounts were lower than in the SLD group (Table 1). According to the gelatin zymography results, the pro-MMP-9 levels were increased in the MCT group and were decreased in all treatment groups ($p<0.05$) (Fig. 6) (Table 2). In the SLD, SLD+ALE, EVE and SLD+EVE groups, the MMP-9 activity was decreased ($p<0.05$) (Table 1). The gelatin zymography results also indicated similar results with activity assays. Decreased active-MMP-9 levels were observed in the SLD+EVE group compared with the SLD group ($p<0.05$) (Table 2).

**TIMP-1 and TIMP-2 levels**

The TIMP-1 levels were increased in the MCT group ($p<0.05$). The levels of TIMP-1 were decreased in the SLD, SLD+ALE and SLD+EVE groups compared with the MCT group ($p<0.05$) (Table 1). The TIMP-2 levels were increased in the MCT group compared with the control group ($p<0.05$). The levels of TIMP-2 were significantly decreased in all treatment groups compared with the MCT group and also decreased to near-control values in the SLD+ALE and SLD+EVE groups (Table 1).

**Ratios of MMP-2 to TIMP-2 and MMP-9 to TIMP-1**

We also calculated MMP-2:TIMP-2 and MMP-9:TIMP-1 ratios to show vascular structural remodeling according to the previous studies (26, 27). When the MMP2/TIMP2 and MMP9/TIMP1 ratios were compared among groups, a significantly increased value was observed in the MCT group compared with the control group. This ratio was decreased in the SLD, ALE, SLD+ALE and SLD+EVE groups compared with the MCT group ($p<0.05$). In the SLD+ALE and SLD+EVE groups, decreased values were observed compared with the SLD group ($p<0.05$) (Table 1).

**Light microscopic analysis**

**Light microscopic analysis of the pulmonary artery**

The endothelial, tunica medial, and tunica adventitial layers appeared to be normal. Subendothelial and endothelial disintegrations were present in the MCT group. In addition, hypertrophy and vacuolisation were observed in the smooth muscle cells of the pulmonary arteries of the MCT group. In the SLD group, the levels of hypertrophy in the intimal and medial layers were reduced compared with the MCT group, whereas disintegration and medial vacuolisation were present. In the adventitial layer, hypertrophy was present. In the ALE group, intraparenchymal arteriolar structures and the parenchymal relationship were disintegrated. The pulmonary endothelium was intact and medial hypertrophy was reduced, but minimal hypertrophy and adventitial inflammation were present. In the pulmonary artery samples of the EVE group, adventitial and medial hypertrophy and also subintimal muscularisation were observed. In the SLD+ALE group, the vascular structure, thought to be protected by the treatment, was observed to be very close to the normal structure except for minimal medial hypertrophy and adventitial inflammation. The intimal and subintimal layers exhibited regular aspects. The structure of the pulmonary arteries of the SLD+EVE group was normal; there was no disintegration of the endothelial cells, and the tunica media and tunica adventitial layers were of normal aspect (Fig. 7).

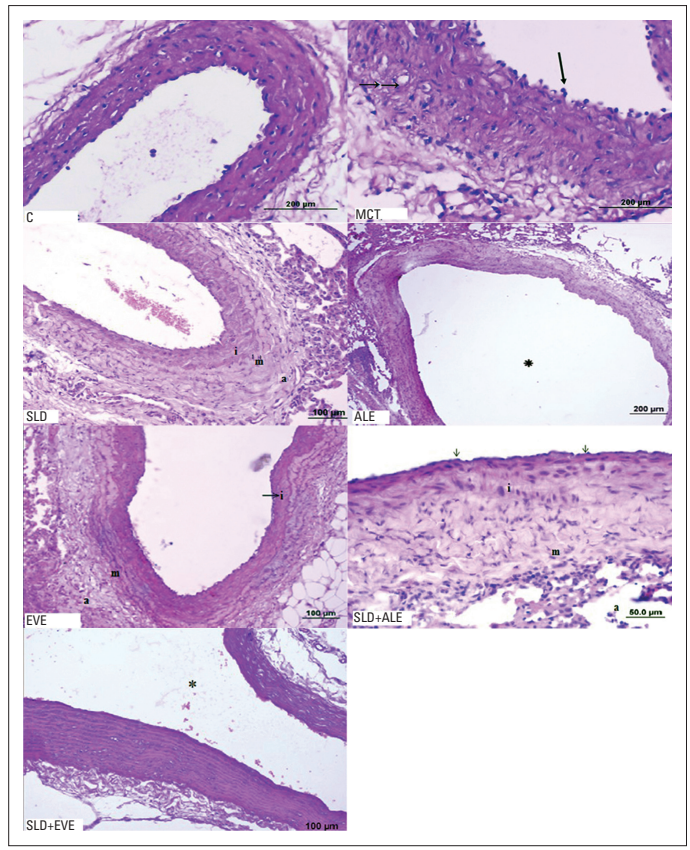


**Figure 7.** Assessment of pulmonary artery light microscopy analysis (n:8)
ALE - alagebrium administered group; C - control group; EVE - everolimus administered group; MCT - group received monocrotaline-injection i.p.; MMP - matrix metalloproteinase; SLD - sildenafil administered group; SLD+ALE - sildenafil and alagebrium combination administered group; SLD+EVE - sildenafil and everolimus combination administered group; TIMP - tissue inhibitors of matrix metalloproteinases. → - endothelium (disintegration in MCT group; subintimal muscularisation in EVE group; regular aspect in SLD+ALE group); →→ - vacuolisation (in MCT group); i - intimal layer; m - medial layer; a - adventitial layer; *: lumen

## Discussion

In this study, the increase in RVSP in MCT-treated rats, which is suggestive of PH, was the result of impaired pulmonary vascular endothelial homeostasis because of the MCT-induced damage. The imbalance between the vasoactive and mitogenic/antimitogenic mediators released from the endothelial layer of the pulmonary arteries resulted in the changes in vascular tone and development of vascular remodeling (28). The RVSP was found to be decreased in the SLD-treated group compared with the MCT group. NO-mediated effect of sildenafil on the pulmonary vasculature also resulted in decreased RVSP against MCT-induced PH

rat model in previous studies (4, 21, 22). RVSP was also significantly decreased in the SLD+EVE-treated group compared with the untreated MCT group and the SLD group. A previous study demonstrated that the effects of everolimus include a reduction in the hypertrophic processes (29). As a result, it was concluded that everolimus alone is not effective in reducing pulmonary artery pressure. However, when used in combination with sildenafil, this drug may have beneficial effects on pulmonary artery pressure because of its anti-inflammatory and anti-proliferative effects. Unlike other immunosuppressants, this drug does not cause any cardiovascular toxicity. In the SLD+ALE group, RVSP was reduced to the control values, supporting the therapeutic effect of the SLD+ALE combination. The regenerative effects of alagebrium (30) on the remodeling of the medial smooth muscle layer and connective tissue components may explain this effect. RVH is seen in the later stages of PH because of an adaptive mechanism induced by the increased pulmonary vascular resistance. In our study, the RV/HW ratio was significantly increased in the MCT group compared with that in the control group. Numerous previous studies have reported that RVH is induced by MCT (13, 18, 21). On the other hand, RVH was significantly reduced in the SLD group compared with the MCT group, which was also demonstrated by other studies (4, 13). Histological sections prepared from our pulmonary artery samples of the SLD group showed reduced hypertrophy and structural improvements compared with those of the MCT group. In the present study, RVH was significantly reduced in the SLD+EVE group compared with the MCT group and even with SLD-only group. In the previous studies of everolimus and also in our preliminary study, the authors reported reduced RVH in rats with MCT-induced PH (21, 31). The significantly decreased RV/HW in the SLD+ALE group compared that in both the MCT and SLD groups may be explained by the fact that alagebrium breaks the cross-links formed in long-lived proteins such as collagen and elastin during the pathological remodeling process. Cross-links in these proteins have a major role in the development of hypertrophy. The effects of alagebrium in improving diastolic functions directed to the cardiac muscle mass (32) are consistent with these findings.

The 5-HT-induced contractile responses of the isolated pulmonary arteries were increased in the MCT group compared with those in the controls. The expression of 5-HT1B receptors has been reported to increase in the MCT-induced experimental PH model (33). Furthermore, one study has shown associations between the increased serotonergic vascular responses and the decreased level of NOS, increased muscular tone and inactivation of intracellular $Ca^{2+}$ and $K^{+}$ channels in the pulmonary arteries (34). In our study, we observed decreased levels of serotonergic contraction in the treatment groups. In SLD+EVE and SLD+ALE groups, the contractile responses were similar to those of the control group suggesting that these agents decrease the serotonergic sensitivity in PH.

ACh-induced endothelium-dependent relaxation of the 5-HT-mediated contractions was significantly decreased in the MCT group compared with those in the control group, indicating that MCT caused endothelial damage. A previous study has also reported decreased relaxation responses to ACh in MCT-induced PH (35). The relaxation responses to the cumulative ACh concentrations in the treatment groups were found to be similar to the controls and higher than those in the MCT group. Furthermore, the relaxation responses were increased in the SLD+EVE and SLD+ALE groups compared with those in the SLD group. It is clear that addition of everolimus or alagebrium to the sildenafil treatment protected the structure of the endothelium and led to a further improvement in functional relaxation responses.

Endothelium-independent responses in the MCT-induced PH experimental model are generally evaluated by the responses to SNP. In the present study, the relaxation responses to SNP were significantly decreased in the MCT group compared with those in the control group. These decreased responses were attributed to the desensitization of soluble guanylate cyclase or decreased cGMP levels in the smooth muscle cells (35). The SNP-induced relaxation responses were increased in our treatment groups, particularly in the SLD+ALE group, suggesting that alagebrium may be beneficial because of the prevention of glycosylated cross-link formation in the medial muscle layer of the arteries, which is a major component of non-endothelial relaxation. It has been demonstrated that intima-media thickening is mediated by AGE and alagebrium reduces the arterial thickening by breaking the glycosylated cross-links and preventing the accumulation of AGEs (36). In conclusion, anti-remodeling and anti-inflammatory agents may provide functional improvements in the relaxation responses in this pathological condition.

There are a limited number of studies on the role of MMP upregulation in the pathogenesis of PH. It has been emphasized that gelatinase production may be related to the several possible mechanisms including the induction of cytokines and mechanical distribution of the forces (vascular tension and pressure changes). Pathogenetic studies on the correlation of intravascular pressure and tension changes with the cell proliferation in the pulmonary vascular tissue and increased protein synthesis in the connective tissue suggest that there may be potential therapeutic targets in these areas (37).

The present study found no alterations in MMP-1 activity in the comparisons among the groups. Previous studies have also noted that the activity of MMP-1 was not altered in other vascular pathologies (7, 9). However, in our study, significantly increased MMP-2 and MMP-9 levels and activities were induced by MCT. Structural remodeling induced by the growth factors in PH caused increased MMP-2 and MMP-9 activities by affecting cell proliferation and ECM turnover in previous studies (2, 18, 37). The levels and activities of MMP-2 and MMP-9 were significantly decreased in the SLD group compared with those in the MCT-treated group in our study. A previous study demonstrated downregulation of the MMPs by cGMP or NO donors (38). Sildenafil treatment has been suggested to improve the hemodynamic parameters by increasing the bioavailability of NO

in the cell as well as causing an NO-induced decrease in the MMP-2 and MMP-9 activities (39). As a novel pathway, it was also suggested that sildenafil enhances Akt activation, therefore decreases inflammatory cell filtration and edema leading to a possible overcome of pathologic remodeling process, independent of its vasodilatory effect (4). It is possible that the hemodynamic and structural improvements induced by the sildenafil treatment in our study may be caused by the decreased MMP-2 and MMP-9 levels and activities resulting from the increased NO levels secondary to the inhibition of the PDE-5 enzyme.

In SLD+EVE group, the MMP-2 and MMP-9 levels and activities were significantly decreased compared with those in the SLD group. It is known that inflammatory cells are a major source of MMPs and release reactive oxygen species and cytokines that affect the vascular cells. Some observations suggest a key role for the regulation of MMP expression in the inflammatory processes in the vascular bed (40). In a study of coronary stenosis, rapamycin treatment resulted in reduced plaque progression by inhibiting MMP-2 and MMP-9 activities. In addition, everolimus has been found to reduce the intimal thickness and prevent the formation of the neointima in coronary arteries (41). It has also been demonstrated that MMP-2 and MMP-9 expression can be regulated by mTOR-dependent signaling (42). At this point, the MMP-2 and MMP-9 levels and activities, which were found to be nearly similar in the control and SLD+EVE groups in our study, were considered to mediate the anti-proliferative effects of everolimus. The inhibition of the increase in the MMP-2 and MMP-9 activities in mTOR-mediated proliferative processes may account for the therapeutic benefit of everolimus in PH. In addition, regardless of the mTOR inhibitory effects, the significant decrease in the MMP-9 activity and level in the SLD+EVE group suggests a possible role for MMP-9 in the inflammatory processes and in the mechanism of action of everolimus. In the SLD+ALE group, the MMP-2 and MMP-9 activities and levels were decreased compared with those in the SLD group. Release of excessive amounts of inflammatory cytokines and MMPs is associated with increased numbers of glycation cross-links (30). Our results may suggest that the breaking of the cross-links by alagebrium causes a decrease in the matrix production in the vascular medial layer and an improvement in the remodeling parameters induced by the ongoing stimulation in this pathological condition.

In the MCT-induced PH model, increased pressure and shear stress lead to a significantly thickened pulmonary artery wall and increased resistance. At this point, our results showing significant decrease of MMP-3 in MCT group, are consistent with previous studies that indicated a significantly increased level of MMP-3 in aneurysms induced by an excessive decrease in vascular tissue resistance and a significantly decreased level of MMP-3 in vascular tissues with no aneurysms (7). It has been demonstrated that collagen and fibronectin accumulation increases with the decreased MMP-3 activity in pathological conditions that are characterized by increased vascular pressure that occurs in the remodeling process in the MCT-induced experimental PH model (43). In addition, MMP-3 has been shown to be a precursor activator for MMP-9 in previous studies. However, the fact that MMP-9 was significantly increased in our study suggests that this activator activity may not be a requirement (44). The significantly increased MMP-3 activity in the treatment groups also suggests that the treatment may have a protective effect in cardiovascular pathologies. The addition of everolimus to the treatment led to a significantly increased MMP-3 activity compared with the other groups, suggesting that this enzyme may play a crucial role in inflammatory processes. In our study, the zymographic measurements demonstrated that the pro- and active-forms of MMP-2 and MMP-9 were significantly increased in PH, and the combination therapies that produced improvements in the PH decreased the activity of these enzymes. These results also suggest that the ELISA and activity measurement methods are reliable and correlate with the results obtained from conventional standard gelatin zymography method.

In the present study, the TIMP-1 and TIMP-2 levels were significantly increased in the MCT group compared with those in the control group. Another study has also demonstrated significant increases in TIMP-1 and TIMP-2 levels in hypoxia- or MCT-induced experimental PH models (45). It has been suggested that there is an imbalance between the MMPs and TIMPs in the pulmonary vascular remodeling process in patients with IPAH (9). Maintenance of TIMP/MMP balance is thought to be preventive against cardiovascular disorders via their crucial role in ECM remodeling (8, 46). It has also been hypothesized that the increased TIMP levels may be an adaptive response to the increased MMP-2 levels (4, 47). It is also possible that the significant decrease in the TIMP-1 and TIMP-2 levels in the SLD+EVE and SLD+ALE groups represent an adaptive response.

There are very limited studies of the MMP enzyme family, which plays major roles in connective tissue remodeling of vascular bed, in the pathogenesis and during standard treatment strategies in PH. In addition, the inflammatory process, which begins as a result of the increased pressure in the vascular bed, and structural changes of connective tissue components are the targets for treatment strategies for PH. Further, our findings demonstrating that the anti-remodeling effects of everolimus and alagebrium involved the MMPs, indicated that in addition to vasodilator therapy, MMP-targeted treatment strategies will inhibit mortality and morbidity by revitalizing vascular bed hemodynamically.

Histopathological examination of pulmonary arteries from the MCT group showed endothelial injury, degeneration of the internal elastic membrane, subintimal muscularisation, marked hypertrophy of the medial layer and associated adventitial hypertrophy and inflammation. In the SLD group, the beneficial effects of the NO-cGMP-mediated decrease in pressure during the remodeling process caused a structural improvement in the intimal layer. However, a complete regression was not observed in the medial layer and adventitia of the pulmonary arteries. The histological sections showed significant improvements in the medial layer of the alagebrium-treated and in the adventi-

tia of the everolimus-treated groups compared with those in the MCT group. It was observed that the matrix accumulation was decreased in all endothelial, subendothelial and medial layers and irregular laminae disappeared in the SLD+ALE and SLD+EVE groups. The adventitial inflammation was significantly improved, particularly in the SLD+EVE group.

## Study limitations

The limitation of our study was that non-invasive pressure monitoring was not present because of the limited application of Doppler technique in rats. In addition, telemetry monitoring may also be preferred for a 24-h monitoring of cardiovascular events related to PH. A comparison between the right and left ventricular function could also give additional information regarding the effects of everolimus or alagebrium.

## Conclusion

The results of the present study suggest that addition of everolimus and alagebrium to the vasodilator therapy may limit the tissue changes in PH. The use of alagebrium to break protein-glucose cross-links and induce anti-hypertrophic effects may significantly improve remodeling process and hemodynamic cardiovascular parameters as well as the morbidity and mortality rates. The addition of everolimus suppressed the inflammatory responses induced in response to the pressure increases in the vascular bed. It is likely that the changes of MMPs and their regulation may have beneficial therapeutic effects in PH. Furthermore, the addition of the anti-remodeling agents such as everolimus and alagebrium to the standard therapy produced significant changes in MMPs in our study. In conclusion, treatment strategies targeting these enzymes in cardiovascular pathologies may be useful in developing effective anti-remodeling agents and may limit the progression of the disease significantly.

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