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*Original Article*

# Sildenafil and metformin combination attenuates monocrotaline-induced pulmonary hypertension via TNF-α and ERK1/2 modulation

[Combinación de sildenafil y metformina atenúa la hipertensión pulmonar inducida por monocrotalina mediante la modulación de TNF-α y ERK1/2]

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**Abstract**

*Context*: Pediatric pulmonary hypertension (PH) is associated with poor outcomes and limited US-approved pharmacotherapies.

*Aims*: To evaluate the effects of sildenafil, metformin, and their combination on hemodynamics, inflammatory mediators, and vascular remodeling in monocrotaline (MCT)-induced PH.

*Methods*: Male Wistar rats received MCT (60 mg/kg, s.c.). Beginning 24 h later, animals were assigned to MCT-control, sildenafil (5 mg/kg), metformin (100 mg/kg), or combined therapy for 21 days. On day 22, right-heart catheterization was performed. Serum TNF-α and ERK1/2 were measured by ELISA, and pulmonary arterial remodeling was assessed by HE and α-SMA staining with intima-media thickness measurements.

*Results*: Relative to MCT-control, all treatments lowered mPAP; the combination group exhibited the most considerable reductions in TNF-α and ERK1/2, and a partial reduction in intima-media thickness.

*Conclusions*: Sildenafil and metformin, particularly in combination, improved pulmonary hemodynamics and attenuated inflammation in MCT-PH. Confirmation in lung tissue (e.g., p-ERK1/2) and cell-specific analyses is warranted.

*Keywords*: extracellular signal-regulated kinase; metformin; monocrotaline; pulmonary hypertension; sildenafil; tumor necrosis factor-α.

**Resumen**

*Contexto*: La hipertensión pulmonar (HP) pediátrica se asocia con malos resultados y farmacoterapias limitadas aprobadas en EE. UU.

*Objetivos*: Evaluar los efectos del sildenafilo, la metformina y su combinación sobre la hemodinámica, los mediadores inflamatorios y la remodelación vascular en la HP inducida por monocrotalina (MCT).

*Métodos:* Ratas Wistar machos recibieron MCT (60 mg/kg, s.c.). Veinticuatro horas después, los animales fueron asignados a un grupo control con MCT, a un grupo con sildenafilo (5 mg/kg), a un grupo con metformina (100 mg/kg) o a un grupo con terapia combinada durante 21 días. El día 22, se realizó un cateterismo cardíaco derecho. Se midieron los niveles séricos de TNF-α y ERK1/2 mediante ELISA, y la remodelación arterial pulmonar se evaluó mediante tinción de HE y α-SMA con mediciones del espesor de la íntima-media.

*Resultados*: En comparación con el grupo control con MCT, todos los tratamientos redujeron la PAPm; El grupo de combinación mostró las reducciones más considerables de TNF-α y ERK1/2, así como una reducción parcial del grosor íntima-media.

*Conclusiones*: El sildenafil y la metformina, especialmente en combinación, mejoraron la hemodinámica pulmonar y atenuaron la inflamación en pacientes con HP-MCT. Se justifica la confirmación en tejido pulmonar (p. ej., p-ERK1/2) y en análisis celulares específicos.

*Palabras Clave*: factor de necrosis tumoral-α; hipertensión pulmonar; metformina; monocrotalina; quinasa regulada por señales extracelulares; sildenafil.

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**Abbreviations:** α-SMA: Alpha-smooth muscle actin; BPD: Bronchopulmonary dysplasia; BW: Body weight; CDH: Congenital diaphragmatic hernia; DSCF: Dwass-Steel-Critchlow-Fligner; dPAP: Diastolic pulmonary arterial pressure; ERK1/2: Extracellular signal-regulated kinase 1/2; ET-1: Endothelin-1; FDA: Food and Drug Administration; FFPE: Formalin-fixed, paraffin-embedded blocks; HE: Hematoxylin-eosin; HUVE: Human umbilical vein endothelial; IL-6: Interleukin-6; IMT: Intima–media thickness; MCT: Monocrotaline; mPAP: Mean pulmonary arterial pressure; MAPK: Mitogen-activated protein kinase; PA: Pulmonary artery; PAH: Pulmonary arterial hypertension; PASMC: Pulmonary artery smooth muscle cell; PDGF: Platelet-derived growth factor; p-ERK1/2: Phosphorylated extracellular signal-regulated kinase 1/2; PH: Pulmonary hypertension; RHC: Right heart catheterization; RV: Right ventricle; RVSP: Right ventricular systolic pressure; sPAP: Systolic pulmonary arterial pressure; $SpO_2$: Peripheral oxygen saturation; TNF-α: Tumor necrosis factor-alpha; VEGF: Vascular endothelial growth factor.

## INTRODUCTION

Pulmonary hypertension (PH) is a progressive disorder with high morbidity and mortality. It is characterized by a mean pulmonary arterial pressure (mPAP) >20 mmHg, measured at rest by right heart catheterization (Humbert et al., 2023). An epidemiological study found that pulmonary arterial hypertension (PAH) occurs in 4 to 5 cases per million among children (Deng et al., 2025). One-third of pediatric patients are also reported to undergo transplantation or die within 10 years after diagnosis (Constantine et al., 2022).

Numerous drugs have been developed as a result of advancements in PH medical therapy. However, the Food and Drug Administration (FDA) authorizes only two drugs—bosentan and sildenafil (oral suspension LIQREV®; injection/oral Revatio®)—for pediatric patients with PH (Sullivan et al., 2023). Because bosentan access is limited in some settings and sildenafil alone may be insufficient in certain pediatric phenotypes, adjunctive strategies targeting inflammatory/proliferative signaling were explored (Cohen et al., 2019; Lilyasari et al., 2019). To improve the clinical condition and prognosis of patients with PH, a combination of two drugs is required (Durongpisitkul et al., 2021).

Metformin, an antidiabetic drug, has the potential to reverse PH pathobiology and lower endothelin-1 (ET-1) levels (Yoshida et al., 2020). Moreover, it can reduce proinflammatory cytokines (Ranchoux et al., 2019). Metformin is also reported to block ERK1/2-dependent B cell-activating factor (BAFF) activation (Chen et al., 2021). Sildenafil was shown to share similarities with metformin in suppressing proinflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-6 (Di Luigi et al., 2020; Zych et al., 2019). Sildenafil also inhibits pulmonary vascular remodeling by reducing p-ERK1/2 (Li et al., 2019).

It was hypothesized that sildenafil and metformin, alone or in combination, would reduce pulmonary arterial pressure and vascular remodeling by lowering TNF-α and ERK1/2 signaling. Therefore, this study seeks to analyze the effects of sildenafil and metformin on the progression of PH in a model focusing on TNF-α, ERK1/2, PASMCs proliferation, and intima-media thickness (IMT) of PA.

## MATERIAL AND METHODS

### Study design and setting

This study was authorized by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, through a letter of approval no. 2.KEH.161.10.2023. An experimental study was conducted from April 2024 to June 2024 at the Animal Laboratory of the same faculty using *Rattus norvegicus* strain Wistar as a model of PH. After inducing the model with monocrotaline (MCT), the rats were randomly categorized into four groups (control, sildenafil, metformin, and sildenafil + metformin). After 24 h, rats were treated for 21 days; on the 22$^{nd}$ day, right heart catheterization was performed, and the rats were sacrificed.

### Animal preparation and sampling

A healthy *Rattus norvegicus* Wistar strain was obtained from PT Riset AIRC, Surabaya, East Java, Indonesia. The minimum sample size calculation for this study was 9 per group, based on the Higgins-Kleinbaum formula with $\alpha = 0.05$, $\beta = 0.20$, SD = 0.535, and d = 0.77. To account for a 20% dropout rate, the animal requirement was 12 rats per group.

### Animal eligibility and randomization

The study included healthy, 12-week-old male Wistar rats weighing 117-193 g. Rats that died during the study were excluded from the analysis. After MCT injection, the samples were assigned to 4 groups: control, sildenafil, metformin, and sildenafil + metformin. Animals were randomized by computer-generated sequences; allocation was concealed. Outcome assessors were blinded (Townsend et al., 2025). The absence of a female cohort was a limitation of the study.

### Animal acclimatization and housing

The animals used in this study were acclimatized for 1 week prior to the intervention. They were housed in a room under standard conditions (24°C room temperature, 50–70% humidity), following a 12-h light/dark cycle, and were supplied with standard laboratory food and water; each rat was housed in a single cage. The single housing is a limitation of the study.

### Animal model and intervention

Pulmonary hypertension was induced by subcutaneous monocrotaline (60 mg/kg). (MedChemExpress, catalog number HY-N0750) (Yoshida et al., 2020). One molar hydrochloric acid was added to MCT, and the mixture was mixed with 1 M sodium hydroxide to achieve a pH of 7.4. After this procedure, the samples were randomly assigned to 4 groups (control, sildenafil, metformin, and metformin + sildenafil). Sildenafil (Revatio oral suspension, 10 mg/mL) was purchased from Pfizer, and metformin (Glucophage) was sourced from Merck. The doses used for sildenafil and metformin were 5 mg/kg body weight (BW) and 100 mg/kg BW, respectively. Treatments were administered once daily for 21 consecutive days, beginning 24 h after MCT; the administration route and dosing schedule are to be specified (oral gavage in a vehicle of bidestilled water, 5 mL/kg, at 09.00 AM; for the combination group, drugs were co-administered). On the 22$^{nd}$ day, pulmonary arterial pressure was evaluated via right heart catheterization.

Intramuscular anesthesia using ketamine (50 mg/kg BW) and xylazine (5 mg/kg BW) was administered before the catheterization procedure (Cahyono et al., 2025). The animals were positioned lying on their backs, and a surgical procedure was performed to expose the heart silhouette after shaving the fur. After heparin wash, an intravenous catheter (22G) was inserted into the fourth intercostal space on the right and directed to the right ventricle. Zeroing of the fluid-filled catheter was always performed before the catheterization procedure. Pulsatile dark red blood was expelled from the right ventricle, after which the catheter was connected to the transducer and Mac 51 cable. The pulmonary artery (PA) was reached by advancing the catheter approximately 5 mm and maneuvering it. The correct position of the catheter was confirmed by the waveform observed on the DASH 4000 monitor. The pressure readings for mPAP, diastolic pulmonary arterial pressure (dPAP), and systolic pulmonary arterial pressure (sPAP) were taken after the pressure was stable. Other clinical data, such as heart rate and peripheral oxygen saturation ($SpO_2$), were also recorded. After catheterization, blood was exsanguinated intracardially for approximately 3.5 mL. The aorta was cut to perform euthanasia under anesthesia, after which the lungs were harvested, placed in formalin, and embedded in paraffin. Before the lungs were harvested, they were inflated with 25 cm $H_2O$ pressure via tracheal instillation. A refrigerated centrifuge (Kubota K3520) was used to centrifuge blood at 4°C and 3,500 rpm for 10 minutes. Prior to ELISA analysis, the serum was stored in a refrigerator at -20°C.

### Histological analysis

Formalin-fixed, paraffin-embedded blocks (FFPE) of the right and left lungs were cut to a thickness of 5 µm. These sections were deparaffinized by washing them with xylene three times for 5 min each. The xylene was then removed with methanol, and the sample was washed in water for 1 min. After this procedure, the sample was stained with hematoxylin for 10 min and eosin for 6 min at room temperature. Small arteries (50–200 µm) were sampled by systematic random sampling (≥8 vessels/animal). The ratio of pulmonary artery smooth muscle cells was measured relative to the arterial area. Analyses were performed blinded. Additionally, four pulmonary arteries from each lung were randomly selected. For each artery, IMT was measured at four orthogonal quadrants per vessel. The samples were stained with an alpha-smooth muscle actin (α-SMA, GeneTex, catalog number GTX636885) antibody. After the procedure, cell-type quantification based solely on α-SMA was considered insufficient; additional markers would be needed to distinguish PASMCs from (myo)fibroblasts (Ju et al., 2024).

### ELISA assay

A rat TNF-α ELISA kit (Catalog number E0764Ra, Bioassay Technology Laboratory) was used to measure serum TNF-α concentrations. Meanwhile, a rat ERK1/2 ELISA kit (Catalog number E2815Mo, Bioassay Technology Laboratory) was used to measure serum ERK1/2 concentrations.

The ELISA assay began with the thawing of frozen serum samples. For TNF-α measurement, the plate was washed twice before the addition of the standard and sample. Each well containing 40 µL of standard or sample was then mixed with 40 µL of biotin-labeled antibody working solution. After shaking the plate for 1 minute, it was statically incubated at 37°C for 45 minutes, then washed three times and bathed for 1 min each. Each well was then added to 100 µL of HRP-Streptavidin Conjugate working solution. Subsequently, the plate was sealed and incubated statically at 37°C for 45 min, followed by five washes and a 1-min bath. The plate was then added to 90 µL of TMB substrate solution and sealed, then incubated at 37°C for 10 to 20 minutes. Finally, the absorbance was measured immediately at 450 nm after the addition of 50 µL of the stop solution. Standard curves were fit by 4-parameter logistic regression, and samples were run in duplicate. The procedure for measuring ERK1/2 mirrored the procedure for TNF-α.

### Statistical analysis

Data were expressed as mean ± SD, and normality of their distribution was assessed using the Shapiro-

Wilk test. A one-way ANOVA was employed to compare normally distributed data among groups, with Tukey's family-wise α control post hoc test for further pairwise comparisons. Non-parametric data were analyzed with the Kruskal-Wallis test, followed by the Dwass-Steel-Critchlow-Fligner pairwise comparisons. A $p<0.05$ was considered statistically significant, and all analyses were conducted using Jamovi 2.4.14.

## RESULTS

### Characteristics of the animal

A total of 7 rats died (3 in the control group, 2 in the sildenafil group, and one each in the metformin and sildenafil + metformin groups) out of 48 rats during the study, leaving 41 rats. The rats died due to pulmonary hemorrhage. The survival analysis is shown in the Kaplan-Meier curve (Fig. 1). The overall comparison was not significant (p = 0.593). Data collected from the experimental rats, including body weight, heart rate, sPAP, dPAP, mPAP, and $SpO_2$, from the four groups are presented in Table 1. For hemodynamics (sPAP, dPAP, mPAP), the effect size indicated a significant effect.

Body weight, heart rate, and $SpO_2$ were similar across the four groups. However, sPAP, dPAP, and mPAP were notably reduced in the treatment groups compared with the control group. Post hoc analysis revealed substantial differences in these parameters across the four groups. The systolic PAP of sildenafil was the lowest among the treatment groups, but it did not reach significance relative to the metformin (p = 0.427) and sildenafil + metformin (p = 0.491) groups. In this study, the diastolic PAP with sildenafil was also the lowest among the treatment groups, although it did not reach significance compared with the metformin (p = 0.963) and sildenafil + metformin (p = 0.865) groups. Moreover, mPAP was lower in all treatment groups versus MCT control (sildenafil: ΔmPAP [19.7 mmHg, p = 0.001; metformin: ΔmPAP [15.96 mmHg, p = 0.009; sildenafil + metformin: ΔmPAP [16.86 mmHg, p = 0.002). Differences among treatment groups were not statistically significant. The representative pulmonary arterial waveforms per group are shown on Fig. 2.

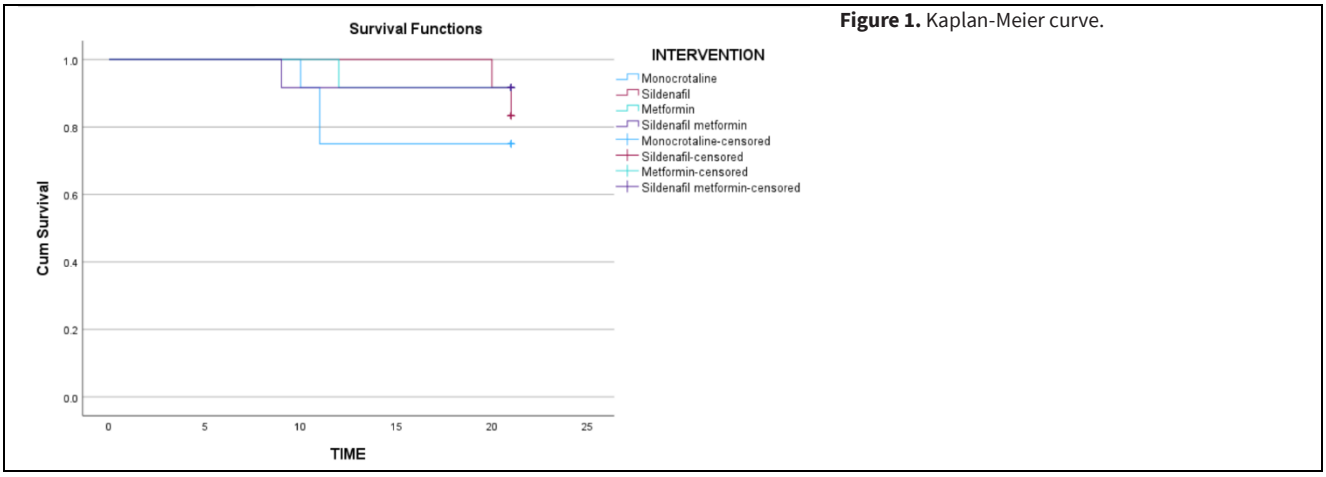


**Figure 1.** Kaplan-Meier curve.

**Table 1**. Clinical data of experimental rats.

| Variable | Control (n = 9) | Sildenafil (n = 10) | Metformin (n = 11) | Sildenafil + metformin (n = 11) | Shapiro-Wilk (p-value) | p-value | Effect size |
|---|---|---|---|---|---|---|---|
| Bodyweight (g) | 160 ± 10.9 | 156 ± 23.3 | 150 ± 22.2 | 169 ± 15.3 | 0.01 | 0.135* | 0.139[1] |
| Heart rate (x/minute) | 270 ± 18.9 | 255 ± 30.5 | 275 ± 23.2 | 271 ± 17.6 | 0.95 | 0.224† | 0.110[2] |
| sPAP (mmHg) | 42.2 ± 7.53 | 18.8 ± 4.05 | 26.9 ± 14.2 | 24.5 ± 11.5 | 0.0005 | 0.001* | 0.406[1] |
| dPAP (mmHg) | 21.6 ± 9.71 | 8.4 ± 3.95 | 9.27 ± 4.15 | 9.82 ± 4.96 | 0.35# | 0.004* | 0.329[1] |
| mPAP (mmHg) | 32.8 ± 6.96 | 13.1 ± 4.53[a] | 16.8 ± 8.54[b] | 15.9 ± 6.19[c] | 0.0003 | 0.0003* | 0.459[1] |
| $SpO_2$ (%) | 91 ± 6.1 | 93.8 ± 5.57 | 92.5 ± 3.8 | 96.1 ± 3.11 | 0.30 | 0.114† | 0.147[2] |

*Kruskal-Wallis, †ANOVA, #the varians was not homogen, [1]$\varepsilon^2$, [2]$\eta^2$, post hoc with DSCF: [a]$p$=0.001, [b]$p$=0.009, [c]$p$=0.002. Note: dPAP: diastolic pulmonary arterial pressure; mPAP: mean pulmonary arterial pressure; sPAP: systolic pulmonary arterial pressure; $SpO_2$: peripheral oxygen saturation.


**Figure 2.** Representative of pulmonary arterial wave per group.
**(A)** MCT control, **(B)** sildenafil, **(C)** metformin, **(D)** sildenafil + metformin.

**Table 2.** Histology and ELISA data.

| Variable | Control (n = 9) | Sildenafil (n = 10) | Metformin (n = 11) | Sildenafil + metformin (n = 11) | Shapiro-Wilk *p*-value | *p*-value | Effect size |
|---|---|---|---|---|---|---|---|
| IMT (μm) | 36.8 ± 10.7 | 46.6 ± 12 | 47.7 ± 14.5 | 27 ± 10.3 | 0.02 | 0.001* | 0.399[1] |
| PASMCs (%) | 53.9 ± 12.4 | 64.5 ± 15.2 | 42.7 ± 11 | 44.8 ± 6.37 | 0.28 | 0.0004† | 0.341[1] |
| TNF-α (pg/mL) | 218 ± 34.8 | 217 ± 25.1 | 235 ± 31.5 | 157 ± 29.7 | 0.01 | 0.0002* | 0.487[1] |
| ERK1/2 (ng/mL) | 45.9 ± 5.74 | 40 ± 5.3 | 39.7 ± 7.47 | 22.8 ± 6.22 | 0.03 | 0.00002* | 0.605[1] |

*Kruskal-Wallis, †ANOVA, [1]ε²

Note: ERK1/2: extracellular signal-regulated kinase 1/2; IMT: intima–media thickness; PASMC: pulmonary artery smooth muscle cell; TNF-α: tumor necrosis factor-alpha.

## Histology and ELISA data

Table 2 presents histology and ELISA data. Substantial variations were noted among IMT, PASMCs, fibroblasts, TNF-α, and ERK1/2 based on ANOVA and Kruskal-Wallis analysis. Post hoc analysis revealed that the IMT in the sildenafil + metformin group was thinner than in all other groups. For IMT, PASMC ratio, TNF-α, and ERK1/2, the effect sizes indicated large effects.

Meanwhile, the metformin group exhibited a thicker IMT than the control group. The combination of sildenafil and metformin was able to significantly decrease IMT thickness as compared to the sildenafil or metformin group and partially decreased IMT thickness as compared to the control group (p = 0.094) (Fig. 3). The metformin and sildenafil + metformin groups had a significantly lower PASMCs ratio than the sildenafil group (Fig. 4).

Both TNF-α and ERK1/2 levels were considerably lower in the sildenafil and metformin group than in the other groups (Figs. 5 and 6). Fig. 7 shows representative PA histology, with panels A-D depicting a comparison of IMT in each group stained with hematoxylin-eosin and panels E-H showing a comparison of the PASMCs ratio in each group stained with α-SMA. The intima-media was stained purple in HE, and the

thickness in the sildenafil or metformin group was thicker than in the other groups, while the IMT in the sildenafil + metformin group was thinner than in all other groups. The PASMC ratio in each group was also compared using α-SMA staining. In the sildenafil group, PASMCs were more prominent and could be clearly distinguished from the other treatment groups.

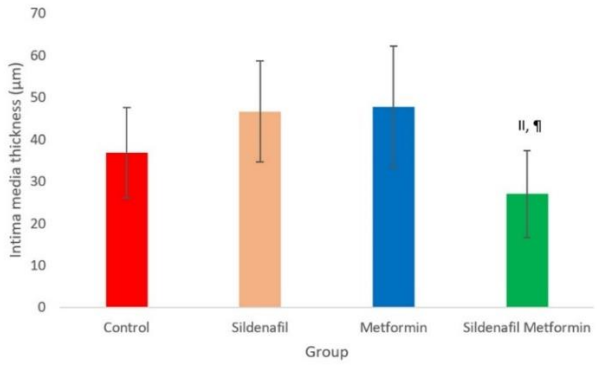


**Figure 3.** Effect of sildenafil and metformin on IMT of the pulmonary artery.

Results are presented as mean ± SD. $^{II}p = 0.01$ in relation to sildenafil. $^{¶}p = 0.01$ in relation to metformin. Post hoc test by DSCF.

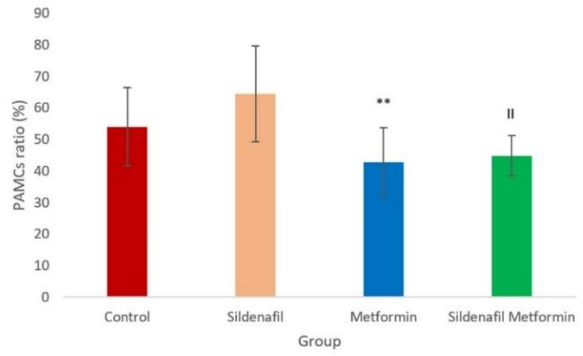


**Figure 4**. Effect of sildenafil and metformin on PASMCs of the pulmonary artery.

Results are presented as mean ± SD. $^{**}p = 0.0006$ for sildenafil in relation to sildenafil. $^{II}p = 0.002$ for sildenafil metformin in relation to sildenafil. Post hoc test by DSCF.

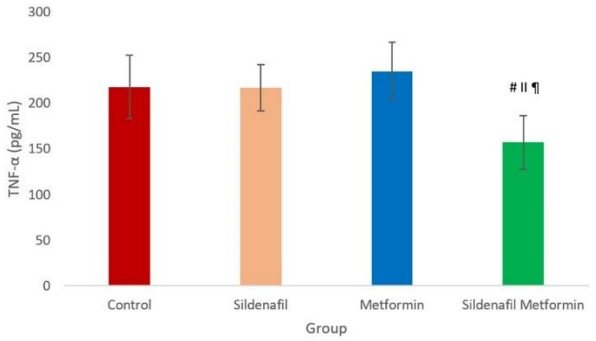


**Figure 5.** Effect of sildenafil and metformin on TNF-α.

Results are presented as mean ± SD. $^{#}p = 0.01$ compared with control. $^{II}p = 0.003$ in relation to sildenafil. $^{¶}p = 0.001$ in relation to metformin. Post hoc test by DSCF.

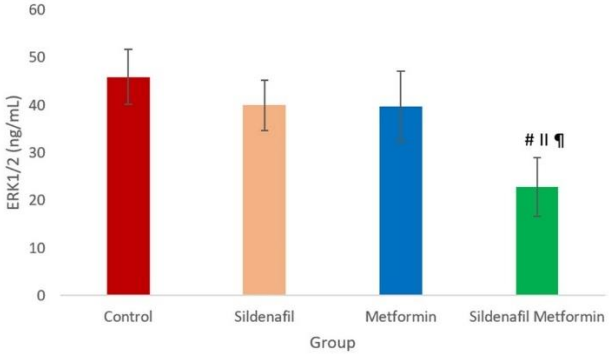


**Figure 6.** Effect of sildenafil and metformin on ERK1/2.

Results are presented as mean ± SD. $^{#}p = 0.001$ in relation to control. $^{II}p = 0.001$ in relation to sildenafil. $^{¶}p = 0.001$ in relation to metformin. Post hoc test by DSCF.

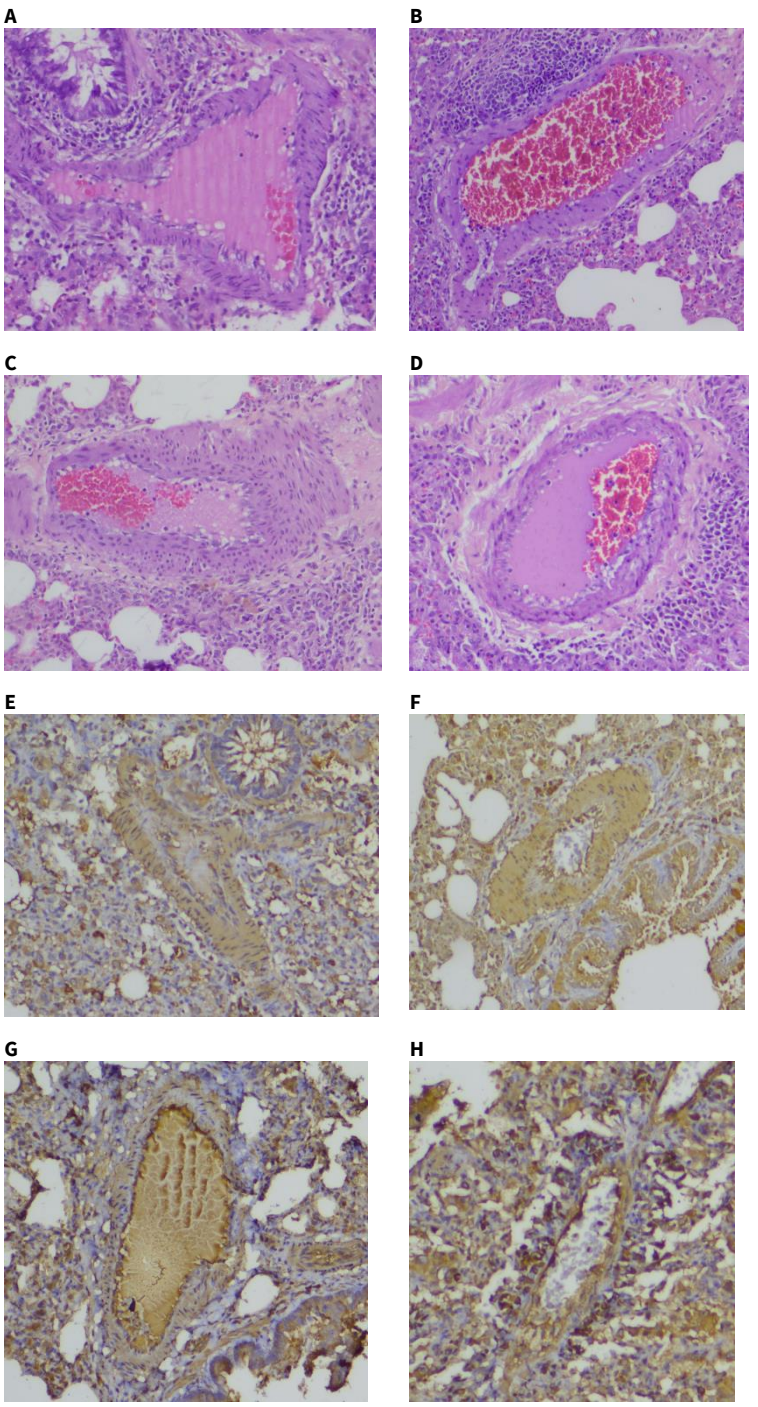


**Figure 7.** Representative PA histology.

**A-D**: Intima-media thickness of each group on hematoxylin-eosin staining: **(A)** control, **(B)** sildenafil, **(C)** metformin, **(D)** sildenafil + metformin. **E-F**: Ratio of PASMCs-of each group on α-SMA staining: **(E)** control, **(F)** sildenafil, **(G)** metformin, **(H)** sildenafil + metformin. Pulmonary artery smooth muscle cells were stained brown.

## DISCUSSION

PH pathobiology involves inflammatory, vasoconstrictive, and proliferative pathways that remodel the pulmonary vasculature. A mean mPAP greater than 20 mmHg, measured via right heart catheterization, confirms the diagnosis of PH (Humbert et al., 2023). PA remodeling results from the orchestration of proinflammatory cytokines and proliferative agents (Guo et al., 2022). Studies using animal models could uncover the molecular and pathological abnormalities associated with PH.

Reducing mPAP is a primary target in PH treatment and could improve the prognosis of patients with PH (Karyofyllis et al., 2023). In this study, sPAP, dPAP, and mPAP were reduced toward healthy ranges relative to MCT- control; comparison with a naïve control is required to confirm normalization. The sildenafil group showed a lower mPAP than the other treatment groups, but the difference was not substantial. This suggests that sildenafil and metformin, either alone or

in combination, were effective in improving pulmonary arterial pressure. Sildenafil is known to reduce pulmonary arterial pressure in PH models and in humans (Ang et al., 2022; Shrestha et al., 2017). However, metformin did not affect right ventricular systolic pressure (RVSP) in humans with PH, although it was shown to reduce RVSP in a PH model (Brittain et al., 2020; Yoshida et al., 2020). Further investigation is essential to verify the effect of metformin on human PH.

TNF-α is a key mediator in PH, and a serum TNF-α level of 218 pg/mL in this study induced mild to moderate PH in rats. Zhang et al. (2020) reported that MCT-induced rats had serum TNF-α levels of up to 120 pg/mL. The difference in the TNF-α concentrations can be strain-related.

In this study, the sildenafil group exhibited TNF-α levels comparable to the levels in the control group. This indicates that a single dose of sildenafil did not suppress TNF-α levels in PH. Previous studies have shown that sildenafil is beneficial for PH and has anti-inflammatory effects. Mediators blocked by sildenafil include TNF-α, IL-6, caspase-3, ERK1/2, Bax, and matrix metalloproteinase (MMP)-2 (Bae et al., 2016; Di Luigi et al., 2020; Li et al., 2007; Zych et al., 2019). Nevertheless, some studies found that sildenafil did not significantly suppress TNF-α expression. Tsai et al. (2006) showed that sildenafil did not suppress TNF-α expression in hypoxia-induced PA. Similarly, in a murine model of allergic asthma, Clayton et al. (2004) reported that sildenafil did not significantly suppress TNF-α production. Human studies demonstrated that sildenafil increased TNF-α production in peripheral blood mononuclear cells (Kaleta et al., 2019).

The metformin group did not show a substantial increase in TNF-α levels compared with the control group. Earlier research has shown that metformin exhibits anti-inflammatory and antivasoconstrictor activities. The anti-inflammatory effect of metformin was indicated by its ability to decrease TNF-α, IL-1, IL-6 (Puşcaşu et al., 2024; Ranchoux et al., 2019; Ye et al., 2018), VEGF, and PDGF (Attia et al., 2019; Wang et al., 2019). The antivasoconstrictor effect of metformin occurs through the reduction of ET-1 levels (Yoshida et al., 2020). However, a human study found that administering metformin for 12 weeks to individuals without obesity or diabetes suffering from coronary heart disease significantly increased TNF-α levels (Carlsen et al., 1998). This dose-dependent TNF-α response to metformin could explain this result (Hattori et al., 2006).

When sildenafil and metformin were combined, TNF-α levels decreased significantly compared with the other groups. Moreover, the combination of the two drugs reduced TNF-α to a greater extent than either agent alone; however, formal interaction testing was not performed. Recently, a few studies have explored the synergistic effects of these drugs. Bruckbauer et al. (2016) reported that a combination of leucine, metformin, and sildenafil decreased serum ALT and improved hepatic steatosis, fat accumulation, hepatic fibrosis, TNF-α, MCP-1, and CRP in a model of nonalcoholic steatohepatitis. These drugs also had synergistic effects in reducing pain, TNF-α, and IL-6 in diabetic neuropathy rats (Puşcaşu et al., 2024).

In this study, neither sildenafil nor metformin treatment resulted in a notable reduction in ERK1/2 levels, suggesting that MCT induced ERK1/2 activation, which was not fully suppressed by three weeks of sildenafil or metformin administration. The reduction in ERK1/2 in these groups was insufficient to prevent IMT thickening. Sildenafil at 5 mg/kg in this study was not sufficient to inhibit PA remodeling. Additionally, it reduced ERK1/2 phosphorylation in PASMC cultures in a dose-dependent manner (Li et al., 2007). Sildenafil inhibited pulmonary vascular remodeling through the reduction of p-ERK1/2 (Li et al., 2007).

The combination of sildenafil and metformin in this study significantly decreased ERK1/2 levels compared with the other groups, underscoring the greater-than-additive effect of these drugs. However, a previous study using an ischemia/reperfusion injury model in rats showed that the combination of these drugs did not significantly alter ERK mRNA expression compared to either drug alone. This study used 4 mg/kg of metformin and 1 mg/kg of sildenafil administered intraperitoneally (Park et al., 2021). The difference in the administration route may explain the different responses (Choi et al., 2006). A pharmacokinetic study of the simultaneous oral administration of metformin and sildenafil in rats demonstrated a reduction in metformin's mean plasma concentration, maximum concentration, area under the curve, and half-life. This study used 200 mg/kg of metformin and 2.5 mg/kg of sildenafil (Dewani et al., 2024). Additional variables that influence the substantial decrease in ERK1/2 in this study require further exploration.

This study provides evidence for the combined action of sildenafil and metformin in decreasing TNF-α and ERK1/2, as well as a partial reduction in IMT (sildenafil + metformin vs. control, $p = 0.094$). Metformin may serve as an alternative to bosentan in combination with sildenafil for PH treatment in the future (Durongpisitkul et al., 2021; Sullivan et al., 2023). Further studies exploring the optimal dosage combination of these drugs for PH are warranted. With these results, the potential for better prognosis in PH patients, particularly those who cannot access ideal treatments, has been opened (Cohen et al., 2019; Constantine et al., 2022; Lilyasari et al., 2019).

## CONCLUSION

Sildenafil and metformin, when used alone or in combination, could reduce mPAP relative to MCT control, with values within ranges reported for healthy rats. When the two drugs were used separately, they could not lower TNF-α, ERK1/2 or IMT. However, when the drugs were combined, mPAP, TNF-α, and ERK1/2 were significantly reduced. The combination of these drugs for preventing PH progression is promising.

## CONFLICT OF INTEREST

The research was conducted entirely free from any involvement in commercial, financial, or personal matters that might be perceived as a possible conflict of interest or that could have influenced the outcomes or interpretation of the study.

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## GENERATIVE ARTIFICIAL INTELLIGENCE (AI)

The authors declare that no generative artificial intelligence (AI) or AI-assisted technologies were used in the preparation, writing, or editing of this manuscript. All content was developed solely by the authors, who take full responsibility for its accuracy and integrity.

## DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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| Contribution | Cahyono A | Irwanto | Rahman MA | Widjiati W |
|---|---|---|---|---|
| Concepts or ideas | x | x | x | x |
| Design | x | x | x | x |
| Definition of intellectual content | x | x | x | x |
| Literature search | x | x | x | x |
| Experimental studies | x | | x | x |
| Data acquisition | x | | x | x |
| Data analysis | x | x | | |
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