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Progression of pulmonary arterial hypertension: A study in model

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ABSTRACT

Background: The pathophysiology of pulmonary arterial hypertension (PAH) is complex. Pathology and molecular biology signatures during its progression are interesting to study.

Aim: This study will describe PAH progression from the first until the fourth week in a model focussing on endothelin-1 (ET-1), tumor necrosis factor α (TNF-α), extracellular signal-regulated kinase 1/2 (ERK1/2), intima media thickness (IMT), and proliferation of pulmonary arterial smooth muscle cells (PASMCs) and fibroblast.

Methods: Six male Wistar rats aged 4 months old with a range bodyweight (BW) of 180–230 g were used in this experiment. Rats were injected with Monocrotaline (MCT) 60 mg/kg of BW subcutaneously to induce PAH. Rats were anesthetized with Ketamin 50 mg/kg BW and Xylazin 5 mg/kg BW intramuscularly before catheterization. Right heart catheterization was performed at days 1st, 2nd, 4th, 9th, 16th, and 23rd after MCT injection. After completion of catheterization, intracardiac exsanguination was performed and blood serum was analyzed by ELISA for ET-1, TNF-α, and ERK1/2. Lungs were harvested and parafinated blocked before being analyzed for IMT and proliferation of PASMCs and fibroblast.

Results: At first until the second week after MCT injection, mean pulmonary arterial pressure fluctuated. However, after 3rd until 4th week after MCT injection, its value becomes established over 40 mmHg. This is also followed by the level of ET-1 over 78 pg/ml, level of TNF-α over 223 ng/l, level of ERK1/2 over 47 ng/ml, IMT over 42 µm, ratio of PASMCs over 65%, and ratio of fibroblast 30%–35%.

Conclusion: Pulmonary hypertension was established at week 3rd-4th after MCT injection in a model.

Keywords: Pulmonary hypertension, Progression, Model.

Introduction

Pulmonary hypertension (PH) is defined by a mean pulmonary arterial pressure (mPAP) of more than 20 mmHg at rest (Humbert *et al.*, 2023). Its prevalence in the adult population is 5 per million adults and in the pediatric population is 2–16 per million children (Hansmann, 2017). As in adults, pediatrics has a similar pathobiology of PH. However, idiopathic pulmonary arterial hypertension, pulmonary arterial hypertension (PAH) associated with congenital heart disease, and developmental lung diseases are predominant in children (Rosenzweig *et al.*, 2019). The pathobiology of PH is complex. It is a progression from the subclinic to the clinic phase that is subtle. One may notice PH when it comes to an advanced degree of severity (Humbert *et al.*, 2006; Ivy *et al.*, 2013; Babu, 2019).

Understanding the pathobiology of PAH is important to find potential novel treatments (Babu, 2019). The basic pathobiology of PAH is endothelial dysfunction. This will later cause pulmonary vascular remodeling. The remodeled vascular is more vasoconstrictive and has a thicker wall. The proliferation of pulmonary artery smooth muscle cells (PASMCs) and fibroblasts plus increasing of vasoreactive modulators contribute to this event. When the causes are persistent, the remodeling process will be progressive and at some point, irreversible. Mediators are many to cause remodeling and their role in the pathobiology of PAH is more understandable recently (Ivy *et al.*, 2013; Humbert *et al.*, 2019; Zahid *et al.*, 2020, Sweatt *et al.*, 2021).

Many researches try to uncover the pathobiology of PAH. From the research, some key mediators have been discovered. Interleukin-1 (IL-1) is responsible in remodeling process by induction of fibroblast proliferation (Mitchell *et al.*, 2007; Bui *et al.*, 2019). Meanwhile, Serotonin and Interleukin-6 (IL-6) are important mediators for PASMCs proliferation (Savale *et al.*, 2009; West *et al.*, 2016; Sakarin *et al.*,

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2020). Furthermore, tumor necrosis factor α (TNF-α), Vascular Endothelial Growth Factor (VEGF), tumor growth factor β (TGF-β), and Platelet Derived Growth Factor (PDGF) are responsible in this process by induction of fibroblast and PASMCs proliferation (Farkas *et al.*, 2009; Biernacka *et al.*, 2011; Zhao *et al.*, 2013; Pilling *et al.*, 2015; Li *et al.*, 2016; Rieg *et al.*, 2018; Bell *et al.*, 2020; Winter *et al.*, 2020; Takamura *et al.*, 2021). Lack of Nitrit Oxyde (NO) and increased level of Endothelin-1 (ET-1) will cause vasocontriction (Klinger *et al.*, 2013; Chester and Yacoub, 2014; Klinger and Kadowitz, 2017). ET-1 itself acts as vasoconstrictor and inducer of fibroblast and PASMCs proliferation (Meoli and White, 2010; Duangrat *et al.*, 2023).

From the aforementioned mediators, ET-1 and TNF-α are interesting to study. These two mediators act in more than one way to cause vascular remodeling. Together, these two mediators inducing extracellular signal regulated kinase 1/2 (ERK1/2) to cause the proliferation of fibroblast and PASMCs with the end results of pulmonary arterial wall thickening (Meoli and White, 2010; Wu *et al.*, 2011; Wang *et al.*, 2017; Duangrat *et al.*, 2023). ET-1 is also known as the strongest endogenous vasoconstrictor (Chester and Yacoub, 2014). Using a model will clearly depict PAH progression that is similar in pediatric patients since in humans the beginning of PAH is difficult to determine. This study will describe PAH progression from the first until the fourth week in a model focusing on ET-1, TNF-α, ERK1/2, and pulmonary artery remodeling.

Materials and Methods

Animal studies

Six male Wistar rats aged 4 months old with a range bodyweight (BW) of 180–230 g were used in this experiment. Rats were housed at 24°C, 50%–70% humidity in room oxygen with a 12-hour light/dark cycle, and given free access to standard laboratory food and water. Rats were injected with Monocrotaline (MCT, MedChemExpress, catalog number HY-N0750) 60mg/kg of BW subcutaneously. MCT was diluted in 1 M Hydrocloride acid and 1 M Sodium hydroxide was added to create a pH of 7.4.

Right heart catheterization was performed at days 1st, 2nd, 4th, 9th, 16th, and 23rd after MCT injection. Rats were anesthetized with Ketamin 50 mg/kg BW and Xylazin 5 mg/kg BW intramuscularly. After that, the rats were put on a supine position. Fur was shaved and skin was incised vertically from 1st to 5th costae until the pectoralis muscle was seen. Pectoralis muscle was bluntly put aside and retracted laterally until the intercostalis muscle is seen. Intravenous catheter no 22G (after washed with heparin) was then introduced through 4th parasternal intercostalis space to the right ventricle. The dark red blood will come out from the right ventricle and immediately, the catheter is connected to tranducer and Mac 51 cable. The cathether then directed to the pulmonary artery and the pulmonary arterial wave will be seen on DASH 4000 monitor. Pulmonary arterial pressure was then recorded.

After completion of cathetherization, a blood sample was taken from the intracardiac (±3.5 ml). The rats were euthanized by this exsangunation. The lungs were harvested and put into formalin before processed to paraffin block. Blood was then centrifuged by refrigerated centrifuse (Kubota K3520) with 3,500 rpm and 4°C for 10 minutes. The serum was kept in −20°C refrigerator before ELISA analysis.

The study was conducted from March to April 2024 at the Animal Laboratory Faculty of Veterinary Medicine Airlangga University, Surabaya, East Java, Indonesia.

Histological analysis

The Parrafin block of each lung lobe was sliced to 5 µm thickness. These slides were washed with xylene for 5 minutes three times to clear parrafin then dripped three times into methanol and washed 1 minute in the water. After the procedure, the slides were stained with hematoxylin and eosin at room temperature for 10 minutes and 6 minutes consecutively. A pulmonary artery with a diameter of 50–200 µm were evaluated using a light microscope. Eight pulmonary artery, for each rat, were randomly selected. Four areas of intima media thickness (IMT) were measured at x400 magnification for their thickness and count for their average. Slides were also stained with alpha smooth muscle actin (α-SMA, GeneTex, catalog number GTX636885) antibody to evaluate the ratio of the smooth muscle cell and fibroblasts.

ELISA assay

Serum ET-1 concentrations were measured using a Rat ET-1 competitive ELISA kit (catalog number ER0019, Fine Test), and serum TNF-α concentrations were measured using Rat TNF-α ELISA kit (Catalog number E0764Ra, Bioassay Technology Laboratory). Meanwhile, serum ERK1/2 concentrations were measured using Mouse ERK1/2 ELISA kit (Catalog number E2815Mo, Bioassay Technology Laboratory).

For ET-1 measurement, frozen samples were thawed at room temperature before ELISA assay. The plate was washed twice before adding the standard and sample. Fifty microliters of biotin-labeled antibody working solution was added to each well that contained 50 µl of standard or sample. The plate was then shaked for 1 minute and statically incubated at 37°C for 45 minutes. After that, the plate was washed 3 times and immersed for 1 minute each time. One hundred microliters of HRP-Streptavidin Conjugate working solution was added into each well and then the plate was sealed and statically incubated at 37°C for 45 minutes. The plate was washed five times after that and immersed for 1 minute each time. Ninety TMB substrate solution was then added and the plate was sealed and incubated at 37°C for 10–20 minutes. Eventually, 50 µl stop solution was added and read

at 450 nm immediately. Regression equations were formulated from a standard curve of optical density and standard solution concentrations. The equation was used to calculate sample concentration. For TNF-α and ERK1/2 measurement, the step was similar to that of ET-1. The serum volume required for measurement was 40 µl.

Ethical approval

This study was approved by the Animal Care and Use Committee Faculty of Veterinary Medicine Airlangga University no 2.KEH.161.10.2023.

Results

Clinical data

Data of rat's clinical condition at catheterization is described in Table 1. On 1st day after MCT injection, mPAP reaches 24 mmHg, which is consistent with PAH definition. However, between 1st and 2nd week, mPAP is still fluctuative. When entering 3rd and 4th week, mPAP is established at 44 and 47 mmHg.

IMT and PASMCs/Fibroblast ratio IMT is described on µm and PASMC/fibroblast ratio is described on percentage. The progression of PAH development is depicted on Figure 1. Figure 2a–c are representative of histological views of IMT (day 1st, day 16th, and day 23rd consecutively), and d, e, f are representative of PASMCs/Fibroblast ratio (day 1st, day 16th, and day 23rd consecutively) ELISA assay.

Inflammatory mediators characterize PAH. ET-1, TNF-α, and ERK1/2 fluctuate during the progression of PAH. ERK1/2 is the least fluctuative mediator. However, at day 23rd, these 3 mediators are higher when compared to day 16th (Fig. 3).

Discussion

PAH is an interesting subject to study. Clinical manifestations, pulmonary arterial pathology, and molecular abnormalities characterize PAH progression (Humbert *et al.*, 2006; Ivy *et al.*, 2013; Hansmann, 2017; Babu, 2019). However, it is not always possible to study the progression PAH in human subjects, since the onset of the disease is less predictable (Humbert *et al.*, 2006). An animal model is chosen for the study to understand the pathobiology of PAH. Wistar rats are commonly used for PAH model since their pulmonary vascular shares similarities with human. MCT was chosen to induce PAH in a model due to its effectiveness (Boucherat *et al.*, 2022). To illustrate PAH progression, which is the dominant type of PH in pediatrics population (Rosenzweig *et al.*, 2019), 16-week-old rats were used in this research. A 4-week-old rats is equal to a 2.5-month-old human, it means 16 weeks rats is equal to 10 years 10-year-old human (Andreollo *et al.*, 2012).

mPAP measurement using right heart Catheterization confirmed evidence of PAH at day 1 after MCT injection, since PH definition is when mPAP of more than 20 mmHg at rest (Humbert *et al.*, 2023). Even though the value fluctuates at the following day, it is established at day 16th and 23rd. This fact reflects that vasoconstriction is might be dominant at the first day and proliferation of PASMCs and fibroblast is more apparent at 3rd and 4th week (Yoshida *et al.*, 2020). Pathobiology of PAH consists of vasoconstriction, proliferation of PASMCs and fibroblast. These entity due to stimulation of mediators involved in PAH (Humbert *et al.*, 2006; Ivy *et al.*, 2013; Babu, 2019).

From hystological views, IMT and proliferation of PASMCs and fibroblast depict the pathology of PAH. Intima media reflects the most area of the pulmonary artery that are affected by either PASMCs or fibroblast proliferation (Humbert *et al.*, 2019; Zahid *et al.*, 2020). However, since PASMCs and fibroblast is proliferating in PAH (Humbert *et al.*, 2019; Zahid *et al.*, 2020; Sweatt *et al.*, 2021), we conducted a study to measure PASMCs and fibroblast ratio in the pulmonary artery. The study showed us that IMT fluctuates from the 1st day until 23rd day after MCT injection. PASMC became prominent at day 16th and 23rd after the MCT injection. This fact is also underscored by the decreasing of fibroblast ratio at the same period. During PAH progression, none of the rats showed obvious clinical signs. This can be explained by the fact that the pulmonary artery has not been fully remodeled (Humbert *et al.*, 2006).

Fluctuation of mediators also accompanied IMT and mPAP. Tumor necrosis factor alpha (TNF-α) and ERK 1/2 fluctuated in accordance with IMT. Meanwhile,

Table 1. Clinical data of experimental rats.

Number	Day	Age (weeks)	BW (gram)	mPAP (mmHg)	Heart rate (bpm)
1	1	16	181	24	185
2	2	16	187	15	206
3	4	16	211	29	262
4	9	16	185	13	179
5	16	16	238	47	138
6	23	16	228	44	163

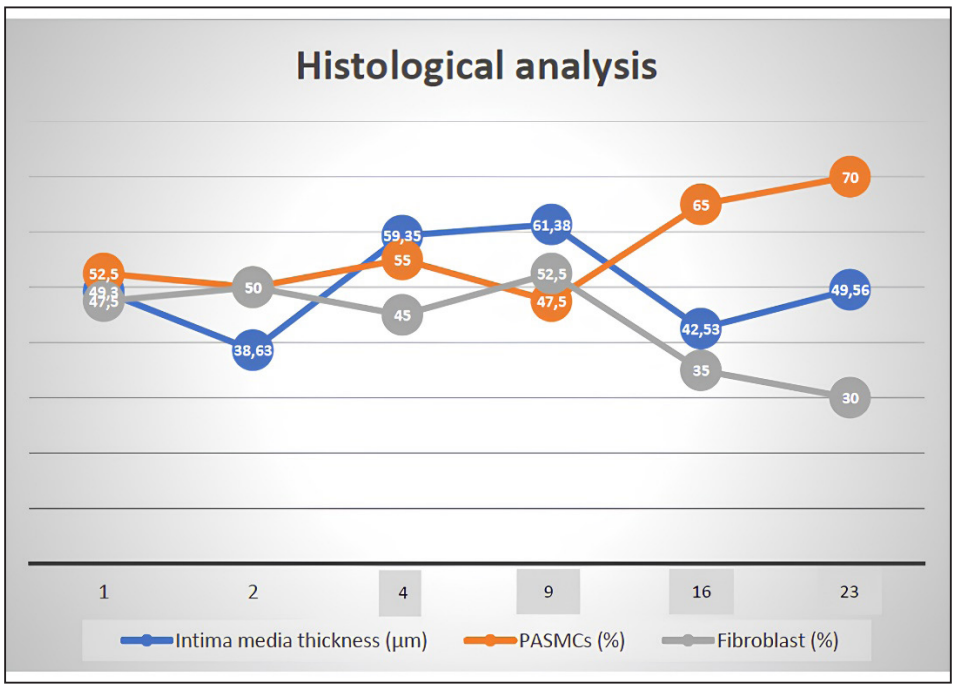

Fig. 1. Histological analysis. This graphic depicts progression of pulmonary arterial from first day to 23rd day of MCT injection. PASMCs: pulmonary arterial smooth muscle cells.

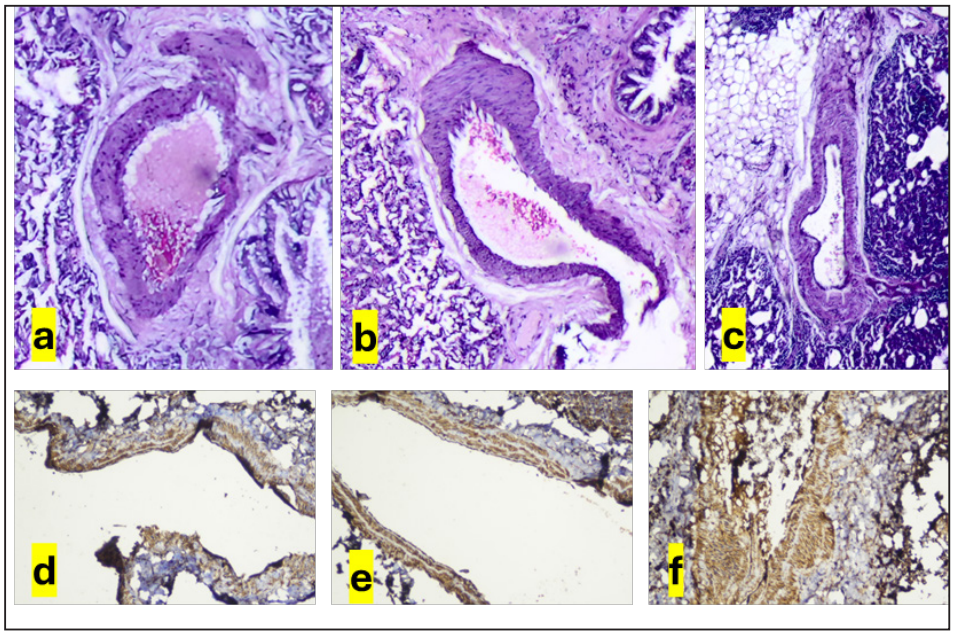

Fig. 2. Intima media and PASMCs/Fibroblast picture. Hematoxylin and eosin staining (a, b, c) depicts IMT at day 1st, 16th, and 23rd consecutively and α-SMA staining (d, e, f) depicts PASMCs/Fibroblast ratio at day 1st, 16th, and 23rd consecutively. At 23rd day, IMT become prominent and arterial wall is dominated by PASMCs. IMT, purple colored; PASMCs brown colored.

ET-1 fluctuated in accordance with mPAP. These facts showed that TNF-α and ERK 1/2 were mediators more related with IMT and ET-1 was a mediator related with mPAP. Previous research showed TNF-α and ET-1 act together to induce ERK 1/2 with the end result of pulmonary vascular thickening. ET-1 also known as

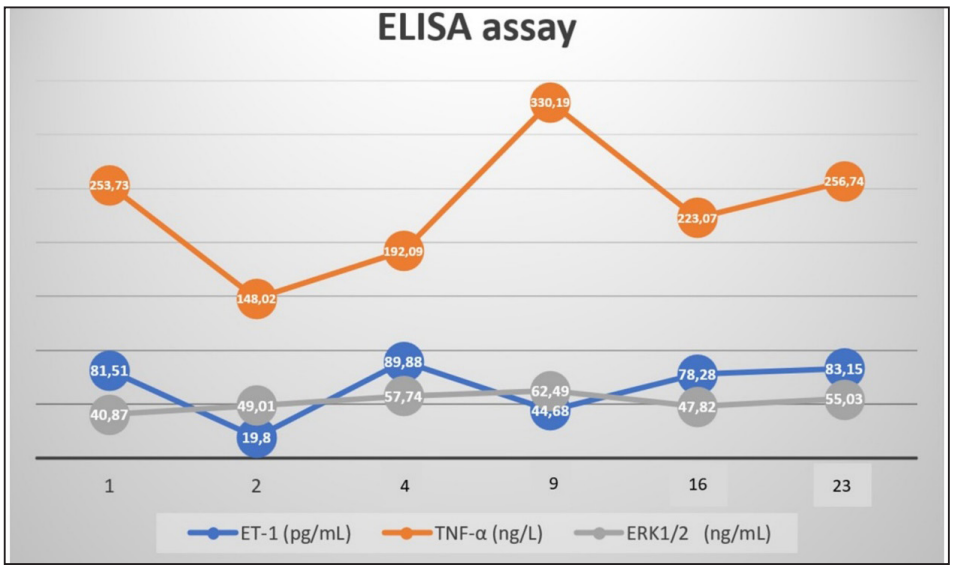

Fig. 3. ELISA assay. This graphic depicts inflammatory mediators fluctuation during PAH progression. At first until 3rd week ET-1, TNF-α, and ERK1/2 are fluctuating. However, at 4th week, these 3 mediators are simultaneously increased.

the strongest vasoconstrictor. These 3 mediators are important in PAH (Meoli and White, 2010; Wu *et al.*, 2011; Chester and Yacoub, 2014; Wang *et al.*, 2017; Duangrat *et al.*, 2023).

We focused on the study of 3 mediators and their effects on the progression of PAH. The role of other mediators such as IL-1, IL-6, Serotonin, VEGF, TGF-β, PDGF, and lack of NO are also significant in the pathobiology of PAH. Their orchestration cause remodelling of pulmonary arterial wall (Mitchell *et al.*, 2007; Farkas *et al.*, 2009; Savale *et al.*, 2009; Biernacka *et al.*, 2011; Klinger *et al.*, 2013; Klinger and Kadowitz, 2017; Zhao *et al.*, 2013; Pilling *et al.*, 2015; Li *et al.*, 2016; West *et al.*, 2016; Rieg *et al.*, 2018; Bui *et al.*, 2019; Bell *et al.*, 2020; Sakarin *et al.*, 2020; Winter *et al.*, 2020; Takamura *et al.*, 2021). IL-1will induce fibroblast proliferation (Mitchell *et al.*, 2007; Bui *et al.*, 2019); meanwhile, Serotonin and IL-6 will induce PASMCs to proliferate (Savale *et al.*, 2009; West *et al.*, 2016; Sakarin *et al.*, 2020). The proliferation of PASMCs and fibroblast simultaneously will be induced by the work of VEGF, TGF-β, and PDGF (Farkas *et al.*, 2009; Biernacka *et al.*, 2011; Zhao *et al.*, 2013; Pilling *et al.*, 2015; Li *et al.*, 2016; Rieg *et al.*, 2018; Bell *et al.*, 2020; Winter *et al.*, 2020; Takamura *et al.*, 2021). On the other hand, a lack of NO will cause the pulmonary artery to easily constrict (Klinger *et al.*, 2013; Klinger and Kadowitz, 2017). Further research to recognize these mediators role in PAH progression is widely open.

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Conflict of interest

There is no conflict of interest.

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Authors' contributions

AC, I, and MAR developing concept and methodology. AC, MAR, and W performing the investigation. MAR and I performing data curation. AC and MAR writing the original draft. I and W reviewing and editing draft. All authors have reviewed and approved the final manuscript.

Data availability

All data supporting the findings of this study are available within the manuscript.

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