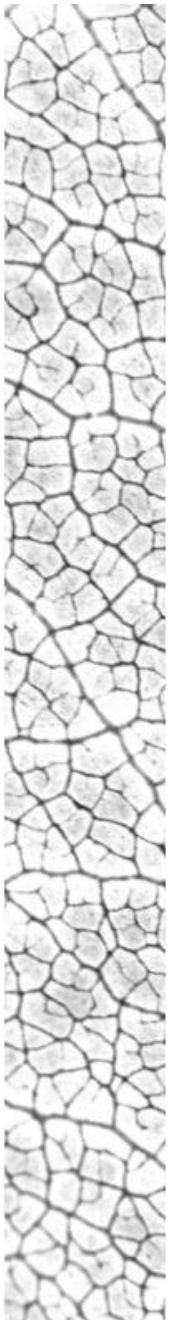

EurAsian Journal of BioSciences
Eurasia J Biosci 14, 3633-3639 (2020)

Potency of pomegranate extract (*Punica Granatum L*) to heat shock Protein27, Laminin 5 γ2, and thickening of pulmonal artery smooth muscle in pulmonary arterial hypertension rat models

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Abstract

Background: Congenital Heart Disease's related Pulmonary Arterial Hypertension (PAH) frequently develops into progressive, causing vascular proliferation and ending with heart failure and death. Definitive therapy on anatomical defect often delayed due to facilities and infrastructure. Medical therapy is needed to decrease morbidity and mortality during awaiting definitive therapy. Pomegranate extract has antiproliferation activity through antiinflammation, ACE inhibitor, and antioxidant mechanism. The antifibrotic effect can be evaluated by the decreasement of cells expressing HSP27 and Laminin 5 γ2, and thickening of smooth muscle pulmonal artery.
Purpose: Knowing the effect of pomegranate extract (*Punica Granatum L*) in cells expressing HSP27 and Laminin 5 γ2, and thickening of smooth muscle PAH rat models.
Method: This randomized post-test only control group carried out on 24 paraffin block from monocrotaline induced male *Sprague Dawley rats.* The monocrotaline dose is 60 mg/kg body weight. The amount of cells expressing HSP27 and Laminin 5 γ2, and thickening of smooth muscle evaluated on week 2 and week 4.
Result: There are significant difference on cells expressing HSP27 and Laminin 5 γ2, and thickening of smooth muscle between 2 weeks treatment by pomegranate extract group and 4 weeks treatment group lower than controls.
Conclusion: Pomegranate extract decrease cells expressing HSP27 and Laminin 5 γ2, and inhibit thickening of smooth muscle on PAH rat models.

Keywords: pulmonary arterial hypertension, PAH rat model, pomegranate extract, HSP27, Laminin 5 γ2, smooth muscle thickness

Rahman MA, Djalilah GN, Utamayasa IKA (2020) Potency of pomegranate extract (*Punica Granatum L*) to heat shock Protein27, Laminin 5 γ2, and thickening of pulmonal artery smooth muscle in pulmonary arterial hypertension rat models. Eurasia J Biosci 14: 3633-3639.

INTRODUCTION

Cardiovascular disease is a high urgency disease in the world (Widiyanti, et al. 2016). The most common cardiovascular risk factor is hypertension (Maharani, et al. 2019). Pulmonary artery hypertension (HAP) is one of the complication that often occurs in congenital heart disease (CHD), characterized by an increase in pulmonary artery pressure that can occur progressively and can cause right heart failure and premature death (Abman, & Ivy, 2011). Pulmonary artery hypertension often occurs in congenital heart disease (CHD) in children where the incidence of CHD in Indonesia is 8 per 1000 live births (Vidyasagar, Wilson, & Djamali, 2012). The proliferation of cardiac smooth muscle cells through the mechanism of endothelin activates p38MAPK and HSP27 accompanied by migration of smooth muscle cells characterized by increased biomarkers of laminin5. Increased HSP27 activity results in proliferation of smooth muscle of the heart. HSP27 is a specific protein in cardiac smooth muscle cells. Laminin 5 activates the results of extracellular matrix resulting in migration of pulmonary smooth muscle cells. Laminin 5 is activated by the p38-MAPK channel door when shear stress arises (Tuder, et al. 2013; Chang, et al, 2016).

The use of pomegranate extract as HAP therapy still needs to be studied further, especially its use as an antioxidant, anti-inflammatory, anti-proliferative, and anti-fibrotic which is expected to be used as an alternative therapy to prevent the progression of HAP so as to reduce patient mortality (Asgary, S., et al. 2013,

Received: November 2019
Accepted: March 2020
Printed: September 2020

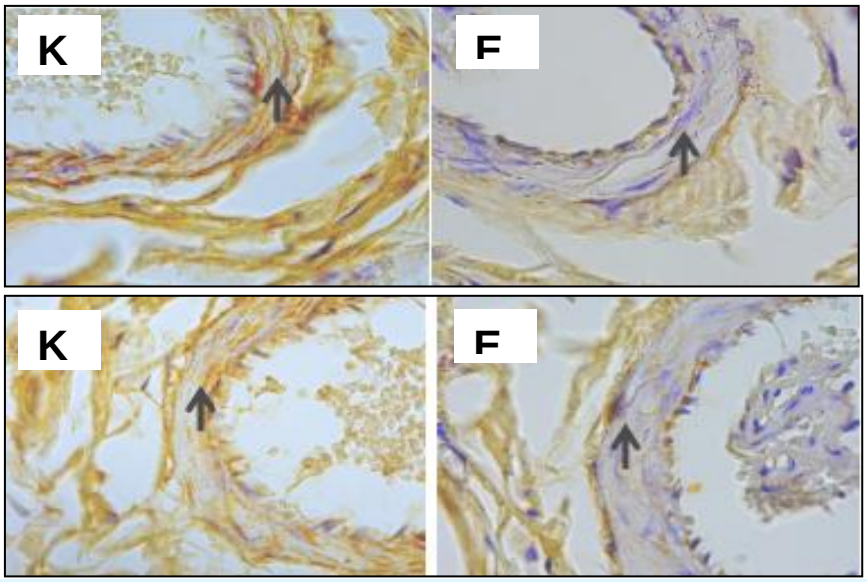

Fig. 1. HSP27 expression in tunica intima and tunica media of mouse HAP pulmonary artery media

Asgary, et al. 2014). Pomegranate is commonly used as an herbal medicine. Extracts from all parts of this fruit have a protective effect against damage in the body, including a decrease in the regulation of apoptotic mechanisms due to ellagic acid compounds (Anggraini, & Hendarto, 2018).

Purposes of this research to explain the mechanism of smooth muscle thickening inhibition as a result of proliferation of pulmonary artery smooth muscle cells expressed by HSP27 and Laminin 5 γ2 in the intima and the pulmonary artery media of the mouse model of pulmonary artery hypertension given pomegranate extract. Proving that pomegranate extract (Punica Granatum L) decrease cells expressing HSP27 and Laminin 5 γ2, and thickening of smooth muscle PAH rat models.

METHOD

This type of research stems from a previous study namely experimental studies in animal models of mouse pulmonary hypertension carried out by making immunohistochemical preparations. Samples are measured and identified well. This randomized post-test only control group carried out on 24 paraffin block from monocrotaline induced male Sprague Dawley rats. The monocrotaline dose is 60 mg/kg body weight. The amount of cells expressing HSP27 and Laminin 5 γ2, and thickening of smooth muscle evaluated on week 2 and week 4.

Population and sample

The paraffin block in this study is 24 divided into 4 groups, with each group of 6 paraffin blocks. Four groups were divided into control groups and treatment groups. Group I (K1) paraffin block control group, group II (E1) paraffin block treatment in observation 2 weeks, group III (K2) paraffin block observation control group 4 weeks, and group IV (E2) paraffin block treatment each each at 4 weeks observation. The subjects of the study were observed HSP27 expression and the expression of laminin 5 γ2 expression, as well as thickening of the pulmonary artery smooth muscle.

Data analysis

Analysis of research data is done by testing the normality first with the results of the data obtained are normally distributed. After that the homogeneity test was carried out using one-way ANOVA method on the body weight of the model rats and obtained homogeneous data. ANOVA and LSD tests were conducted because the data obtained were normally distributed and homogeneous.

RESULTS

Figure 1 was taken with a 400x magnification on a Sony Nex7 digital imaging light microscope, while cell calculations of HSP27 expression were calculated on endothelial cells expressing brown using a micrometer device mounted on a microscope lens with 1000x magnification of 20x field of view. The black arrow on the K1 image shows HSP27 expression on the tunic intima and the tunic media of the mouse pulmonary artery in the control group model on 2 weeks observation that appears more when compared to the one shown in E1 image, the group given EBD at 2 weeks observation. Black arrows on K2 images that showed HSP27 expression in tunica intima and mouse pulmonary artery media in the control group model on 4 weeks observation which also showed cells expressing HSP27 appeared to be much more than in E2 image, ie groups of mouse models given EBD on 4 weeks observation.

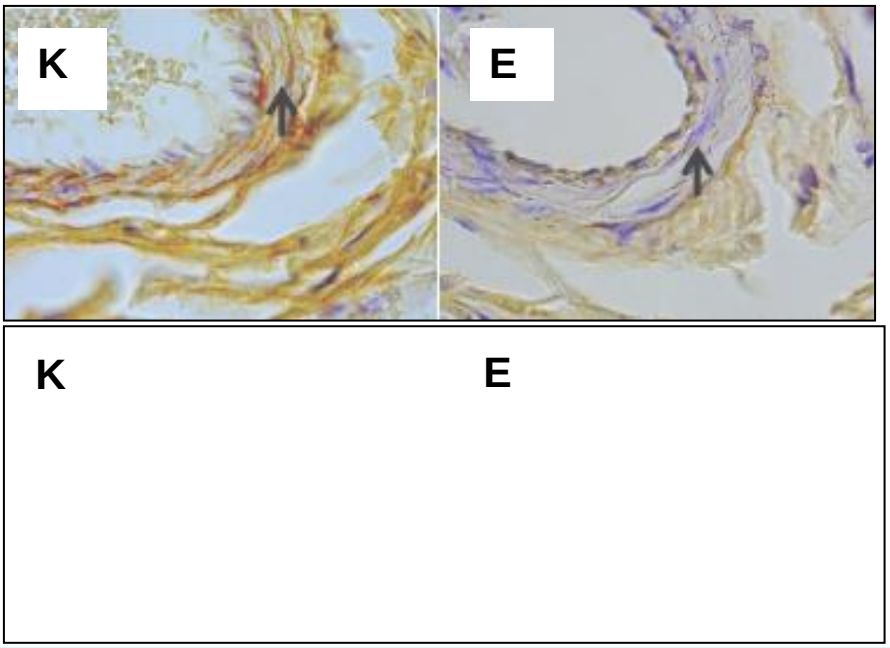

Fig. 2. Laminin 5 γ2 expression on tunica intima and tunica media of mouse HAP pulmonary artery media

Table 1. The average number of cells expressing HSP27

Treatment group	HSP27 (cell/hpf)				P
	Mean	SD	Minimum	Maximum	
K1	10^{a}	1,581	8	12	<0,001
E1	$4,6^{b}$	1,517	3	7	
K2	$18,4^{c}$	2,191	15	21	
E2	$6,2^{d}$	0,837	3	21	

Different ^{abcd} *superscripts* show that there are significant differences between groups based on the LSD double comparison test (p <0.05).

Table 1 explains the number of cells expressing HSP27 that there was a decrease in the EBD (E1) administration group to the control group (K1), as well as a decrease in the EBD group (E2) to the control group (K2). Mean control group 2 weeks (K1) from the 4-week control group (K2), as well as the group given EBD 2 and 4 weeks. b illustrates the significant difference between the groups given EBD 2 weeks (E1) from the 4-week EBD group (E2), and the control groups 2 and 4 weeks. c illustrates the significant difference in the 4-week (K2) control group from the 2-week control group (K1), as well as the group given EBD 2 and 4 weeks. d illustrates the significant difference from the 4-week EBD group (E2) from the 2-week EBD group (E1).

Figure 2 was taken with 400x magnification on a Sony Nex7 digital imaging light microscope, while cell calculations from Laminin 5 γ2 expression were calculated on endothelial cells expressing brown color using a micrometer device mounted on a microscope lens with 1000x magnification of 20x field of view. The black arrow in the K1 image shows the number of cells expressing Laminin 5 γ2 in the tunica intima and the pulmonary artery media in the mouse group in the control model on 2 weeks observation. The number of cells appeared more when compared to the number of cells expressing Laminin 5 γ2 in the tunnels of the intima and pulmonary artery media in the model mouse group given EBD in the 2 weeks observed by the black arrow in image E1. The same thing can be seen in the K2 image where the black arrow showing the number of cells expressing Laminin 5 γ2 in tunica intima and pulmonary artery media in the control group mouse model at 4 weeks observation appeared to be much more than in cells expressing Laminin 5 γ2 in tunica intima and Pulmonary artery media in the model mouse group that was given EBD in the 4-week observation shown by the black arrow on E2 image.

Table 2. The average number of cells expressing HSP27

Treatment groups	Laminin 5 γ2 (cells/hpf)				P
	Mean	SD	Minimum	Maksimum	
K1	11^{a}	1,581	9	13	<0,001
E1	$5,2^{b}$	1,304	4	7	
K2	$15,4^{c}$	2,408	12	18	
E2	$7,2^{ab}$	1,643	5	9	

Different ^{abcd} *superscripts* show that there are significant differences between groups based on the LSD double comparison test (p <0.05).

Table 2 explains the number of cells expressing Laminin 5 γ2 that there was a decrease in the EBD (E1) group of the control group (K1), as well as a decrease in the EBD group (E2) to the control group (K2). Significant differences in the 2-week (K1) control group from the 4-week control group (K2), as well as the groups given EBD 2 and 4 weeks. b illustrates the significant difference between the groups given EBD 2 weeks (E1) from the 4-week EBD group (E2), and the control groups 2 and 4 weeks. c illustrates the significant difference in the 4-week (K2) control group from the 2-week control group (K1), as well as the group given EBD 2 and 4 weeks. ab illustrates the significant non-significant difference of the 4-week EBD group (E2) from the EBD

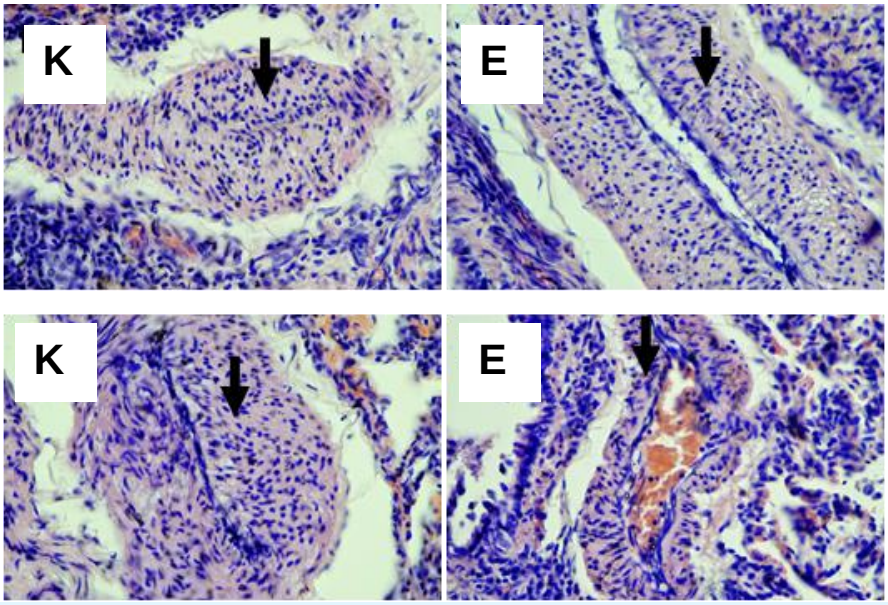

Fig. 3. The thick smooth muscle of the HAP model of the mouse pulmonary artery

group 2 weeks (E1) but still shows a decrease in the number of laminin 5 γ2 expressions so that in the HAP process it can inhibit HAP from worsening.

Pulmonary histology preparations made using hematoxylin-eosin (HE) staining were performed to measure the thickness of smooth muscle of the HAP model animal pulmonary arteries. This measurement aims to see the proliferation process in the smooth muscle of the pulmonary artery wall from the mice of the control and experimental group models

Figure 3 was taken with 400x magnification on a Sony Nex7 digital imaging light microscope, while the calculation of endothelial cells using a micrometer device mounted on the ocular lens from a microscope with 1000x magnification, cell counting was calculated by calculating pulmonary artery smooth muscle cells in 100 cells from the area the tunica edge of the pulmonary artery media, with 1000x magnification, in 10 visual fields. The black arrows in image K1 showed the thickness of the pulmonary artery smooth muscle of the control group rats in 2 weeks observation, black arrows were also shown towards smooth muscle cells in the group given EBD (E1). The picture above does not clearly show the number of smooth muscle cells because shooting is only 400x, while the calculation of cell counts uses 1000x enlargement, but from the field of view it looks different in the control group (K1) and the EBD (E1) administration group, as well in the control group (K2) observation with the group given EBD (E2) at 4 weeks observation.

Table 3 explains that the mean thickness of pulmonary artery smooth muscle in the EBD treatment group at 2 weeks observation (E1) was lower than the control group paraffin block in 2 weeks (K1) and significantly different observations, as well as 4 weeks observation, the number of muscle cells plain in the EBD (E2) treatment group was less than the control group (K2) The description of a, b, c superscripts where a illustrates the significant difference in the 2-week (K1) control group from the 4-week control group (K2), as well as the groups given EBD 2 and 4 weeks. b illustrates the significant difference between the groups given EBD 2 weeks (E1) from the 4-week EBD group (E2), and the control groups 2 and 4 weeks. c illustrates the significant difference in the 4-week (K2) control group from the 2-week control group (K1), as well as the group given EBD 2 and 4 weeks. ab illustrates the significant non-significant difference of the 4-week EBD group (E2) from the EBD group 2 weeks (E1) but still shows a decrease in the number of smooth muscle cells so that in the HAP process it can prevent HAP from worsening.

Table 3. The average thickness of the smooth muscle of the pulmonary artery wall

Treatment groups	Thick smooth pulmonary artery muscles(μm)				P
	Mean	SD	Minimum	Maksimum	
K1	1,92[a]	0,38	1,50	2,50	<0,001
E1	0,55[b]	0,24	0,25	1,00	
K2	5,75[c]	1,04	4,00	7,00	
E2	1,12[ab]	1,21	0,20	3,50	

Different [abcd] *superscripts* show that there are significant differences between groups based on the LSD double comparison test (p <0.05).

DISCUSSION

Administration of EBD can cause a decrease in HSP27 expression through p38-MAPK activation in paraffin blocks derived from monocrotalin-induced HAP model mice. Starting from endothelin is one of the vasoactive mediators that play a role in the formation of HAP. Endothelin in HAP is converted into endothelial cells to ET-1 by ECE. Endothelin-1 increases the

expression of endothelin A (ETA) and endothelin B (ETB) which eventually leads to contraction, proliferation and hypertrophy of pulmonary artery smooth muscle cells (Kingsley, et al. 2002).

Administration of EBD for 2 weeks was significantly different with HSP27 expression with 4 weeks of EBD administration. This shows that EBD can inhibit the progression of HAP, but more research is needed to evaluate the effectiveness of use.

Pomegranates (punica granatum) contain polyphenols which function as anti-inflammatory and antioxidant. Among them are ellagic acid (EA) and flavonoids, tanins, and anthocyanins (Kiss, et al. 2014. Lévy, et al. 2013). Antioxidant effects on tannins contained in pomegranates cause a decrease in angiotensin II levels but the mechanism of action still cannot be explained (Li, et al. 2015).Pomegranate extract can inhibit liver and heart fibrosis in animal models, can function as an anti-oxidant, anti-inflammatory, anti-proliferative, and as apoptosis, and can inhibit p38-MAPK and endothelin activity (Grinnan, et al. 2014. putri, 2019. Li, et al. 2015. Kingsley, et al. 2002). Cells expressing ET-1 by decreasing mRNA upregulation, EBD also decreases the number of cells through the p38-MAPK canal, and also directly decreases HSP27 22 HSP27 expression can be reduced through the inhibition of ET-1 production by substance NO and also through p38-MAPK. Several studies have shown pomegranate juice can increase the expression of eNOs and NO release, and can protect NO degradation. (Nogueira-Ferreira, Ferreira, & Henriques-Coelho, 2014. Pugliese, et al. 2015).

The role of laminin 5 in the pathophysiological process of HAP occurs in the endothelin (ET) pathway through p38-MAPK activation and inflammatory cells. Initially ET-1 increases the expression of ETA and endothelin B (ETB), which increases intracellular calcium (Ca +), causing pulmonary artery vasoconstriction (Simonneau, et al. 2013. Taguchi, 2013). Endotelin A will activate protein kinase C which then activates the p38-MAPK path through the PLC. When p38-MAPK is active, it also directly causes the migration of laminin 5γ2 to pulmonary artery smooth muscle cells. This will stimulate the migration of pulmonary artery smooth muscle cells (Kingsley, et al. 2002. Kiss, et al. 2014. Khan, et al. 2012. Kingsley, & Plopper, 2005. Takatsuki, & Ivy, 2013).

The administration of EBD for 2 weeks to Laminin 5γ2 expression was not significantly different from EBD 4 weeks. This shows that EBD administration can inhibit the progression of HAP, but more research is needed to evaluate the effectiveness of use. EBD which is rich in polyphenols can inhibit inflammation by mast cells so that it can inhibit laminin5γ2 activation through p38-MAPK by other Rasheed studies conducted in 2016(Li, et al. 2015. Grinnan, et al. 2014). A 2005 study by Ahmed et al. Found that EBD had an effect on inhibiting laminin 5γ2 activation through p38-MAPK and NF-β in human in vitro chondrocytes (Kiss, et al. 2014(Lévy, et al. 2013).

This study showed that the mean thickness of pulmonary artery smooth muscle in the paraffin block group given EBD in 2 weeks and 4 weeks observation was significantly lower than in the control group paraffin block. While the mean smooth muscle thickness between the two groups that received EBD was not statistically significant, it was found to have significant differences between the two control groups. This shows that thickening of the pulmonary artery smooth muscle blocks paraffin originating from HAP mice, thickening of the pulmonary artery smooth muscle is increasing following the progression of HAP occurrence in paraffin blocks originating from HAP mice, the administration of EBD can prevent thickening of the pulmonary artery smooth muscle of the paraffin block originating from HAP mice, giving EBD can inhibit HAP progression.

Pomegranate extract has been widely investigated in its effect of inhibiting cell proliferation associated with the process of proliferation itself. The effect of EBD in inhibiting the proliferation and thickening of pulmonary artery smooth muscle walls associated with the role of endothelin can occur through a decrease in HSP27 expression and Laminin expression5γ2. The effect of EBD on the increase of NO so as to inhibit the proliferation of pulmonary artery smooth muscle in accordance with Ignarro's research and De Nigris's study which showed that the effects of EBD administration can increase the activity of eNOs (Tang, et al. 2015. putri, 2019.)

Pomegranate extract can inhibit the proliferation of smooth muscle cells by increasing prostacyclin, decreasing TXA2, inhibiting TGF-β1, and the role of inflammatory mediators. Pomegranate seeds and skin extracts inhibit fibrosis through the cytokine regulatory pathway with antioxidant activity, reduce TGF-β1 levels and inhibit collagen synthesis as the results of the study obtained by Wei et al. Long-term administration of EBD is safe to use because EBD comes from fruits which also have antioxidant effects and provide protection to vascular smooth muscle cells of cigarettes.

CONCLUSION

In this study, pomegranate extract could decrease cells expressing HSP27 and Laminin 5 γ2, and inhibit thickening of smooth muscle on PAH rat models.

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