Profile of Apoptotic Marker Genes and Histopathology of the Placenta in Pregnancies with Pre-Eclampsia

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Abstract

Background: Pre-eclampsia (PE) is a hypertensive disorder in pregnancy and a significant cause of maternal and perinatal mortality and morbidity. Failure of spiral artery remodeling due to abnormal apoptosis, triggers disturbances in the mother and the baby's growth. This study aimed to identify the profile of apoptotic marker genes and histopathological features of the placenta in pregnancies with pre-eclampsia.

Methods: This study had used case-control method. Samples were taken from normal pregnancies (n=25) and pregnant women with pre-eclampsia (n=25) using a purposive sampling method from several hospitals in Jambi. qRT-PCR was used to examine apoptotic gene expression from placental tissue and hematoxyline eosin staining to view the placenta's microscopic appearance. The targeted genes were BCL2-associated X (BAX) and B-cell lymphoma 2 (BCL-2). Histopathological changes of the placenta observed were syncytial node, cytotrophoblast, villous edema, hypervascularization, fibrosis stroma, atherosis, infarction, and thrombosis.

Results: Relative BAX genes expression were increased once in placenta pre-eclampsia compared to controls, but not statistically significant (p-value>0.05). There was no difference between the decline of BCL-2 gene expression in pre-eclampsia placenta compared to the control (p-value >0.05). Histopathological changes in the placenta were syncytial node and cytotrophoblast (25 of 25), villous edema (19 of 25), hypervascularization (24 of 25), fibrosis stroma (22 of 25), atherosis (12 of 25), infarction (17 of 25), and thrombosis (24 of 25).

Conclusion: The expression of BAX genes in pre-eclampsia tends to increase compared to normal pregnancy, and the expression of BCL-2 decreases. The histopathological features of pre-eclampsia pregnancy placenta are mostly syncytial nodes, cytotrophoblasts, stromal fibrosis, and thrombosis.

Keywords: BAX, BCL-2, histopathology placenta, pre-eclampsia

Introduction

Maternal mortality rate (MMR) in developing countries is still a problem. The MMR ratio for the Southeast Asian region in 2015 was 164 deaths for every 100,000 live births. In Indonesia, the MMR in 2017 was 177 for every 100,000 live births;[1] whereas in Jambi Province in 2020 was 94 per 100,000 live births.[2] The causes of maternal death in Indonesia are bleeding (30%), pre-eclampsia (27.1%), infection (7.3%), and other causes (40.8%). It is obvious that pre-eclampsia is the second leading cause of maternal death after bleeding.[3]

Pre-eclampsia is a hypertension and a renal organ dysfunction that causes proteinuria after 20 weeks of gestation, and usually appears during the gestational period. Hypertension during pregnancy is characterized by systolic

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blood pressure in excess of 140 mmHg and diastolic blood pressure in excess of 90 mmHg or both.[4] Among all pregnant women worldwide, 5–8% suffer from Pre-eclampsia, which is associated with an increased risk of maternal and infant morbidity and mortality.[5,6]

The exact pathogenesis of pre-eclampsia is still unknown. In a normal pregnancy, apoptosis plays an essential role in regulating placental development. Trophoblast apoptosis increases with placental growth and advancing gestation. Pre-eclampsia is commonly associated with multiple theories, such as vascular disease and placental endothelial dysfunction.[7] Epidemiological studies in previously different populations has shown that placental apoptosis is primarily associated with pre-eclampsia related pregnancy complications of various etiologies, including oxidative stress and hypoxia.[8] Damage to the structure and reduced number of endothelial cells of the trophoblast will result in hypoperfusion of blood to the placenta. This process inhibits the implantation, regulation and proliferation of trophoblast development.[9,10]

Placental hypoxia induces apoptosis through two mechanisms; the initiation phase (activation of caspase) and the execution phase. The initiation phase occurs through the intrinsic pathway (mitochondria pathway) and the extrinsic pathway (death receptor pathway). Placental hypoxia mainly leads to apoptosis through the intrinsic pathway. In the intrinsic pathway, the apoptosis signal is mediated directly from the mitochondria in response to cellular stress, such as DNA damage that will initiate the activation of the apoptosis signal. There is thus an imbalance between the pro-apoptotic protein (BAX) and the antiapoptosis (Bcl-2). Trophoblasts exposed to hypoxia causes an increase in Bax expression and a decrease in Bcl-2 expressions.[8,9] This apoptotic expression might serve as a marker for pre-eclampsia.

Histopathological examination of the placenta with pre-eclampsia has shown infarcts, and sclerotic lesions, also narrowed arteries and arterioles.[11] The lesions are characterized by atherosis, infarction, and thrombosis of the placenta. Therefore, this study aimed to identify apoptotic marker gene profiles and histopathological features of the placenta in pregnancies with pre-eclampsia.

Methods

The research was a descriptive-analytic study with a case-control approach, including pregnant women diagnosed with pre-eclampsia according to the American College of Obstetricians and Gynecologists 2013, such as hypertension (systolic blood pressure ≥ 140 mmHg and or diastolic blood pressure ≥ 100 mmHg) after 20 weeks of pregnancy. The respondents were recruited by purposive sampling method at Raden Mattaher Hospital Jambi and Mitra Hospital Jambi City. As for control group, normotensive healthy women with gestational age matched with the preeclamptic group were recruited. The exclusion criteria were twin pregnancy, the mother with signs of active clinical infection, pregnancy with assisted technology for fertilization, and kidney as well as hepatic failure history. All respondents were followed until childbirth.

Placental specimens were taken after birth, from the full thickness section from maternal and fetal side, in 2 cm x 2 cm size, then put into a pot containing 10% buffer formalin. The placental tissues were then made into a paraffin block to proceed to the microscopic examination stage. The hematoxyline-eosin staining was performed to view the placenta's macroscopic appearances. After staining, the image J application was used for counting cells and another microscopic finding in magnification 40 times. The histopathological variables evaluated in the placenta were syncytial node, cytotrophoblast, villous edema, hypervascularization, fibrosis stroma, atherosis, infarction, and thrombosis.

All examinations were carried out in The Biomolecular Laboratorium Faculty of Medicine and Health Sciences. This research had received ethical approval from the Medical and Health Research Ethics Committee from the Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada with Ref. No. KE/FK/1189/EC/2020.

The identification of apoptotic gene profiles was examined using qRT-PCR. RNA isolation from placenta samples was carried out according to the manufacturer's protocol using an RNA extraction kit (Promega, USA). Before RNA isolation, the placental tissue was put into RNA lysis buffer, and then the tissue was homogenized using a sterile pestle, followed by centrifugation at 14,000 rpm for 10 minutes at 40 to separate the insoluble material. Reverse transcription was performed on approximately 7 µg of each RNA sample to synthesize complementary DNA (cDNA). The cDNA was prepared using Arctic Thermal Cycler PCR (Thermo Fisher, USA) and a cDNA synthesis kit (Promega, USA). The

Table 1 Primers and Sequences in Realtime PCR Studies

Genes Name	Forward Primer	Reverse Primer
BAX	5'-CCTTTTCTACTTTGCCAGCAAAC-3'	5'-GAGGCCGTCCCAACCAC-3'
BCL-2	5'-ATG TGT GTG GAG AGC GTC AAC C-3'	5'-TGA GCA GAG TCT TCA GAG ACA GCC-3'
GAPDH	5'-AGC CAC ATC GCT CAG ACA C-3'	5'-GCC CAA TAC GAC CAA ATC C-3'

thermal cycle used for cDNA synthesis was performed at 25°C for 5 minutes, 42°C for 60 minutes, and 70°C for 15 minutes. Synthesized cDNA was stored at -20 °C until later use. Gene expression was then analyzed using the cDNA template for qRT-PCR reactions

The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a standard to normalize the relative expression of the BAX and BCL-2 genes. Amplification of the BAX and BCL-2 genes was performed in a 20 µl reaction mix containing the following reagents: 2 µl of BAX and BCL-2 forward primer, 2 µl of BAX and BCL-2 reverse primer, 10.0 µl of sybr green master mix (Promega, USA), 2.8 µl of nuclease-free water, 0.2 µl of DNA dye, and 3 µl of cDNA. All the qRT-PCR was performed according to the manufacturer's instructions using the PicoReal96 real-time PCR system (Thermo Fisher, USA). Thermal cycling conditions for the BAX and BCL-2 genes were 1 cycle for 2 min at 95°C, followed by 40 cycles for the denaturation process at 95°C for 15 sec and 1 min for annealing at 60°C, and for dissociation at 60°C for 2 min. Thermal cycling conditions for GAPDH were 1 cycle at 95°C for 5 min, followed by 55 cycles of denaturation process at 95°C for 10 sec, annealing at 60°C for 20 sec, and elongation at 72°C for 10 seconds. Fold changes in relative expression levels of BAX and BCL-2 were quantified using the 2-ΔΔCq method (Livak), using an average of a housekeeping gene as a reference.

Statistical analysis used was independent student T-test for parametric data and a Mann-Whitney test for non-parametric to find the differences between the PE and normal pregnancy groups. The data were considered statistically significant if $p<0.05$.

Results

In total, pre-eclampsia (PE) pregnant women (n=25) and the normal (normotension) pregnant women (n=25) were included. Maternal characteristics showed that the age of pregnant women in the PE group was higher than the control, however, the difference was not significant (Table 2). The proportion of PE was higher in women with primigravida

Table 2 Characteristic Research Subject

Characteristic		Control (n=25)	PE (n=25)	p-value
Maternal ages (year)	Mean	29 ± 6.16	29.28 ± 6.65	0.924*
	High risk, n	8	10	0.556***
	Low risk, n	17	15	
Blood Pressure (BP)	SBP (mmHg)	110 (100–130)	170 (150–210)	< 0.001**
	DBP (mmHg)	70 (60–80)	100.00 (80–150)	<0.001**
Gravidity, n	Primigravida	10	13	0.426***
	Multigravida	20	17	
Parity, n	Nulliparous	11	14	0.432***
	Multiparous	19	16	
Gestasional age, n	Pre term	5	14	0.012***
	A term	25	16	
Birth weight	IUGR	1	12	0.000***
	Normal	24	13	

Note: PE=Pre-eclampsia, SBP=Systolic blood pressure, DBP= Diastolic blood pressure, IUGR=Intrauterine growth retardation, * Independent t-test, ** non-parametric test-MannWhitney Test, ***chi-square test

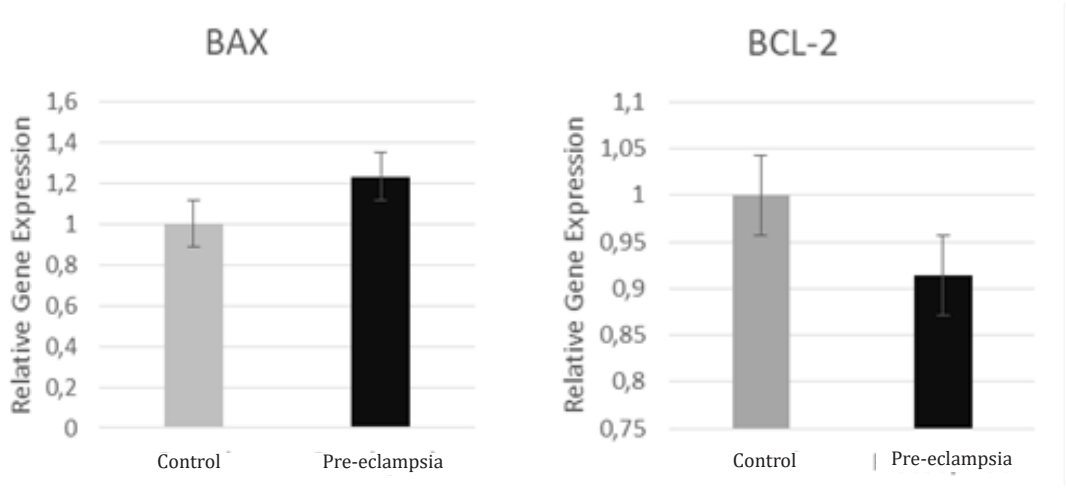

Figure 1 Relative Expression of BAX and BCL-2 Genes between Pregnancies with Pre-eclampsia and Normal Pregnancies. P-value BAX and BCL-2 Genes >0.005

or nulliparous, but again was not significantly different. Gestational characteristics such as gestational age at birth was a term, and low birth weight or known as intra uterine growth retardation (IUGR) showed a higher percentage in infants born to pregnant women with PE compared to infants born to normotensive pregnant women.

The mean value of BAX gene expression was higher in the case group; whereas the mean value of BCL-2 gene expression was lower in the case group, however, the statistical test (independent t-test) showed no significant difference (p-value >0.005). Furthermore, the Cq value from running qPCR was lower in pre-eclamptic pregnancies than in normotensive pregnancies, with fold changes indicating the Bax gene increased one time in the pre-eclamptic pregnancy group compared to the normotensive pregnancy group. Cq values of BCL-2 were lower in pre-eclamptic pregnancies than in normotensive pregnancies, with fold changes indicating the BCL-2 gene increased one time in the normal pregnancy group compared to the pre-eclamptic pregnancy group. The expression of the BCL-2 gene in pregnancies with PE was even lower than in normal pregnancies (Figure 1).

The histopathology of the placenta of PE patients after staining with hematoxylin-eosin showed syncytial nodes, cytotrophoblast cells, villous edema, hypervascularization, stromal fibrosis, atherosis, infarction, and thrombosis. Most PE subjects in the pregnancy

Table 3 Overview of Pre-eclampsia Placental Histopathological Changes

Characteristics	Pre-eclampsia	Normal pregnancy
	n	n
Syncytial knot	25	25
Cytotrophoblast cells	25	25
Villous edema	19	9
Hypervascularization	24	12
Stromal Fibrosis	22	8
Atherosis	12	5
Infarction	17	2
Thrombosis	24	12

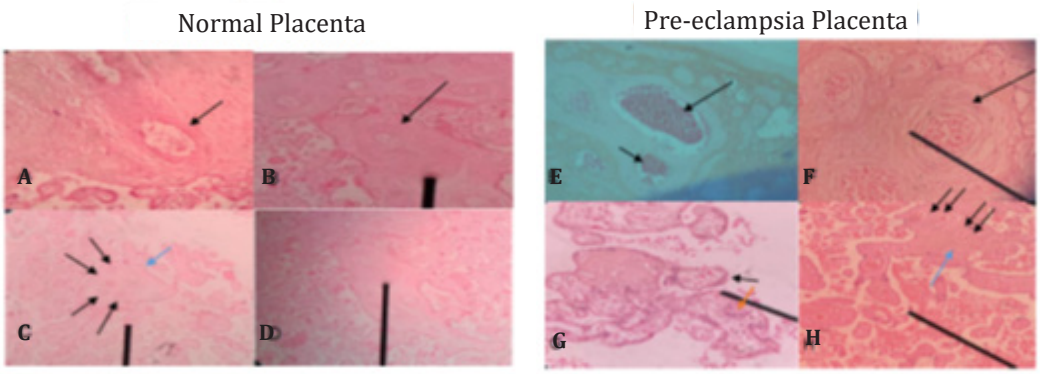

Figure 2. Histopatological of Placenta. (A) Placental Vascular in Normal Pregnancy. (B) Cytotrophoblast Cells. (C) Syncytial Knot in Normal Pregnacy. (D) Placental Villous. (E) Infarction in PE Placenta (F) Hypervascularisation in PE. (G) Syncytial Knot in PE. Original magnification x40

group had syncytial nodes, cytotrophoblast cells, hypervascularization, stromal fibrosis, infarction, and thrombosis. Some subjects also showed atherosis and villous edema in their histopathological placental samples.

Discussion

Data from maternal characteristics in this study showed that 10/25 of pregnancies with PE were in the high-risk age range, conform a high risk of pregnant women suffering from PE at the age of <20 years or >35 years.[12] The reproductive organs in women aged <20 years are still immature, and not ready for a pregnancy process, that is at risk for an abnormal placenta. In the other side, the female reproductive organs older age at >35 years begin to develop a degenerative process that interferes the implantation process and the development of trophoblasts in the placenta. Poor trophoblast invasion will further interfere with the remodeling of spiral arteries, followed by ischemic placenta and inflammation, which is one of the pathogenesis of PE. Moreover, a woman at this age has a 5% chance of giving birth to a baby with a chromosomal abnormality.[13]

Our results has shown that PE pregnant women are mostly nulliparas, conforms to another study that also found a relationship between nullipara and PE.[14] Pre-eclampsia is at risk for nulliparas or primigravidas, as endothelial dysfunction in the placenta causes vasoconstriction, resulting in increased of blood pressure and PE.[15]

In addition, the increased of blood pressure associated with PE occurs during pregnancy and resolves after birth. In pregnancy, apoptosis plays a role in placental growth. Apoptosis is a cell regulation in maintaining homeostatic conditions of tissue remodeling. Apoptosis plays a role during the attachment process, followed by trophoblast invasion, spiral artery transformation, trophoblast differentiation, and the process of immune tolerance to paternal antigens expressed by trophoblast cells. Apoptotic trophoblast cells are also found in normal pregnancy placentas on both the maternal and perinatal sides.[11,16]

In response to genotoxic or cellular stress, abnormal placental apoptosis affects the placenta's endothelial cells and induces extensive placental apoptosis.[17] Hypoxia-induced cellular stress is the most frequent factor triggering increased p53 gene expression in PE.[18] Increased expression of the pro-apoptotic gene p53 leads to excess apoptosis leading to trophoblast damage. Another study found a change in the expression of the p53 gene from the placenta of pregnant women with PE compared to the normotensive pregnancy group.[19] In pre-eclampsia, increased p53 expression occurs in the trophoblast layer due to the previous transcription of other pro-apoptotic proteins, including p21 and BAX. [19,20,21]

The expression imbalance between pro-apoptotic and anti-apoptotic genes is associated with excessive apoptosis and syncytial degeneration. The expression of Bcl-2, an anti-apoptotic gene, is higher than the expression of the BAX gene, which is an essential pro-apoptotic gene for normal placental development and pregnancy continuity. On the other hand, overexpression of BAX in placental tissue may lead to failure of placental development through excessive

apoptotic mechanisms.[22,23] The results obtained in this study showed an increase in BAX gene expression levels one time higher in pregnancies with PE compared to normal pregnancies. The increased apoptosis in PE occurs via an intrinsic pathway in response to cellular stress. This condition initiates the pro-apoptotic BAX protein, which is produced further to suppress the regulation of the anti-apoptotic gene BCL-2, so that the expression of the BCL-2 gene is lower than the pro-apoptotic gene expression. This study found that the level of BCL-2 gene expression was one time lower in pregnancies with PE compared to normal pregnancies. So that in pregnancies with PE, the ratio of BAX gene expression compared to BCL-2 is 1:1, even though not statistically significant. In previous studies, the intrinsic and extrinsic pathways are related because p53 can also increase the expression of several death receptors through the extrinsic pathway.[24,25]

Histopathological examination of PE placenta showed placental infarction and sclerosis, causing narrowing of the arteries and arterioles. Another factor that triggers PE is apoptosis, which results from placental hypoxia leading to placental ischemia. Furthermore, there are changes in syncytial nodes, cytotrophoblasts, villous edema, hypervascularization, atherosis, stromal fibrosis, infarction, and thrombosis, similar to other study.[26] The PE may thus harm placental morphology and consequently affects the fetus.[26] Several other studies have proofed the significance relationship between the microscopic changes of the placenta with the incidence of PE.[27,28,29] The changes are due to a lack of oxygen perfusion in the preeclamptic placenta. In the mature placenta, cytotrophoblast cells will be reduced, but in PE, many new villi are formed to support the lack of placental perfusion. The more trophoblast cells and new villi are formed. Inflation of trophoblasts that will become cytotrophoblasts occurs due to inadequate oxygen perfusion. The function of the cytotrophoblast itself is for gas exchange, where the cytotrophoblast will replace the endothelial function of the arterioles. The typical mature placenta in each villus only has 20% of cytotrophoblasts; while in PE cytotrophoblasts that functions as gas exchange is increased to compensate the hypoxic state.[29]

The ischemic process in pregnant women with PE triggers the inflammatory process in the endothelial wall. This proliferation is characterized by the thickening of the tunica intima, resulting in constriction of blood vessels. Stromal fibrosis is also found in the placentas due to the presence of tissues that have not experienced a lack of oxygen perfusion. The formation of stromal fibrosis is associated with impaired vascularization. However, fibrosis can be referred as a repair process in damaged tissue with fibroblastic proliferation due to chronic inflammation due to hypoxia and a lot of tissue necrosis in blood vessels in the long term.[28]

In pre-eclampsia placenta, there is a change in vascularization in the form of hypervascularization, which is due to angiogenesis or the formation of new vessels. Angiogenesis occurs due to reduced oxygen perfusion and is a normal response of oxygen-deprived tissues. Vascular endothelial growth factor (VEGF) is essential in forming new blood vessels. The placenta is in inadequate perfusion in PE until the spiral arteries change. The placenta in this state undergoes hypoxia and ischemia resulting in necrosis and infarction.[25]

Atherosis is also found in preeclamptic placentas caused by abnormalities of blood vessels, blood flow, and blood composition. Vascular abnormalities in the PE placenta are caused by the absence or only part of the spiral arteries being invaded by trophoblast cells. Abnormal blood flow in PE affects the spiral arteries, where the spiral arteries are arteries that supply the villi. The reduced oxygen supply of the villi causes smooth muscle cells to move toward the tunica intima. The proliferation of smooth muscle cells and deposition of extracellular matrix by smooth muscle in the intima transforms fatty patches into mature fibrofatty atheroma and plays a role in the progressive growth of atherosclerotic lesions. Oxygen deprivation due to damage to spiral arteries causes necrosis, and hypoxic trophoblasts secrete thromboxane as a vasoactive factor, leading to thrombosis. Thromboxane is produced as a vasoactive agent that compensates for endothelial damage.[27]

The limitation of the study is that the pregnancy is a late pregnancy. Ideally, the respondents are from several trimesters, to determine the histopathological results and apoptosis pattern at each pregnancy stage.

In conclusion, pre-eclampsia pregnant women are mostly delivered a term with intra uterine growth. Expression of BAX gene in PE is higher and of the BCL-2 gene is lower than in normotensive pregnancies. The histopathological findings

of most PE pregnancies have syncytial nodes, cytotrophoblasts, stromal fibrosis, and thrombosis.

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