RESEARCH ARTICLE

Bax mRNA Expression as A Potential Biomarker of Placental Apoptosis in Early-onset Preeclampsia

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Abstract

BACKGROUND: Early-onset preeclampsia is characterized by higher oxidative stress and apoptosis level than late-onset one. Studies comparing the expression of the *Bcl-2* family protein in early and late-onset preeclampsia are still lacking and show inconclusive evidence. This study aimed to compare the expression of *Bax* and *Bcl-2* messenger RNA (mRNA) as a biomarker of placental apoptosis between early-onset and late-onset preeclampsia.

METHODS: A cross-sectional study was conducted using formalin-fixed, paraffin-embedded preeclamptic placental samples and dividing them into early-onset and late-onset preeclampsia groups. *Bax* and *Bcl-2* mRNA expressions were assessed using the quantitative real-time polymerase chain reaction method. Apoptosis was assessed through DNA fragmentation examination by the ligation-mediated real-time polymerase chain reaction method.

RESULTS: Thirty early-onset and 30 late-onset preeclamptic placental samples were included. The mean fold change *Bax* mRNA in early-onset was higher than in late-onset preeclampsia (6.02±3.59 *vs.* 2.82±1.97; p=0.00). The mean fold change *Bcl-2* mRNA early-onset was not different from late-onset preeclampsia (31.20±17.94 *vs.* 31.01±27.60; p=0.98). The mean DNA fragmentation cycle threshold in early-onset preeclampsia was lower than in late-onset preeclampsia (28.07±0.64 *vs.* 30.63±0.96; p=0.00). A weak negative correlation exists between fold change Bax mRNA and DNA fragmentation cycle threshold (r=-0.30; p=0.02).

CONCLUSION: *Bax* mRNA showed significant correlation in DNA fragmentation compared to *Bcl-2* mRNA; hence, might show more role in apoptotic pathway. Early-onset preeclampsia has higher *Bax* mRNA relative expression and apoptosis than late-onset preeclampsia. Therefore, *Bax* mRNA can be potential biomarker in early-onset preeclampsia.

KEYWORDS: mRNA, *Bax*, *Bcl-2*, apoptosis, DNA fragmentation, early-onset, preeclampsia

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Introduction

Preeclampsia is a specific syndrome in pregnancy that causes a high risk of maternal morbidity and mortality. (1-3) The preeclamptic placenta undergoes morphological alterations, including placental villi necrosis, enhanced trophoblast apoptosis, fibrin deposition, and shallow trophoblast invasion.(4) Increased apoptosis in the preeclamptic placenta may be caused by the activation of the mitochondrial apoptosis pathway that is triggered due to increased oxidative stress, which is not balanced by the antioxidant capacity and the proliferation mechanism.(5,6)

Based on the gestational age at which clinical preeclampsia first develops, preeclampsia can be classified into early-onset (occurring before 34 weeks of pregnancy) and late-onset (occurring at or after 34 weeks of gestation) subtypes.(7,8) Early-onset preeclampsia is associated with worse maternal and perinatal outcomes.(9) This subtype has the characteristics of more prolonged trophoblast cell oxidative stress, related to the consequences of placental hypoxia since early pregnancy due to shallow placentation and impaired spiral artery remodeling.(10) Early-onset preeclampsia is also associated with a lower compensation of antioxidants and mitochondrial adaptation compared to late-onset preeclampsia.(11)

B-cell lymphoma 2 (*Bcl-2*) family gene regulation is essential in the activation of apoptosis in the placenta of preeclampsia patients.(12,13) Anti-apoptotic *Bcl-2* and pro-apoptotic *Bcl-2* associated X-protein (*Bax*) genes exhibit the most significant impact on placental apoptosis. (14) The simultaneous examination of *Bax* and *Bcl-2* gene expressions allows the analysis of the balance of pro-apoptosis and anti-apoptosis effects, thereby making it easier to explain the phenomenon of susceptibility of a cell to apoptosis.(13,15,16)

Biomarkers study related to placental apoptosis is expected to improve the management and outcome of early-onset preeclampsia. Several studies have examined differences in level of apoptosis by examining *Bcl-2* family protein expression in trophoblast cells between early and late-onset preeclampsia. However, there is still controversy regarding some inconsistent research results with previous placental preeclampsia apoptosis studies.(17-19) Examining different gene expression levels, such as messenger RNA (mRNA) expression, is necessary to clarify these differences. Accordingly, this study aimed to compare the expression of pro-apoptosis (*Bax*) and anti-apoptosis (*Bcl-2*) mRNA as a biomarker of placental apoptosis between early-onset and late-onset preeclampsia.

Methods

Study Design and Sample Collection

A cross-sectional study was conducted at the Biomolecular Laboratory, Department of Anatomical Pathology, Faculty of Medicine, Public Health and Nursing of the Universitas Gadjah Mada, Yogyakarta, Indonesia. Approval for the study protocoal was obtained from the Medical and Health Research Ethics Committee, Universitas Gadjah Mada (No. KE/FK/1145/EC/2022).

Samples were collected from formalin-fixed, paraffin-embedded (FFPE) preeclamptic placentas originating from previous studies' sample preparations, which were taken in March-June 2019 and May-July 2020 at Dr. Sardjito Hospital, Yogyakarta, Indonesia.(17-21) Samples were divided into early-onset and late-onset preeclampsia groups. Early-onset preeclampsia was identified as preeclampsia occurring before 34 weeks of gestation, while late-onset preeclampsia occurs at/or after 34 weeks. Preeclampsia subjects with diabetes mellitus, chronic hypertension, chronic kidney disease, systemic lupus erythematosus, and chorioamnionitis were excluded from the study.

Examination of *Bax* and *Bcl-2* mRNA Expressions Using Quantitative Real-Time Polymerase Chain Reaction

RNA was extracted from FFPE samples using the RNeasy FFPE Kit (Qiagen, Hilden, Germany) and reverse transcribed using the Universal cDNA synthesis kit II 8-64 rxns (Exiqon, Vedbaek, Denmark). Quantitative real-time PCR was performed using the NEXpro qRT-PCR Master Mix (SYBR) kit (Genes Laboratories, Seongnam, South Korea) following the manufacturer's protocol, using primary sequences as shown in Table 1. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene is a housekeeping gene used as an internal control to assess the expression of target genes. The relative expression of mRNA was presented as fold change according to the Livak calculation.(22)

Apoptosis Assessment Using the Ligation-Mediated Real-Time Polymerase Chain Reaction (LM-RT-PCR) Method

Genomic DNA samples were extracted from the placental tissue using the Exgene clinic SV kit (GeneAll Biotechnology, Seoul, Korea) following the manufacturer's instructions. LM-RT-PCR method performed according to previously described protocols.(23) In the ligation reaction, 500 ng of genomic DNA was added with one nmol of 12 bp (5'-TGCGGTGAGAGG-3') and 24 bp (5'-AGCACTCT CGAGCCTCTCACCGCA-3') oligonucleotides in 2.5 µL DNA ligase buffer (Cosmo Genetech, Seoul, Korea). The mixtures were heated to 55°C per10 minutes, cooled to 10°C per 55 min, then incubated at 10°C per10 min for annealing. Then, the samples were added with 20 U of T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and diluted up to 20 µL volume using purified water. The ligated DNA samples were incubated at 16°C for 12-16 hours and were stored at -80°C until PCR.

Table 1. Primary sequence for real-time PCR.

	Forward	Reverse
Bax	5'-TGGCAGCTGACATGTTTTCTGAC-3'	5'-TCACCCAACCACCCTGGTCTT-3'
Bcl-2	5'-TCGCCCTGTGGATGACTGA-3'	5'-CAGAGACAGCCAGGAGAAATCA-3'
GAPDH	5'-AGCCACATCGCTCAGACAC-3'	5'-GCCCAATACGACCAAATCC-3'

The template for RT-PCR was 50 ng of ligated DNA in 20 µL of mixtures containing 10 pmol of the 24 bp oligonucleotide primer, master mix, and purified water. PCR was carried out using reaction conditions: preincubation at 72°C per 7 minutes; enzyme activation at 95°C per 5 min; then 30 cycles at 94°C per 1 min and 72°C per 2 min for denaturation and elongation, respectively; incubation at 72°C per 5 min and 50°C per 30 s.

Apoptosis was assessed by examining DNA fragmentation expression in the cycle threshold (CT) value. The lower the DNA fragmentation CT shows increased DNA fragmentation (apoptosis) expression.

Statistical Analysis

Data were analyzed using SPSS version 25 (IBM Corporation, Armonk, NY, USA). Kolmogorov-Smirnov was used to normality test. Comparisons between the two groups were analyzed using Mann-Whitney tests or independent T-tests, and the association between two variables was analyzed using Spearman's correlation test. Multiple logistic regression was used for the analysis of multivariate variables. A p-value<0.05 was considered statistically significant.

Results

Characteristics of Subjects

Thirty early-onset and 30 late-onset preeclamptic placental samples were analyzed in this study. The characteristics of subjects in the early-onset and late-onset preeclampsia groups are described in Table 2. There was no difference in distribution between the early and late-onset preeclampsia groups regarding maternal age, body mass index (BMI), blood pressure, nulliparity, and delivery method. The classification of the preeclampsia onset group was based on gestational age, so there was a statistically significant difference between the early and late-onset preeclampsia groups in the gestational age variable.

Bax mRNA and *Bcl-2* mRNA Relative Expressions

The fold change of *Bax* mRNA in early-onset was higher than in late-onset preeclampsia (Figure 1A), and the difference was statistically significant (6.02±3.59 *vs.* 2.82±1.97; p=0.00). The fold change of *Bcl-2* mRNA in early-onset preeclampsia was higher than in late-onset preeclampsia (Figure 1B), but the difference was not

Table 2. Characteristics of subjects.

Characteristics	Preeclampsia		*p*- value
	Early-onset (n=30)	Late-onset (n=30)	
Maternal age (year), mean±SD	30.40±5.16	29.20±5.98	0.42[a]
Body mass index (kg/m^2), mean±SD	30.07±4.40	28.63±5.25	0.16[a]
Gestational age (week), mean±SD	31.43±1.94	37.40±1.89	0.00[a,*]
Blood pressure, mean±SD			
Systolic (mmHg)	172.07±19.90	164.53±16.43	0.25[a]
Diastolic (mmHg)	103.53±11.28	101.30±12.50	0.47[b]
Mean arterial pressure (mmHg)	126.33±12.67	122.47±12.54	0.24[b]
Nulliparity, n (%)			
Yes	16 (53.30%)	14 (46.70%)	0.60[c]
No	14 (46.70%)	16 (53.30%)	
Delivery method, n (%)			
Vaginal	6 (20%)	12 (40%)	0.09[c]
Cesarean section	24 (80%)	18 (60%)	

Data are represented in mean±standard deviation. [a]Tested with Mann-Whitney test; [b]Tested with Independent T-test; [c]Data frequency in percentage, tested with Chi-Square test. *Significant if p<0.05.

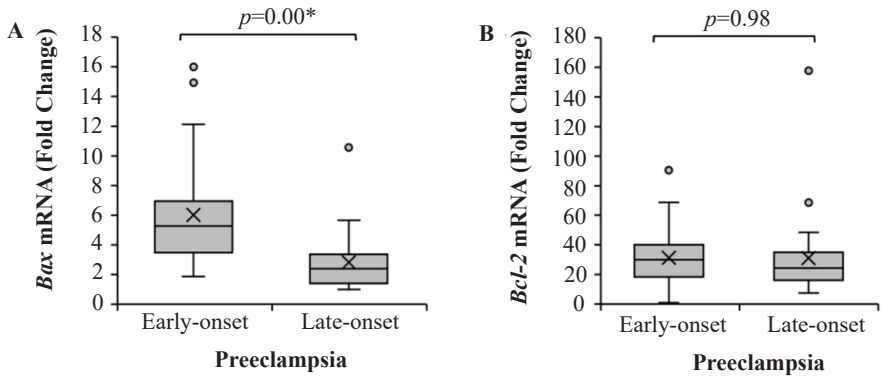

Figure 1. *Bax* mRNA (A) and *Bcl-2* mRNA (B) fold change in early-onset and late-onset preeclampsia. *Significant if $p<0.05$, tested with Mann-Whitney test.

statistically significant (31.20±17.94 *vs.* 31.01±27.60; $p=0.98$).

Logistic regression analysis among maternal characteristics, *Bax* mRNA, and *Bcl-2* mRNA fold change variables showed that only the Bax mRNA fold change significantly affects the onset of preeclampsia (Table 3).

DNA Fragmentation Relative Expressions

The CT of DNA fragmentation in the early-onset was lower than in the late-onset preeclampsia (Figure 2). The mean difference was statistically significant (28.07±0.64 *vs.* 30.63±0.96; $p=0.00$). It could be interpreted that in this study, early-onset preeclampsia had a higher relative expression of DNA fragmentation than late-onset preeclampsia.

Correlation of *Bax* and *Bcl-2* mRNA Relative Expressions with DNA Fragmentation in Preeclampsia

There was a statistically significant negative correlation between *Bax* mRNA fold change and DNA fragmentation CT with weak correlation strength (Table 4). Meanwhile, there was no significant correlation between *Bcl-2* mRNA fold change and DNA fragmentation CT. Thus, there was a weak positive correlation between the relative expression of Bax mRNA and DNA fragmentation in this study.

Discussion

Early-onset and late-onset preeclampsia have distinct pathogenesis and clinical manifestations. Hypoxic events occurring since the first trimester of pregnancy and excessive placental apoptosis characterize early-onset preeclampsia.(9) Consequently, this event impairs trophoblast invasion and spiral artery remodeling.(4,11) These disturbances lead to prolonged placental stress induced by hypoxia, further excessive apoptosis, placental dysfunction, and release of systemic inflammatory factors, thus developing into early-onset preeclampsia.(10)

Messenger RNA is the RNA (ribonucleic acid) involved in the translation process to synthesize protein.(5,6) The balance between pro-apoptotic *Bax* and anti-apoptotic *Bcl-2* protein plays an important role in regulating placental apoptosis by the mitochondrial pathway. Increased *Bax* mRNA and decreased *Bcl-2* mRNA due to hypoxic stress on trophoblast cells can trigger the apoptotic cascade in the placenta.(4,24) In this study, the relative expression of *Bax* mRNA in early-onset was higher than in late-onset preeclampsia. Meanwhile, relative expression of *Bcl-2* mRNA in early-onset was not different from late-onset

Table 3. Multivariate analysis candidates.

Variable	OR	95%CI	*p*- value
Maternal age (year)	1.04	0.95-1.14	0.40
Body mass index (kg/m^2)	1.07	0.96-1.19	0.26
Nulliparity	0.77	0.28-2.11	0.60
Bax mRNA (fold change)	1.78	1.26-2.51	0.01[a]*
Bcl-2 mRNA (fold change)	1.00	0.98-1.02	0.98

[a]Variable was included in the multivariate analysis if $p<0.25$; *Significant if $p<0.05$, tested with logistic regression analysis. OR: Odds ratio; CI: Confidence interval.

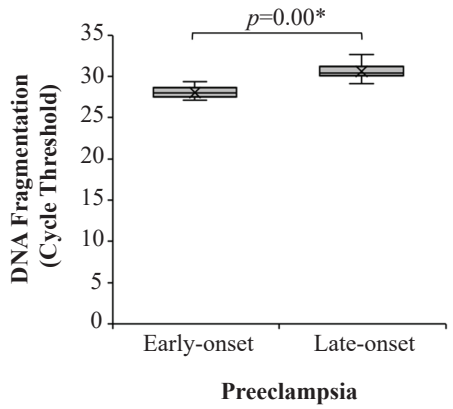

Figure 2. DNA fragmentation cycle threshold in early-onset and late-onset preeclampsia. *Significant if $p<0.05$, tested with Independent T-test.

preeclampsia. Logistic regression analysis showed that the maternal characteristics variables do not significantly affect preeclampsia onset. Moreover, it appears that the relative expression of *Bax* mRNA also has a more significant effect than the relative expression of *Bcl-2* mRNA. These results indicate that in early-onset preeclampsia, pro-apoptotic activity predominates and is more susceptible to placental apoptosis.

Evidence regarding differences in *Bax* and *Bcl-2* apoptotic markers between early-onset and late-onset still finds inconsistent results. Previous study found a significant increase in *Bax* mRNA and a decrease in *Bcl-2* mRNA under conditions of prolonged hypoxia.(25) This prolonged hypoxic condition is similar to that in early-onset preeclampsia. Another study also found that the *Bax/Bcl-2* ratio increased in preterm preeclampsia and decreased in term preeclampsia.(26) However, several studies do not align with this study. The study conducted using a mouse animal model showed no significant difference in the expression of fold change *Bax* and *Bcl-2* mRNA between the early-onset and late-onset preeclampsia groups.(27) At the proteomic level, studies found that *Bcl-2* protein expression was lower in early-onset preeclampsia.(20,28) Whereas the more recent study showed that BAX protein expression did not differ between early and late-onset preeclampsia.(19)

Apoptosis plays a critical role in the homeostasis regulation of normal placental development. However, excessive placental apoptosis leads to placental dysfunction, which may consequence in pregnancy disorders such as preeclampsia and intrauterine growth restriction.(13,24) The terminal stage of the molecular apoptotic cascade is apoptotic DNA fragmentation.(29) This study shows apoptosis occurring more predominantly in early-onset preeclampsia, characterized by a higher expression of DNA fragmentation. The results of this study are similar to the previous study, which found that early-onset preeclampsia had a higher apoptosis index by TUNEL assay.(30)

In the apoptotic cascade of the mitochondrial pathway, an increase in *Bax* expression or a decrease in *Bcl-2* will increase the cell's susceptibility to apoptotic events. In this study, the relative expression of *Bax* mRNA had a weak positive correlation with the relative expression of DNA fragmentation. Meanwhile, *Bcl-2* mRNA did not have a significant correlation with DNA fragmentation. These results indicate that *Bax* mRNA does not solely determine apoptosis susceptibility. This study cannot determine the possibility of dominance by other factors. Several possibilities may cause this discrepancy of correlations. First, other apoptotic pathways components have a more dominant role than *Bax* and *Bcl-2* mRNA. Secondly, post-transcriptional modification processes occur in *Bax* and *Bcl-2* mRNA resulting in *Bax* and *Bcl-2* mRNA degradation and causing inhibition of the *Bax* or *Bcl-2* protein translation process.

Several studies have shown the contribution of other *Bcl-2* family members in the pathogenesis of apoptosis in preeclampsia. Studies show that Bcl-X_L protein expression and the *Mtd/Mcl-1* ratio balance are essential in preeclamptic trophoblastic cell apoptosis.(31,32) In addition, some evidence suggests that epigenetic factors are regulated in post-transcriptional modification involving microRNAs (miRNA).(33,34) The dysregulation of these miRNAs will

Table 4. Correlation of the relative expression of *Bax* mRNA and *Bcl-2* mRNA with DNA fragmentation in preeclampsia.

	DNA Fragmentation (Cycle Threshold)		
	n	**R**	***p*-value**
Bax mRNA (fold change)	60	-0.3	0.02*
Bcl-2 mRNA (fold change)	60	-1.67	0.20

*Significant if $p<0.05$, tested with Spearman correlation test.

disrupt placental development and pregnancy, such as fetal growth restriction and preeclampsia.(35) Studies show that miR-29b, miR-34a, and miR-128a have a role in increasing the apoptosis of trophoblastic cells in preeclampsia by regulating the expression of *Mcl-1*, *Bcl-2*, and *Bax* proteins, respectively.(36-38)

In this study, the classification of the preeclampsia onset group was based on gestational age. The gestational age variable cannot be excluded as a confounding factor that could influence the results because apoptosis-related markers also undergo dynamic changes as gestational age increases. Therefore, further research is still needed to comprehensively assess the role of *Bax* mRNA expression in the onset of preeclampsia, using a control group adjusted for gestational age and involving other factors that influence the apoptotic cascade, such as other *Bcl-2* family members and miRNA expression. In addition, a cohort study using blood plasma samples from patients with preeclampsia also might be considered for developing *Bax* mRNA as a potential biomarker in early-onset preeclampsia.

Conclusion

Bax mRNA showed significant correlation in DNA fragmentation compared to *Bcl-2* mRNA; hence, might show more role in apoptotic pathway. Compared to late-onset, early-onset preeclampsia has higher *Bax* mRNA relative expression and apoptosis. This suggest that *Bax* mRNA can be potential biomarker in early-onset preeclampsia. This study may contribute to understanding the pathogenesis of the placental apoptosis pathway to develop a new potential biomarker in patients with early-onset preeclampsia.

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Authors Contribution

MJI collected the sample, analyzed the research data, and drafted the manuscript. DRH and DSH were involved in designing the protocol, interpreting the result, and supervising the study. All authors discussed and provided feedback on the manuscript.

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