

## Apoptotic, MDA, and FGF2 Level of Quercitrin Treatment on Hypoxic-induced EA.hy926 Cell Line

### Abstract

**Background:** Preeclampsia (PE) is pregnancy disorder that is characterized by hypertension, proteinuria, and an enhanced maternal systemic inflammatory response. PE affects 5% to 10% of all pregnancies and remains a leading factor of fetal and maternal morbidity and mortality. The existence of oxygen deprivation is involved in PE. Inflammation is a requisite to the pathogenesis of PE. Quercitrin belongs to the flavonoid group that is known to have antioxidant and anti-inflammatory activity. **Aims:** This study aims at determining the potential of quercitrin to reduce the percentage of apoptosis, levels of lipid peroxidase (MDA), and FGF2 in the human endothelial cell (EA.hy926) line that is induced by hypoxia (2% O<sub>2</sub>) as a PE model. **Materials and Methods:** Five treatments were used in this study (negative control, vehicle control, hypoxia control, quercitrin 25 µg/mL, and quercitrin 6.25 µg/mL) to determine the live, necrotic, and apoptotic cells percentage; MDA and FGF2 levels toward hypoxia-induced endothelial cells as a PE model. ELISA method was used to measure the MDA and FGF2 levels. Live, necrotic, and apoptotic cells were measured by using the flow cytometry method. **Result:** Quercitrin was capable of decreasing the MDA and FGF2 levels compared with hypoxia control; of increasing live cells percentage; and of decreasing apoptotic and necrotic cells percentage compared with hypoxia control cells. **Conclusion:** This study showed that quercitrin possesses antioxidant and anti-inflammatory properties that can decrease the percentage of the apoptotic cells, suppress MDA levels and FGF2 levels, and increase live cells percentage in hypoxia-induced endothelial cells as a PE model.

**Keywords:** Apoptotic, hypoxia, preeclampsia, quercitrin

### Introduction

PE is a pregnancy syndrome that is characterized by hypertension and proteinuria after 20 weeks of gestation.<sup>[1,2]</sup> PE is a very significant disease that affects 5% to 7% of pregnant women worldwide per year. PE is a fairly common pregnancy condition and it is defined as maternal hypertension, endothelial dysfunction, proteinuria, and increased systemic inflammatory response.<sup>[3,4]</sup> PE often occurs with premature delivery of the fetus and is often a significant cause of fetal and perinatal morbidity and mortality.<sup>[5]</sup> The causes of PE are complex and partially understood; there is a consensus that factors released by the placenta, which are possibly a consequence of faulty placentation, may cause the onset of maternal disease by causing endothelial dysfunction and trigger hypertension and proteinuria as a result.<sup>[3,6]</sup>

PE can be triggered by placental or endothelial apoptosis, which could be linked to local

hypoxic conditions and also with the oxidative stress caused by free radicals.<sup>[7]</sup> Numerous studies have reported on the correlation between apoptosis and PE. Apoptosis happens during normal embryonic development and also in the transition of mature tissues. However, apoptosis is often present in tissues that are exposed to exogenous stimuli, such as cytotoxic agents or hypoxia conditions. Fibroblast growth factor (FGF) is a growth factor that has shown potential effects on the repair and regeneration of tissues. FGF2 is one of the FGFs subfamily that promotes the angiogenesis process. Specifically, FGF1 and FGF2 induce the promotion of endothelial cells proliferation and the physical organization of endothelial cells into tube-like structures.<sup>[8]</sup> In a recent study, increased FGF2 production by endothelial cells under hypoxic conditions was related with PE condition.<sup>[9]</sup>

PE is also caused by oxidative stress, which is linked to the free radicals.<sup>[7]</sup> During PE, the production of lipid peroxide is increased as a result of increased oxidative stress.<sup>[10]</sup> In

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Chrismis Novalinda Ginting<sup>1</sup>,  
I Nyoman E. Lister<sup>1</sup>,  
Ermi Girsang<sup>1</sup>,  
Dwi Surya Artie<sup>2</sup>,  
Jenifer Kiem Aviani<sup>2</sup>,  
Wahyu Widowati<sup>3</sup>

<sup>1</sup>Faculty of Medicine, Universitas Prima Indonesia, North Sumatera, <sup>2</sup>Biomolecular and Biomedical Research Centre, Aretha Medika Utama, West Java, <sup>3</sup>Faculty of Medicine, Maranatha Christian University, West Java, Indonesia

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### Address for correspondence:

Dr. Chrismis Novalinda Ginting,  
Faculty of Medicine, Universitas  
Prima Indonesia, Jl. Belanga  
No. 1, Medan 20118, North  
Sumatera, Indonesia.  
E-mail: [chrismis@unprimdn.ac.id](mailto:chrismis@unprimdn.ac.id)

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this case, antioxidant compounds were chosen to handle this imbalance of oxidative stress in a pregnant woman with PE. In a human body, endogenous antioxidants had already been present but the exogenous antioxidants are still needed for balancing the number of free radicals and antioxidants for a proper physiological function.<sup>[11]</sup> Antioxidants inhibit the oxidation by reacting to reactive free radicals, which form reactive substances that are relatively stable. Nowadays, a natural antioxidant is chosen more than a synthetic antioxidant due to its side effects and being less toxic when consumed over a long term. Several studies have determined that phytochemical compounds contain rich pharmacological properties. Quercitrin belongs to the flavonoids group, one of the phytochemical compounds that is easy to get and that is mainly presented in nature and also considered to have positive effects on health. Quercitrin shows strong antioxidant activity by maintaining oxidative balance.<sup>[12]</sup> Previous studies have shown that quercitrin exerts downregulation activity in inflammatory responses.<sup>[13,14]</sup> Numerous molecular markers of PE have been studied in the past; nevertheless, the importance of oxidative stress and apoptosis has not been well recorded. In this research, we presumed that the administration of quercitrin as a flavonoid compound that contains high antioxidant activity<sup>[12]</sup> could reduce the percentage of apoptosis, lipid peroxidase (MDA), and FGF2 in EA.hy926 cell line modeled as hypoxia-induced PE.

## Materials and Methods

### Cell culture

The EA.hy926 (ATCC® CRL-2922TM) cells were obtained and cultured in Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Biowest, L0104-500) supplemented with 10% fetal bovine serum (FBS) (Biowest, S181-500), 1% nanomycopolitine (Biowest, L-X16-100), 1% antibiotic/antimycotic (ABAM) (Biowest, L0010-100), 1% Amphotericin B (Biowest, L0009-050), and 0,1% Gentamicin (Gibco, 15750060), respectively. The cells were incubated at 37°C with 5% CO<sub>2</sub>. The cells were treated with quercitrin (Chengdu Biopurify Phytochemicals Ltd, BP1192) (25 µg/mL and 6.25 µg/mL; this concentration is determined from optimization) and incubated in hypoxia condition (2% O<sub>2</sub>) at 37°C for another 24 h. The treatments were as follows: negative control (untreated-Ea.Hy926 cells), hypoxic (2% O<sub>2</sub>), vehicle control (Ea.Hy926 cells + DMSO 1%), hypoxia + quercitrin 25 µg/mL, and hypoxia + quercitrin 6.25µg/mL. Each treatment was carried out thrice.<sup>[14]</sup>

### Hypoxia-induced EA.hy926 (2% O<sub>2</sub>)

EA.hy926 cells were cultured in DMEM high glucose (Biowest, L0104-500) supplemented with 10% FBS and 1% ABAM. The cells were plated in a six-well plate with a density of 150,000 cells/well and incubated at 37°C with 5% CO<sub>2</sub> for 24 h. Then, the cells were incubated in 2% O<sub>2</sub> for the next 24 h to mimic the PE condition. After incubation, the cells were treated with

25 µg/mL and 6.25 µg/mL quercitrin and incubated again in 2% O<sub>2</sub>, at 37°C for another 24 h. Conditioned medium (CM) and cells were collected after treatment for further assay.<sup>[14]</sup>

### Measurement of live, apoptotic, and necrotic cells

Measurement of live, apoptotic, and necrotic cells was determined by flow cytometry. Cells were washed with PBS 1X (Biowest X0515-500) and harvested with trypsin-EDTA (0.25-0.038%) (Biowest, L0931-500). The cells were centrifuged at 1600 rpm for 5 min; then, the supernatant was discarded. The pellet was washed twice using 500 µL Annexin Binding Buffer 1X (Miltenyi Biotec, 130-092-820); then, it was centrifuged in 1600 rpm for 5 min. The pellet was washed using 100 µL Annexin Binding Buffer (Miltenyi Biotec, 130-092-820); then, it was stained with Annexin V-FITC (BioLegend, Part 79998) and Propidium Iodide (PI) (BioLegend, Part 79997). The cells continued to be incubated at 4°C in the darkness and the apoptotic percentage of EA.hy926 cells was analyzed using MACSquant Analyzer 10 (Miltenyi Biotec).<sup>[15]</sup>

### Measurement of malondialdehyde content

MDA content is an index of free radical generation/lipid peroxidation. An MDA assay was measured using MDA Assay Kit (Elabscience, E-BC-K025-S) and this was done according to the manufacturer's protocol. Conditioned medium of the cells was taken and used as test sample. Four group tubes were used and marked as blank, standard, sample, and control. Each tube contained a different composition that was used in the assay and read at 532 nm using the microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Waltham, MA, USA).<sup>[16,17]</sup>

### Measurement of FGF2 levels

CM from the cells was collected and used as a sample test. The FGF2 measurement was done using the human bFGF/FGF2 ELISA Kit (Elabscience, E-EL-H0483) and conducted according to the manufacturer's protocol. The treatment that was used in this assay was as follows: (A) negative control, (B) vehicle control, (C) positive control, (D) positive control + quercitrin 25 µg/mL, and (E) positive control + quercitrin 6.25 µg/mL.<sup>[14,17]</sup>

## Results

### Effect of quercitrin on apoptosis of Eahy.926 cells

This study was conducted to determine the effect of quercitrin on apoptosis of Eahy.926 cells, and this was analyzed using flow cytometry. PI and Annexin surface markers have been used as stains. Five treatments were used (negative control, vehicle control, positive control, positive control + quercitrin 25 µg/mL, positive control + quercitrin 6.25 µg/mL). To ensure that the toxicity of DMSO 1% did not affect the results, DMSO 1% was used as vehicle control. Figure 1A shows the comparison between each treatment based on live cells (I), early apoptosis (II), late apoptosis (III), and necrotic cells (IV). Figure 1B represents the dot plot data from flow cytometry analysis

results that contain the percentage of live cells, early apoptosis cells, late apoptosis cells, and necrotic cells in each treatment.

### MDA analysis

The CM from each treatment was collected and used as a sample in MDA analysis. Figure 2 depicts information about the MDA level that tested the EA.hy926 cell line as a PE model. This result was compared with the negative control and positive control. Both concentrations of quercitrin that were used (25 and 6.25 µg/mL) in this treatment showed a significant difference of MDA content compared with the negative control (normal cells) and positive control (hypoxia control). The total protein assay was used to measure the MDA content by nmol/mg protein. The result shows that there is a decrease of MDA content compared with the positive control, but yet not significantly different for quercitrin 6.25 µg/mL [Figure 2B].

### FGF2 level

Measurement of FGF2 level was performed by Human bFGF/FGF2 ELISA Kit (Elabscience, E-EL-H0483), and this was done according to the manufacturer's protocol. Figure 3A and B depicts information about the FGF2 level on the EA.hy926 cell, which has been modeled as a PE. The result shows a significant decrease of FGF2 level compared with the positive control and a significant difference compared with the negative control. The total protein assay was used to measure the FGF2 level by pg/mg protein [Figure 3A and B].

### Discussion

PE is a chronic condition that is characterized by inflammatory and angiogenic status.<sup>[18]</sup> In this study, we used the EA.hy926 cell line, which is an endothelial cell line that can be modeled as PE with hypoxic condition (2% O<sub>2</sub>) and treated with quercitrin with various concentrations. Apoptosis assay was done by the flow cytometry method.<sup>[15]</sup> The apoptotic percentage of hypoxia-induced EA.hy926 cells shows that hypoxia induction can reduce the cell viability level. It can be seen from the apoptotic assay results that the number of EA.hy926 live cells on induced hypoxia as a PE model has decreased compared with the normal cells without hypoxia induction [Figure 1A]. However, the number of cells undergoing apoptosis in Ea.Hy926 cells with hypoxia induction was the highest compared with the number of cells undergoing apoptosis in other treatments. This study shows that hypoxia induction can cause apoptotic cells in the EA.hy926 cells as a PE model [Figure 4]. The rate of apoptosis is believed to have risen in PE due to hypoxia reperfusion and oxidative stress.<sup>[19,20]</sup>

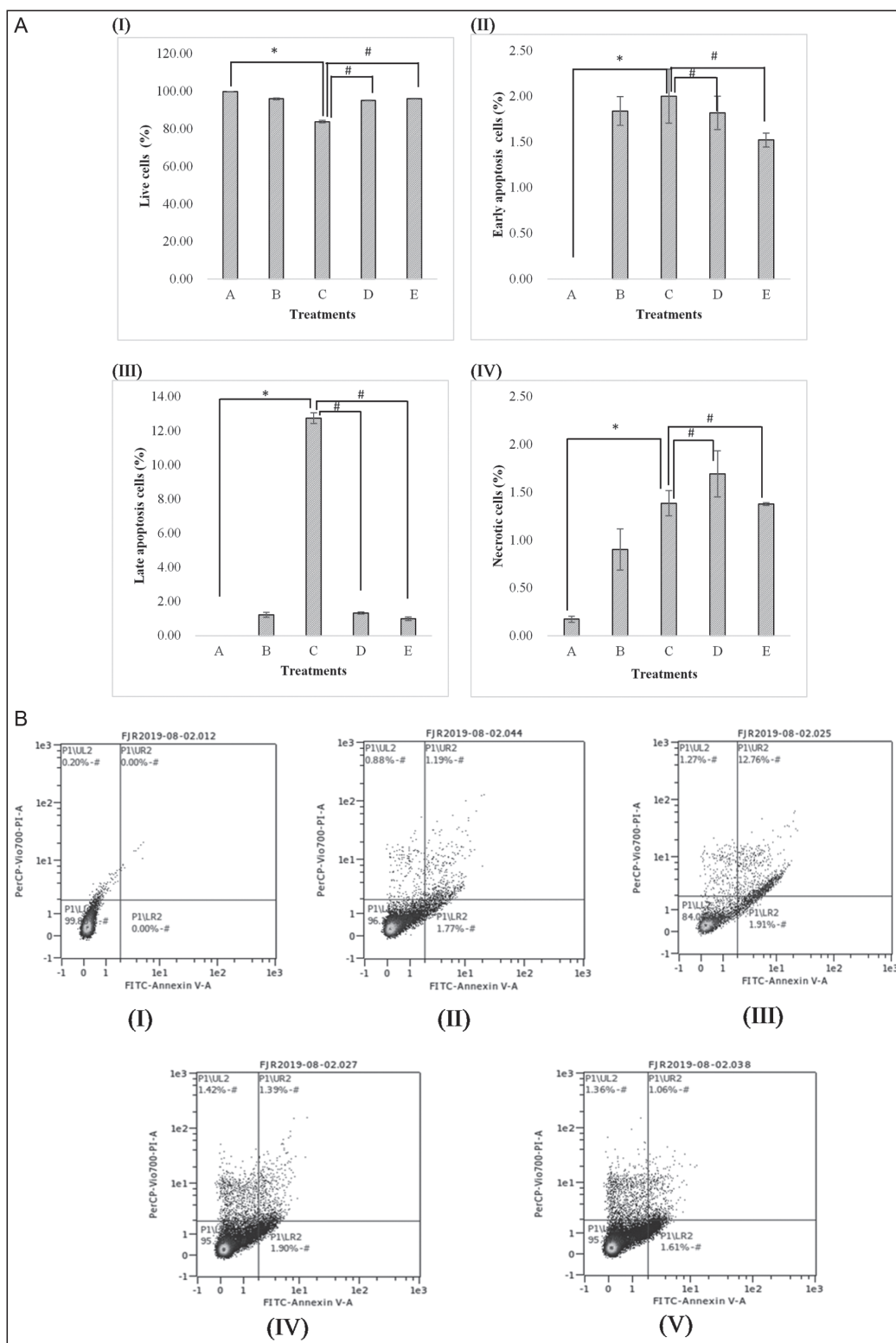
According to a previous study, hypoxia may not be the sole inducer of placental apoptosis. In PE, oxidative free radicals are often increased, although not universally.<sup>[21]</sup> This oxidative stress may be linked with increased placental apoptosis and increased turnover of the syncytiotrophoblast.<sup>[22]</sup> Oxidative stress is known to cause apoptosis in various cell types.<sup>[23]</sup> PE can be triggered by placental or endothelial apoptosis that happened in a pregnant woman.<sup>[7]</sup> Systemic endothelial

disruption tends to be a significant factor in the signs and symptoms of PE. Systemic endothelial damage is caused by microdeposition of syncytiotrophoblast microvillous membrane particles.<sup>[24]</sup> In normal pregnancies, these particles can be detected but they also increase in women with PE; these increases may be caused by enhanced apoptosis at the syncytium. Endothelial tissue apoptosis could be linked to the toxic impact of excessive syncytial particles, but they may also be due to local hypoxic conditions caused by the constriction of efferent blood vessels or to the effect caused by free radicals.<sup>[7]</sup>

In a normal pregnancy, the formation of free radicals and lipid peroxidation occurs at a low level. Lipid peroxidation formation is at its peak when it is in the second trimester of pregnancy. In a woman with a normal pregnancy, lipid peroxidation and free radicals formation are regulated by the adequate response of antioxidants that tries to minimize the cellular damage.<sup>[25]</sup> During PE, oxidative stress progressively increases and the formation of lipid peroxides, reactive oxygen species, and superoxide anion radicals are increased as a result, which leads to endothelial destruction and dysfunction.<sup>[25,26]</sup> Free radicals cause oxidative damage to cellular macromolecules in tissues such as nucleic acids, proteins, and lipids. Free radical-induced oxidation of polyunsaturated fatty acids in cells results in the formation of lipid peroxidation products such as MDA, which is used as a biomarker of lipid peroxidation.<sup>[27]</sup>

In this study, the MDA level in cells induced with hypoxia condition shows a significant difference compared with the normal and vehicle control [Figure 2]. The positive control has the highest MDA level. This showed that hypoxia is a strong stimulator that promotes the production of oxygen-free radicals and it enhances lipid peroxides production. The decrease in MDA levels from quercitrin treatment was significant when compared with positive control, quercitrin administration was successful in reducing MDA levels. These findings are in line with a previous study, which reported that lipid peroxidation is increased during normal pregnancy but the production is more abundant in a woman with PE and may be an important factor in the pathogenesis of PE.<sup>[25,26,28,29]</sup>

Hypoxia condition is stimulated FGF2 expression in the EA.hy926 cell line that is hypoxia induced.<sup>[9,30]</sup> These statements are in line with the research findings that FGF2 levels were increased in hypoxia conditions [Figure 3]. This result is the same as that of Luo *et al.* (2011), hypoxia conditions are stimulated FGF2 expression in both mRNA and protein in HUVEC-12 as an endothelial cell line. However, in this study, EA.hy926 as the other endothelial cell line was used as modeled by PE by hypoxia induction. In positive control, the FGF2 protein level was increased compared with the negative control. As a result, FGF2 level decreased after being treated with quercitrin in both concentrations (25 µg/mL and 6.25 µg/mL). FGF2 has an important role during angiogenesis, in line with FGF1. The FGF1 and FGF2 induced the promotion of endothelial cell proliferation and the physical organization



**Figure 1: A.** Effect of quercitrin on PE cell models toward (I) Live cells, (II) Early apoptosis (Apoptosis), (III) Late apoptosis (Death cells), and (IV) Necrotic cells. Treatments: (A) negative control (untreated cells), (B) vehicle control (cells+DMSO 1%), (C) positive control (hypoxia-induced cells), (D) positive control+quercitrin 25  $\mu\text{g}/\text{mL}$ , and (E) positive control+quercitrin 6.25  $\mu\text{g}/\text{mL}$ . \*Data are presented as mean  $\pm$  standard deviation. Single star (\*) marks significant difference compared with the negative control group, and single hashtag (#) marks significant difference compared with the positive control ( $P < 0.05$ , Tukey's HSD test). **B.** Representation of dot plots on hypoxia-induced Ea.Hy926 cells that are treated with quercitrin in various concentrations toward apoptosis percentage using flow cytometry. (I) Negative control (untreated cells): live cells: 99.80%, necrotic: 20.0%, early apoptosis: 0.0%, late apoptosis: 0.0%. (II) Vehicle control (cells + DMSO 1%): live cells: 96.36%, necrotic: 0.88%, early apoptosis: 1.77%, late apoptosis: 1.19%. (III) Positive control (hypoxia-induced cells): live cells: 84.06%, necrotic: 1.27%, early apoptosis: 1.91%, late apoptosis: 12.76%. (IV) Positive control+quercitrin 25  $\mu\text{g}/\text{mL}$ : live cells: 95.29%, necrotic: 1.42%, early apoptosis: 1.90%, late apoptosis: 1.39%. (V) Positive control+quercitrin 6.25  $\mu\text{g}/\text{mL}$ : live cells: 95.97%, necrotic: 1.36%, early apoptosis: 1.61%, late apoptosis: 1.06%

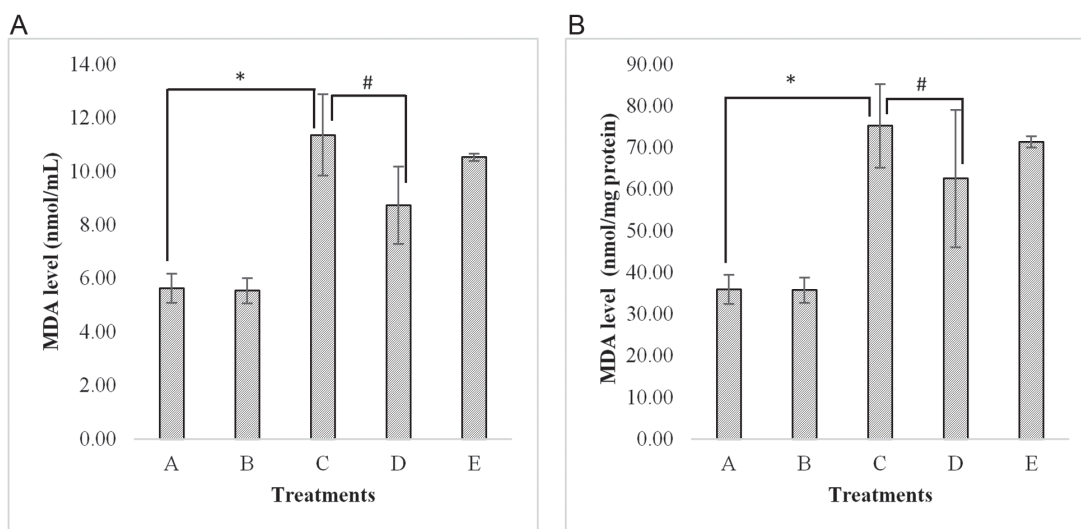


Figure 2: Effect of quercitrin toward MDA level on PE cell models. (I) MDA level (nmol/mL), (II) MDA level (nmol/mg protein). Treatments: (A) negative control (untreated cells), (B) vehicle control (cells + DMSO 1%), (C) positive control (hypoxia-induced cells), (D) positive control + quercitrin 25 µg/mL, and (E) positive control + quercitrin 6.25 µg/mL. \*Data are presented as mean  $\pm$  standard deviation. Single star (\*) marks significant difference compared with the negative control group, and single hashtag (#) marks significant difference compared with the positive control ( $P < 0.05$ , Tukey's HSD test)

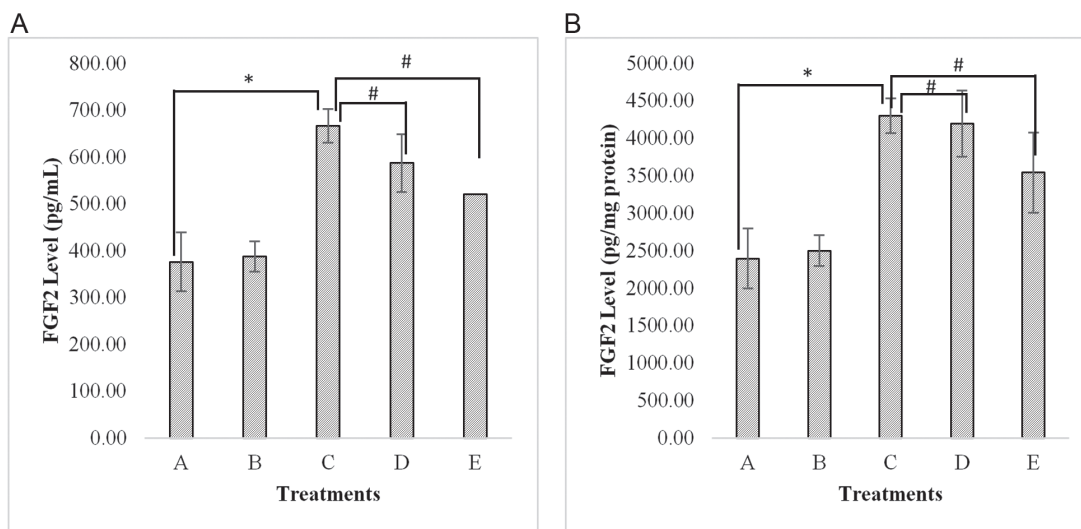


Figure 3: Effect of quercitrin toward FGF2 level on PE cell models. (I) FGF2 level (pg/mL), (II) FGF2 level (pg/mg protein). Treatment: (A) negative control (untreated Ea.Hy926 cells), (B) vehicle control (Ea.Hy926 cells+DMSO 1%), (C) positive control (hypoxia-induced Ea.Hy926 cells), (D) positive control+quercitrin 25 µg/mL, and (E) positive control+quercitrin 6.25 µg/mL. \*Data are presented as mean  $\pm$  standard deviation. Single star (\*) marks significant difference compared with the negative control group, and single hashtag (#) marks significant difference compared with the positive control ( $P < 0.05$ , Tukey's HSD test)

of endothelial cells into tube-like structures.<sup>[31]</sup> Maria *et al.* (2002) found that FGF2/bFGF could not be used as a prognostic factor for serious PE, although there appears to be a substantial correlation between serum FGF2/bFGF and minor PE. There is no significant difference between minor PE women and healthy pregnant controls. Thus, these findings have different results compared with our study, which used the Ea.hy926 cell line that was treated with hypoxia condition as a PE model. Previous research showed that elevated FGF2/bFGF serum concentrations in PE are associated with minor hypertensive disorders in pregnancy, but it does not seem to be a prognostic factor for serious PE.<sup>[31]</sup>

In accordance with Ginting *et al.*, quercitrin belongs to the quercetin-related flavonoids compound that has antioxidant and anti-inflammatory activity. In this study, two concentrations of quercitrin were given to the cell-hypoxia induced EA.hy926. Quercitrin treatment on the cell EA.hy926 can reduce the number of apoptotic cells. Both the concentrations of quercitrin used in this treatment (25 and 6.25 µg/mL) can be used to reduce the percentage of apoptosis compared with the hypoxia control.<sup>[12]</sup> Also, they can reduce the levels of lipid peroxide (MDA) and protein FGF2, which is significantly increased in pregnant women with PE. Antioxidant compounds are often chosen to handle disease with a case of an imbalance of

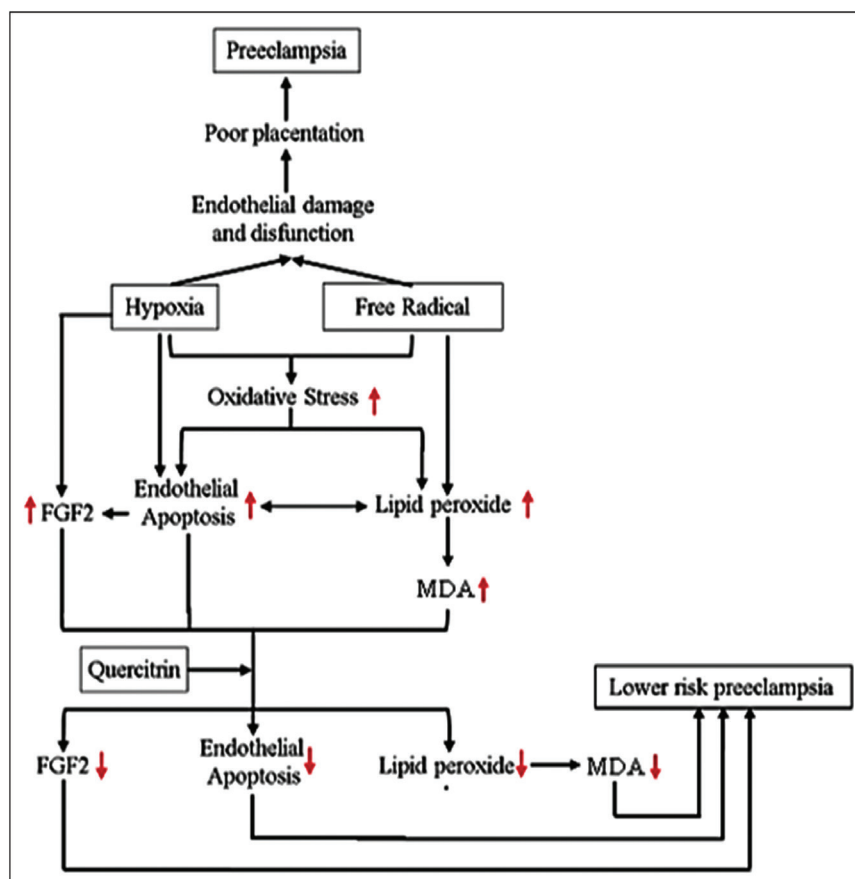


Figure 4: Mechanism of hypoxic effects on apoptosis, MDA, and FGF2 levels in PE cell models. PE is triggered by a free radical, hypoxia condition that induces the increase of FGF2 level, increasing endothelial apoptosis, and increasing lipid peroxidation and MDA level. Quercitrin is a flavonoid compound that has the ability to decrease FGE2 level, endothelial apoptosis, and MDA level, which could lower PE risk.

oxidative stress, which is PE in this study. In a human body, endogenous antioxidants are already present but exogenous antioxidants are still needed for balancing the number of free radicals and antioxidants for a proper physiological function.<sup>[11]</sup> Antioxidants inhibit oxidation by reacting to reactive free radicals to form reactive substances that are relatively stable. The ability of antioxidants to prevent oxidative damage can play a key role in PE.<sup>[20]</sup>

## Conclusion

This study showed that quercitrin possesses antioxidant and anti-inflammatory properties that can decrease the percentage of apoptotic cells, suppress MDA levels and FGF2 levels, and increase live cells percentage in hypoxia-induced endothelial cells as PE model.

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## Conflict of interest

All contributing authors declare no conflicts of interest.

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