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Low Expression of Calcitriol Level and Interleukin-10 and Hypoxia-inducible Factor-1 Alpha Expression on Placenta

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Abstract

BACKGROUND: Preeclampsia (PE) is the disease of theories and the second leading cause of maternal and perinatal morbidities and mortalities worldwide. These pathological disturbances will induce inflammation process, oxidative stress, and poor subsequent growth on the fetus including 32% of intrauterine growth restriction, 22% of prematurity, and 24% of neonatal sepsis and asphyxia. There are many theories about the mechanism of PE. In the molecularly level, it is suspected that the low level of calcitriol and interleukin (IL)-10 expressions and high expression of hypoxia- inducible factor (HIF)-1α are the risk factors of PE.

AIM: The aim of this study was to prove the low calcitriol level, IL-10, and high expression of HIF-1α in the placenta as the risk factors of PE.

METHODS: A nested case–control study was conducted at the Department of Obstetrics-Gynecology Sanglah and Wangaya Hospital Denpasar, Bali, from November 2020 to February 2021.

RESULTS: A total of 64 samples of 20–40 weeks gestation age were selected by purposive consecutive sampling, divided into two groups consist of 32 PE as the cases and 32 non-PE as the controls. The material examination, 3 × 3 cm was isolated from the maternal placental surface, was performed at Laboratorium Biomedik Terpadu, Faculty of Medicine, Udayana University. We performed an ELISA technique to find out the calcitriol level; in the other hand, we used immunohistochemistry for detected expression of IL-10 and HIF-1-α. The results revealed the risk of PE in low placental calcitriol levels about 13.8 times higher than in high calcitriol levels (odds ratio [OR] = 13,801, 95% confidence interval [CI] = 4.048–47,050, p = 0.001. The risk of PE in low placental IL-10 expression about 6.6 times higher than in high IL-10 expression (OR = 6600; 95% CI = 2208–19,728; p = 0.001). The risk of PE in high placental HIF-1-α expression about 5.6 times higher than in low placental HIF-1-α expression (OR = 5.622; 95% CI = 1.922–16.450; p = 0.001).

CONCLUSION: Low calcitriol level, low IL-10, and high HIF-1-α expression on the placenta were proved as significant risk factors for the development of PE.

Introduction

Preeclampsia (PE) is a specific clinical syndrome in gestational age ≥20 weeks which is characterized by maternal blood pressure ≥140/90 mmHg and accompanied by proteinuria, and/or impaired liver, hematological, and kidney function. Until now, PE is still a health problem for pregnant women related to prevalence, morbidity, maternal and perinatal mortality, and risk factors.

The reported prevalence of PE differs from country to country depends on the development of the country and geography. A systematic review of 74 reports and 78 data sets on 39 million women from 40 countries, the prevalence of PE was 4.6% (prevalence ratio 2.7–8.2) [1]. In developed countries, such as Europe and the United States, the prevalence of PE is 2.31–3.8% [2]. Meanwhile, in some developing countries such as South Africa, Egypt, Tanzania, Nigeria, and Ethiopia, the prevalence of PE is 2.0–16.7% [3]. In Indonesia, the prevalence of PE in several teaching hospitals in Indonesia is 3–10% [4] and in Sanglah Hospital Denpasar in 2011, it was 7.31% and in 2013 it was 9.23% [5].

The maternal morbidity of PE, including eclampsia, intra and postpartum bleeding, high care unit care, post-seizure sequelae, and psychiatric problems requires high time and cost. Meanwhile, perinatal morbidity consisted of 22.0% prematurity, 32.0% intrauterine growth restriction, 24.0% neonatal intensive care unit related to neonatal sepsis, and asphyxia with a neonatal mortality rate of ±15%. Most neonatal deaths occur related to low birth weight between 1000 and 2000 g [6], [7]. In addition, the maintenance costs that arise will certainly increase in line with the treatment. PE mortality in the world is 18.0% of the maternal mortality rate (MMR) which is estimated at 62,000–77,000/year [1]. Meanwhile, PE mortality in the United States was 7.4% of MMR for the period 2011–2013 [8]. Meanwhile in Indonesia, MMR related to PE is 27.1% which is the second largest

cause of maternal death after intra and postpartum bleeding [9].

Scientists and clinical practitioners have agreed that the cause of PE is pregnancy itself. However, the mechanism of PE is still in the form of theories involving hormones, antigen-antibody, free radicals, metabolism, spiral artery remodeling, and placental ischemia. All of these theories have not received broad agreement so that until now PE is still a disease of theories; controversy.

The difference in the prevalence of PE which is significantly higher in developing and equatorial countries raises the suspicion that nutritional and environmental factors play an important role as risk factors for PE. In addition, demographic differences, sun exposure, and lifestyle are thought to be related to Vitamin D levels. Levels of several micronutrients such as iron, calcium, phosphorus, iodine, zinc, magnesium, selenium, copper, chlorine, manganese, and cobalt are lower in PE compared to without PE [10], [11]. In addition to the above micronutrients, a nutritional deficiency that is also thought to be associated with the occurrence of PE is Vitamin D. This suspicion arises based on the fact that the incidence of PE is reported to be higher in winter and/or autumn when the sun exposure is low compared to summer. This variation in the incidence of PE is thought to be related to variations in Vitamin D levels which also vary according to the intensity of sun exposure [12]. Singh *et al.*, reported low levels of Vitamin D in the blood serum of pregnant women with PE and non-PE were 82.8% versus 31.25% and 51.78% severe PE found Vitamin D deficiency [13]. However, the role of Vitamin D in the mechanism of PE is still controversial where Murat Bakacak, (2015) and Anupama *et al.*, stated that there is a positive relationship between vitamin D and the occurrence of PE [14]. Otherwise, Goel *et al.*, and dan Umar *et al.*, reported that blood plasma Vitamin D deficiency was not significantly different in PE compared to non-PE [15], [16].

Until now, the placenta is still the center of research on PE. In placental trophoblast cells and endometrial decidua, large amounts of the enzyme 1 hydroxylase (CYP27B1) and Vitamin D receptors were found, leading to the suspicion that calcitriol plays a major role in pregnancy, starting from the placentation process, trophoblast cell invasion, inflammation, angiogenesis and immunomodulator [17]. Calcitriol is thought to increase the ability to invade extravillous trophoblast cells (EVT) into decidual and endothelial cells of blood vessels supplying the uterus. *In vitro* studies showed an increase in the invasion of EVT cells in placental tissue given calcitriol. Calcitriol stimulates mesenchymal differentiation from trophoblast cells by decreasing the expression of E-cadherin (an epithelial marker) and increasing the expression of vimentin (a mesenchymal marker). Calcitriol also activates extracellular-signal-regulated-kinase (ERK) signaling via phosphorylation and facilitates the secretion of matrix metalloprotein-2 (MMP-2) and MMP-9 [18], [19]. Calcitriol is also thought to play a role in the regulation of the immune system where placental calcitriol can trigger Th2 cells to increase interleukin (IL)-10 secretion. The role of IL-10 in pregnancy is an anti-inflammatory in the embryo allograft process.

In PE, it is suspected that there is an increase in the Th1/Th2 ratio so that the inflammatory process will increase. One mechanism by which IL-10 lowers blood pressure is by inhibiting Th1 cytokines, including TNF-, which is a hallmark of PE. Research by Smith *et al.* showed that pregnant women with high levels of IL-10 in the blood circulation had low blood pressure during pregnancy [20]. The role of IL-10 in PE is also controversial. Benian *et al.* found that the levels of IL-10, transforming growth factor beta 1 (TGF-1) and E-cadherin plasma and placental PE were increased along with the increase in diastolic blood pressure in PE [21].

As a result of disruption of trophoblast invasion, there will be disruption of spiral artery remodeling which will cause placental hypoxia. In hypoxic conditions, it will trigger the release of hypoxia-inducible factor-1 alpha (HIF-1α) as a physiological response. Upregulation of HIF-1α which is a transcription factor of a large number of genes will increase the expression of vascular endothelial growth factor (VEGF) [22]. The HIF-1 α protein is known to mediate the delivery of oxygen and other nutrients during the process of angiogenesis and the protein binds to elements that respond to hypoxia in increasing the activity of several genes involved in adaptation to a hypoxic environment [23]. However, if placental hypoxia and ischemia are prolonged due to impaired spiral artery remodeling, HIF-1α will also be expressed at sustained high levels. This will induce the release of sFlt-1 and sEng where sFlt-1 and sEng are anti-angiogenic proteins that play a role in endothelial damage in PE [24].

Thus, calcitriol levels and low IL-10 expression are thought to trigger the failure of the invasion of placental trophoblast cells resulting in hypoxia, ischemia, and placental inflammation. Conditions of hypoxia, inflammation, and high levels of HIF-1 will continuously increase the production of anti-angiogenic factors such as sFlt-1 and sEng and reduce the production of placental growth factor (PLGF) and VEGF. This increase in the sFlt-1/PLGF ratio will be associated with endothelial damage which is the beginning of the clinical syndrome of PE [25].

Based on the background explained above, the problems found are as follows; is low calcitriol level in the placenta a risk factor for PE?, is low IL-10 expression in the placenta a risk factor for PE?, is high HIF-1-α expression in the placenta a risk factor for PE? And which risk factors are most involved and their correlation to the mechanism of PE?

Materials and Methods

Theoretical framework, conceptual framework, and research hypotheses theoretical framework

The levels of calcitriol, IL-10, and HIF-1α in placental tissue are thought to play a role in the mechanism of PE.

Placental calcitriol plays a role in the placentation process and is an immunomodulator through down-regulation of E-cadherin and up-regulation of vimentin, resulting in remodeling of spiral arteries. As an immunomodulator, calcitriol is thought to decrease the Th1/Th2 ratio, increasing IL-10 and other anti-inflammatory cytokines.

The HIF-1α protein as a major marker of the placental ischemia that is prolonged overexpression will lead to increased transcription of genes encoding fms sFlt-1, sEng, and ET-1 associated with PE.

IL-10 expression in placenta is thought to be low in PE due to the Th1/Th2 ratio which acts as an anti-inflammatory related to spiral artery remodeling. This initiates inflammation and oxidative stress that triggers the release of mediators that cause PE.

Research design

The design of this research is an unmatched nested case–control study. The case group was live singleton pregnancies at 20–40 weeks' gestation who suffered from PE who went into labor. The control group was a single live pregnancy at 20–40 weeks' gestation who did not suffer from PE/Non-PE who went into labor. The levels of calcitriol, HIF-1α protein, and IL-10 were measured on postpartum placental tissue material.

This study was conducted in the Maternity Room in the Emergency Installation of Sanglah Hospital and the Wangaya Regional General Hospital Denpasar. The ELISA and immunohistochemistry examinations were carried out at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University Denpasar Bali. This study was conducted from November 17, 2020, to February 11, 2021.

Variables used in this study were as follows:

1. The independent variables were calcitriol levels, HIF-1α expression, and IL-10 expression.
2. The dependent variable was PE.
3. Confounding variables were: Maternal age, parity, gestational age, multiple pregnancies, history of PE, chronic hypertension, chronic kidney disease, heart disease, diabetes mellitus, and mola hydatidosa (molar pregnancy).

The data was recorded on a special research form and analyzed using the SPSS-24.0 version for windows software. The results of the analysis are presented in the form of tables and narratives.

1. Normality of data was tested using Kolmogorov–Smirnov test.
2. Homogeneity of data was tested using Levene's test
3. Calculation of odd ratio using Chi-square test.
4. Multivariate analysis with logistic regression test to determine the most important risk factors.
5. Pathway analysis test to determine the magnitude of the effect of calcitriol on HIF-1 α and IL-10 on the risk of PE.

Results

The data normality test was performed using the K-S test and homogeneity with Mann–Whitney using the SPSS-24 version for windows software. The results of the research data analysis are presented as in Table 1. Table 1 distribution of characteristics of maternal age, parity, and gestational age in the case group and control group.

Table 1: Distribution of characteristics of maternal age, parity, and gestational age in the case group and control group

Variable	Case group (n = 32)	Control group (n = 32)	p
Age (year), mean ± SD	30.8 ± 5.5	26.3 ± 5.6	0.002
Parity, median (IQR)	3 (3)	2 (2)	0.050
Gestational age (week), median (IQR)	36 (3)	37.5 (1)	0.001

SD: Standard deviation, IQR: Interquartile range.

Based on the mean comparison test using the student t-test, the mean maternal age in the case group (30.8 ± 5.5) was significantly higher than the control group (26.3 ± 5.6) with a p-value of 0.002. Similarly, the median value of gestational age using the Mann–Whitney test found that the case group (36, IQR 3) was significantly lower than the control group (37.5, IQR 1) with p = 0.001). There was no statistically significant difference in the median parity value (p > 0.05) between the two groups of subjects.

Discussion

Distribution of characteristics of maternal age, parity, and gestational age, in the case group and control group

In this study, the mean maternal age in the case group (30.8 ± 5.5) was higher than in the control group (26.3 ± 5.6). From the results of the independent T-test on the two parameters, there were statistically significant differences between the two groups

(p = 0.002), thus it was concluded that maternal age affected the occurrence of PE, where PE tended to occur in older mothers. Several studies have proven the relationship between older mothers and poor pregnancy outcomes, including miscarriage, stillbirth, PE, gestational hypertension, and gestational diabetes mellitus [26]. The risk of PE in women aged >35 years is 3.23 times higher compared to women aged 20–24 years (odds ratio [OR]: 3.23 with confidence interval [CI]: 1.58–6.59) with p = 0.001 [27]. At the age of more than 30 years, it appears that increased villous reaction may be a contributing factor to PE [28]. Sheen *et al.* found that the proportion of women with PE among those aged 30–54 years increased from 32.9% to 43.7% [29]. The present study revealed an altered demographic profile of PE, with older women contributing more to the increased proportion of PE and associated adverse effects. In terms of risk factors, PE is classified into two parts, namely, PE that is purely caused by placental factors, and PE caused by maternal factors that worsen placental perfusion [30].

This study also found a statistically significant difference in the median gestational age between the two groups. Based on the Mann–Whitney test, the median gestational age of the case group (36, IQR 3) was significantly lower than the control group (37.5, IQR 1) with a p < 0.001. This shows that gestational age affects the occurrence of PE, where PE tends to occur at a lower gestational age but still above 34 weeks. Of all cases of PE, most occur at gestational age >34 weeks (2.7–88%) and only a small proportion occur at gestational age <34 weeks (0.38–12%), this is related to the extent of vascular lesions on placental villi [31], [32]. Different things were reported by Shiozaki *et al.* who got a PE incidence of 2.7% (6426 patients) with the highest frequency at <32 weeks of gestation, which was 83.3% [33].

In this study, the median parity between the two groups was not significantly different (p = 0.050). This shows that parity did not affect the occurrence of PE. This was different from the theory which states that nullipara is one of the strong risk factors for PE. Large population studies conducted in Denmark and Sweden indicated that nulliparous women had a PE risk of between 4 and 6% whereas in multiparous the risk fell to 2% [34]. Das *et al.* found that primiparous women had a twofold higher risk of developing PE than multiparous women [27]. Different things were reported by Li *et al.*, who found that the risk of PE in nulliparas and multiparas was not statistically significant and Yazdani *et al.* found that the incidence of PE in multiparas was higher than nulliparas. Thus, from the studies mentioned above, it can be concluded that the effect of parity as a risk factor for PE is still controversial [35], [36].

Risk of preeclampsia (PE) at low calcitriol levels

To determine the role of low calcitriol levels on the risk of PE, the Chi-square test was used as shown in Table 2.

Table 2: Distribution of calcitriol, IL-10, and HIF-1 alpha levels in case and control groups

Variables	Group		OR	95% CI	p
	Case	Control			
Calcitriol levels					
Low	27	9	13.801	4.048–47.050	0.001
IL-10					
Low	24	10	6.600	2.208–19.728	0.001
HIF-1 α					
High	22 (68.8)	9 (28.1)	5.622	1.922–16.450	0.001
Low	10 (31.3)	23 (71.9)			

CI: Confidence interval, OR: Odds ratio, IL-10: Interleukin-10, HIF-1α: Hypoxia-inducible factor-1 alpha.

Table 2 shows that in the case group, there were 27 (84.4%) with low calcitriol levels and in the control group, there were 9 (28.1%) with low calcitriol levels. Thus, low calcitriol levels were a risk factor for PE by 13.801 times (OR = 13.801, 95% CI = 4.048 – 47.050, p = 0.001) compared to high calcitriol levels.

In this study, low placental calcitriol levels affected the risk of PE by 13.8 times (OR= 13,801, 95% CI = 4.048–47,050, p = 0.001) compared to high placental calcitriol levels. In the regulation of angiogenesis and vasculogenesis, calcitriol plays a role by increasing the activity of endothelial progenitor cells. In addition, calcitriol is also able to increase proangiogenesis components, namely, endothelial colony-forming cells (ECFCs), which play a role in the regeneration of endothelial cells [37], [38], [39] found that in placental tissue, levels of 25-hydroxy vitamin D3 were found to be strongly correlated (r = 0.83, p = 0.001) with 24, 25-dihydroxy vitamin D3 (a metabolite of calcitriol), thus proving that calcitriol is metabolized in the placenta. Halhali *et al.* proved that placental calcitriol synthesis and IGF-I concentrations affected tissues obtained from pregnancies with PE. In the PE group, placental [3H]-1.25(OH) 2D3 synthesis was significantly lower than in the Normotension group (19.6 ± 6.2 vs. 29.9 ± 8.1 fmoles/200 mg wet weight, p = 0.013) [40]. IGF-I levels were significantly lower in the PE group than in the normotensive group (15.2 ± 3.9 vs. 21.6 ± 4.9 ng/g wet weight, p = 0.012). Xiao *et al.*, obtained the mean ± SD of 25 (OH) D mothers in the control and PE groups were 38.06 ± 6.28 and 33.05 ± 4.10, respectively, significantly different with p < 0.0001 between control and PE in continuous and categorical variables, especially in the early onset severe PE subtype (32.96 ± 4.49). In the 25(OH) D deficiency category (<30 nmol/L), there was an increased risk of PE (OR = 2.83, 95% CI = 1.32–6.08) in both maternal and 25 (OH) D umbilical cord serum. Multivariable logistic regression.

Risk of PE at low IL-10 in case and control groups

To determine the low IL-10 expression on the risk of PE, the Chi-square test was used. The results of the analysis are presented in Table 2.

Table 2 shows that in the case group, there were 24 (75%) with low IL-10 and in the control group, there were 10 (31.3%) with low IL-10. Thus, low IL-10 expression is a risk factor for PE by 6.6 times (OR= 6.600; 95% CI = 2.208 – 19.728; p = 0.001) compared to high IL-10 expression.

In this study, it was found that low placental IL-10 expression increased the risk of PE by 6.6 times (OR= 6,600; 95% CI = 2.208–19.728; p = 0.001) compared to high placental IL-10 expression. IL-10, VEGF, and PlGF levels were decreased in PE compared to normotensive placentas. Both TNF-α/IL-10 and sFlt-1/PlGF ratios were higher in the placental homogenate of early-onset PE compared to late-onset PE and control groups. IL-10 decreases pro-inflammatory cytokines associated with oxidative stress while promoting vascular healing, a process required for spiral artery remodeling and placental perfusion [41]. Serum IL-6 and IL-6:IL-10 ratios were significantly correlated with systolic blood pressure, diastolic blood pressure, and proteinuria levels. This suggests that pro-inflammatory cytokines (IL-6) play a role in the pathogenesis of PE [42]. Fan *et al.* conducted a study of the effect of four single nucleotide polymorphisms in the promoter of IL-6 (−572G/C, −597G/A, and −174G/C) and IL-10 (−592A/C) in susceptible patients [43]. The results showed that compared to the AA genotype, the CC and AC + CC genotypes of IL-10 -592A/C correlated with an increased risk of developing PE, with adjusted odds ratios, 95% CI of 2.45 (1.26–4.72) and 1.71 (1.09–2.68).

Risk of PE at high HIF-1α expression

To determine the role of high HIF-1α expression on the risk of PE, the Chi-square test was used. The results of the analysis are presented in Table 2. Table 2 shows that in the case group, there were 22 (68.8%) with high HIF-1α levels and in the control group, there were 9 (28.1%) with high HIF-1α levels. Thus, high HIF-1α expression was a risk factor for PE by 5.622 times (OR = 5.622; 95% CI = 1.922–16.450; p = 0.001) compared to low HIF-1α expression.

In this study, high HIF-1α expression was a risk factor for PE by 5.6 times (OR= 5.622; 95% CI = 1.922–16.450; p = 0.001) compared to low HIF-1α expression. Failure of spiral artery remodeling followed by placental hypoperfusion will result in a hypoxic microenvironment, which induces HIF-1α and the C/EBP homologous protein (CHOP) pathway of apoptosis [44]. The overexpression of HIF-1α in PE also results in the expression of TGF-β3, a potent inhibitor of trophoblast differentiation. TGF-β3 can inhibit cytotrophoblast differentiation, resulting in impaired extravillous trophoblast invasion in early placentation. HIF-1α which is expressed at high levels can also induce the production of sFlt-1 and sEng, which are anti-angiogenic factors that play a very important role in the occurrence of PE [24], [45]. Rath and Aggarwal, (2014) found in the PE group, significant nuclear and cytoplasmic HIF-1α expression was seen in the syncytiotrophoblast (p = 0.0001) but in the control placenta, localized only to the cytoplasm (p = 0.0001). The intensity of PlGF expression was lower in the cytoplasm of the syncytiotrophoblast (p = 0.0001) in PE cases compared to controls. Zhang *et al.* proved that HIF-1α and FOXO3a are highly expressed in the placental tissue of patients with PE under hypoxic conditions. The results of this study suggest that increased expression of HIF-1α enhances trophoblast apoptosis by regulating FOXO3a, which may be involved in the pathogenesis of PE [46]. Lai and Liu (2018) conducted a study on the expression of HIF-1α and heat shock protein 70 (HSP 70) in the placenta of PE patients. As a result, the positive expression of HIF-1α and HSP70 in placental tissue in the experimental group was 80.00% (40/50) and 78.00% (39/50), while in the control group was 28.00% (14/50) and 32.00% (16/50) (p < 0.05), respectively [22]. Thus, it is thought that HIF-1α affects trophoblast apoptosis by regulating the expression of FOXO3a and HSP 70 under the influence of hypoxia.

Pathway analysis between calcitriol, IL-10, and HIF-1α levels on the risk of PE

To estimate the relationship between low calcitriol and IL-10 levels and high HIF-1α to the risk of PE, a multivariate analysis was carried out through multiple logistic regression tests by including low calcitriol levels, low IL-10 expression, and high HIF-1α. The results of the analysis are shown in Table 3.

Table 3: Results of multivariate analysis of calcitriol levels, IL-10 expression, and HIF-1 alpha as risk factors for pre-eclampsia

Variable	OR	95% CI	p
Low calcitriol	9.6	2.503–36.742	0.001
Low IL-10	4.6	1.232–16.986	0.023
High HIF-1α	3.0	0.824–11.218	0.095

CI: Confidence interval, OR: Odds ratio, IL-10: Interleukin-10, HIF-1α: Hypoxia-inducible factor-1 alpha.

Based on the results of multivariate analysis using the Logistics Regression Test with low calcitriol levels, low IL-10, and high HIF-1α as independent risk factors for PE, the most important factor found was the low calcitriol levels with an AOR of 9.59, 95% CI: 2,503–36,742 with p = 0.001. In addition, the second risk factor that plays a role is low IL-10 expression with AOR: 4.6, 95% CI: 1.232–16.986 with p = 0.023. The analysis also found high HIF-1α with an AOR of 3.0. However, statistically, there is not enough evidence to conclude that high HIF-1α is a risk factor for PE with a 95% CI that exceeds one and p > 0.05.

To determine the role of low calcitriol levels, low IL-10 expression, and high HIF-1α on the risk of PE based on the contribution pathway of each of these variables, they can be simultaneously summarized in pathway analysis as shown in Figure 1.

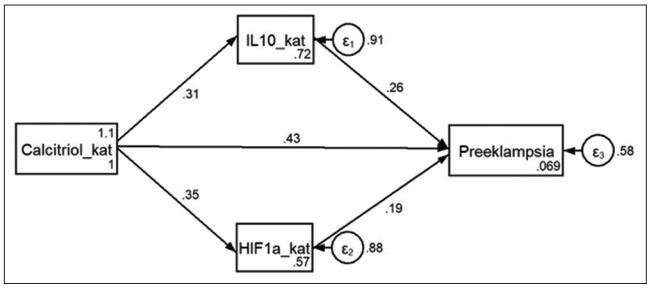

Figure 1: Pathway analysis between calcitriol levels, interleukin-10 expression, and hypoxia-inducible factor-1α expression on preeclampsia

In the model above, three main models can be reported were:

1. Low calcitriol levels had a direct and indirect effect on the risk of PE. Directly, the contribution of calcitriol levels to the risk of developing PE is 43%. Indirectly, the contribution of calcitriol levels was 31% to IL-10 levels and 35% to HIF-1α levels. The total effect of calcitriol levels on the risk of developing PE was 58%.
2. The contribution of IL-10 levels to the risk of PE was 26%.
3. The contribution of HIF-1α expression to the risk of PE was 19%.

Based on the pathway analysis, it can be concluded that the occurrence of PE in this study could be partially caused by low calcitriol levels, low IL-10 expression, and high HIF-1α expression alone or through combinations of these three variables. The contribution of low placental calcitriol levels to HIF-1α and IL-10 can be explained through two mechanisms, namely: First, low calcitriol can lead to superficial trophoblast invasion resulting in impaired spiral artery remodeling. Impaired spiral artery remodeling will lead to ischemia and placental hypoxia. This hypoxic state will stimulate HIF-1α secretion. Overexpression of HIF-1α results in the expression of TGF-β3, a potent inhibitor of trophoblast differentiation. Prolonged expression of HIF-1α also induces increased transcription of genes encoding fms such as tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng), and endothelin-1 (ET-1), all known to contribute to PE [19], [47]. The contribution of low calcitriol levels through HIF-1α to the risk of PE was 35% in this study.

Second, as an immunomodulator, calcitriol decreases the activity of Th1 cells and increases IL-10. One of the inhibitory effects of calcitriol occurs in IFN-γ, IL-2, IL-12, and TNF-α, which has an impact on decreasing Th1 activity. This is followed by an increase in Th2 activity, due to an increase in IL-10 production and a decrease in IL-2 secretion [20]. Calcitriol can also inhibit dendritic cell maturation, leading to an increase in the population of immature dendritic cells, which contributes to the inhibition of CD40, CD80, CD86, and MHC II [20]. Noyola-Martinez *et al.*, demonstrated that in placental cell culture, the pro-inflammatory cytokine TNF- and IL-6 secretion and mRNA expression were downregulated by calcitriol ($p < 0.05$) [48]. Thus, low calcitriol can play an indirect role in the pathogenesis mechanism of PE through low IL-10. In this study, the contribution of calcitriol levels through IL-10 to the risk of PE is 31%.

In the pathway analysis, it is also stated directly that the contribution of calcitriol levels to the risk of developing PE is quite large (43%). This was because calcitriol has a direct effect on vascular endothelium and prevents oxidative stress. Calcitriol plays a role in stimulating VEGF and vascular smooth muscle regulation and increasing proangiogenesis components, namely, ECFCs, which play a role in the regeneration of endothelial cells. PlGF levels were significantly lower in women with 25(OH)D levels <50 nmol/L (low), at 12–18 weeks gestation compared with 25(OH)D levels >50 nmol/L (high), ($p = 0.04$) and also with significantly lower levels of Intercellular Adhesion Molecule-1 (ICAM-1) [49]. Recent studies have shown that calcitriol suppresses the expression of cyclooxidase-2 (COX-2) in cells to promote the action of anti-inflammatory cytokines. Vitamin D has a remodeling function by lowering Angiotensin II type 1 receptors and reducing oxidative stress [50]. Martínez *et al.* (2014) reported that endothelial cells treated with 10 nM 1,25(OH)2D3 significantly increased NO production as well as increased protein and eNOS bioactivity. Low levels of calcitriol in the body are associated with increased ROS production and weakened antioxidant capacity which can cause endothelial dysfunction through inhibited eNOS and NO synthesis. This ROS-induced damage to NO and eNOS synthesis is reduced by vitamin D treatment through the upregulation of antioxidants and the downregulation of ROS production [51].

The role of calcitriol levels, expression of IL-10 and HIF-1α in new mechanisms of PE

In the two-stage theory of PE, it is known that in the first stage, there is a failure of the invasion of trophoblast cells which leads to impaired remodeling of the spiral arteries. Impaired spiral artery remodeling is believed to result from immunological maladaptation between maternal and fetal tissues. In our new theory of the mechanism of PE, we propose impaired spiral artery remodeling caused by low levels of placental calcitriol and IL-10. Low levels of placental calcitriol interfere with the invasion of EVT cells into the spiral artery walls.

In the conventional theory, the increased expression of sFlt-1 and sEng which is the beginning of PE is caused by placental hypoxia and ischemia and also due to oxidative stress and placental inflammation due to failure of spiral artery remodeling. In our new theory, the increased expression of sFlt-1 and sEng is the result of a persistently high expression of HIF-1α, as a result of impaired spiral artery remodeling. The high expression of sflt-1 and sEng will disrupt systemic endothelial function which is the basis of PE.

Low placental calcitriol involvement, low IL-10 expression, and high HIF-1α expression in a pathway have been demonstrated in pathway analysis, where the direct role of calcitriol in the risk of PE is 43%. Meanwhile, the role of low placental calcitriol on the risk of PE through IL-10 and HIF-1α was 31 and 35%, respectively. The direct effect of calcitriol on the occurrence of PE is probably due to the effect of calcitriol which can play a direct role in reducing oxidative stress, suppressing inflammation, and improving endothelial function. Moreover, if there is a deficiency of calcitriol, it can result in increased oxidative stress, inflammation, and disruption of endothelial function. This requires further research (Figure 2).

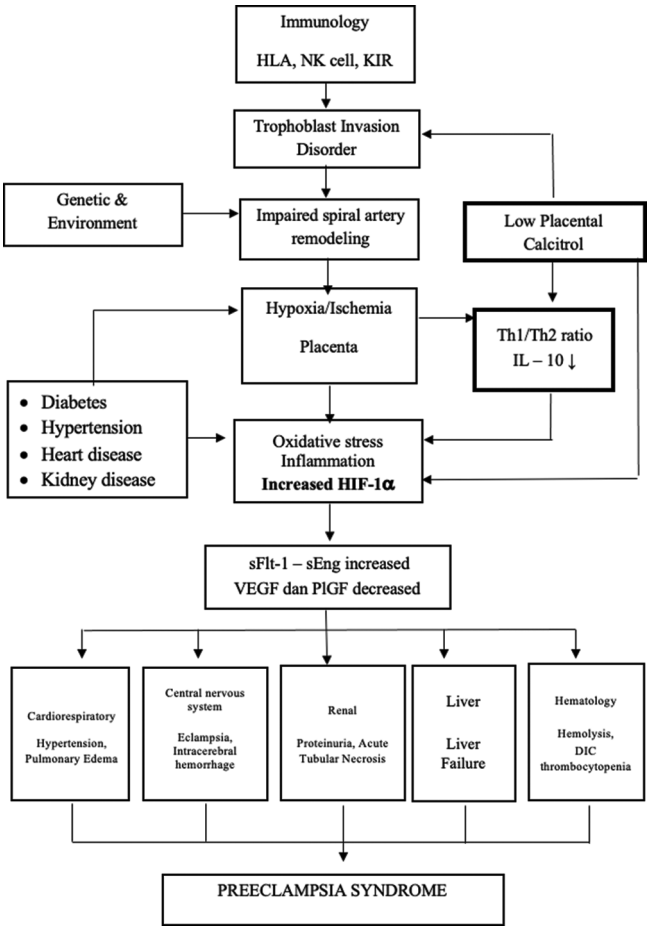

Figure 2: Schematic of a new pathogenesis of preeclampsia involving calcitriol, hypoxia-inducible factor-1α, and interleukin-10

Conclusion

In this study, the following conclusions can be drawn:

1. The risk of PE at low placental calcitriol levels is 13.8 times higher than high calcitriol levels.
2. The risk of PE with low placental IL-10 expression was 6.6 times higher than with high IL-10 expression.
3. The risk of PE with high placental HIF-1α expression was 5.6 times higher than with low placental HIF-1α expression.
4. Low placental calcitriol levels have the greatest effect on the mechanism of PE occurrence with a discriminant coefficient or 58% distribution. Indirectly, the calcitriol levels were distributed by 31% to the expression of IL-10 and 35% to the expression of HIF-1α.

Thus, it has been proven that low calcitriol levels and low IL-10 expression are risk factors for PE. Meanwhile, high HIF-1α expression was not proven as a risk factor for PE.

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