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Pregnancy-Induced Hypertension and Preeclampsia: Levels of Angiogenic Factors in Malaysian Women

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Summary **Preeclampsia (PE) is a major contributor to maternal and fetal mortality. The cause of preeclampsia remains unclear, but oxidative stress on the endothelium leading to endothelial dysfunction is said to be the root cause of the disease. The aim of this study was to measure and determine the plasma levels of key angiogenic factors in pregnancy as an indicator for the early onset of preeclampsia in pregnancy. Plasma levels of circulating a soluble fms like tyrosine kinase-1 (sFlt-1), an anti-angiogenic factor, vascular endothelial growth factor (VEGF) and placental growth factor (PIGF), both pro-angiogenic factors were analyzed in normal pregnant Malaysian women (control group, $n = 34$), women with pregnant induced hypertension (PIH, $n = 34$) and women with preeclampsia (PE, $n = 34$) all at three gestational ages, 24–28 weeks (early pregnancy: EP), 32–36 weeks (late pregnancy: LP) and 6 weeks after delivery (postpartum: PN). The plasma levels of angiogenic factors were determined by ELISA. sFlt-1 levels were elevated in PIH and PE patients as compared to controls. PIGF and VEGF were significantly decreased in PIH and PE as compared to the controls. These results suggest that elevated concentration of sFlt-1 and suppressed levels of PIGF and VEGF may contribute to the development of hypertension in pregnancy which precedes preeclampsia.**

Key Words**: oxidative stress, preeclampsia, angiogenic factors, endothelial dysfunction**

Introduction

Preeclampsia (PE) is a hypertensive disorder in pregnancy associated with high blood pressure and proteinuria which frequently develops after 20 weeks of gestation [*1*]. This disorder affects between 2–7% of pregnancies worldwide and it's the major cause of maternal and fetal mortality [*2*, *3*]. The etiology of this disease remains unclear, however the pathophysiological changes seen in preeclampsia include increased vasoconstriction and coagulation, vascular endothelial dysfunction and reduced placental perfusion [*4*]. Until recently, most investigations on PE were focused on reduced placental perfusion [*5*]. During placental perfusion cytotrophoblast invasion does not occur due to improper differentiation of maternal uterine spiral arteries. This reduces the blood flow in the placenta which subsequently causes poor oxygen and nutrient transfer between mother and fetus [*6*]. Consequently, this leads to endothelial dysfunction which is the primary cause of PE [*7*]. However, in the last few years, there has been increased focus on the role of angiogenic factors in PE.

Recent studies suggested that there are imbalances in the production level of circulating pro- and anti-angiogenic factors and have implicated this in endothelial dysfunction [*8*]. The concentration of the anti-angiogenic factor, soluble fms like tyrosine kinase-1 (sFlt-1) was found to be increased in the plasma of preeclamptic women [*8–10*]. sFlt-1 antago-

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nizes the action of the pro-angiogenic factors, vascular endothelial growth factor (VEGF) and ploncental growth factor (PIGF) [*11–14*] by adhering to the receptor binding domains of VEGF and PIGF. This prevents their interaction with endothelial cell surface receptors and subsequently results in endothelial dysfunction leading to preeclampsia [*15*]. Levels of circulating VEGF and PIGF were found to be significantly lowered in preeclamptic women compared to controls [*8*, *16*, *17*].

The objective of this study was to compare the plasma levels of angiogenic factors in normal pregnant women, pregnant women with induced hypertension and preeclamptic patients at three different gestational ages. We also investigated the potential for these angiogenic factors to serve as a biomarker for early detection of PE.

Materials and Methods

Study Population

The subjects were recruited from the Obstetric Unit of the University of Malaya Medical Centre (UMMC) with the study protocol approved by the UMMC Ethics Committee. Thirty four subjects from each of the three categories: (i) pregnancy induced hypertension (PIH), (ii) preeclampsia and (iii) controls (normal pregnant women) were enrolled in this study. Single and multiparous women participated in this study. Blood samples were collected at three gestational ages: (i) early pregnancy (EP) (24–28 weeks), (ii) late pregnancy (LP) (32–36 weeks) and (iii) postpartum (PN) (6 weeks after delivery). Subjects classified as preeclamptic met one or more of the following diagnostic criteria: systolic blood pressure ≥140 mmHg or a diastolic pressure of ≥90 mmHg taken on two occasions at least six hours apart and proteinuria of ≥0.3 gm/dl in a 24 h urine collection or 2+ based on semiquantitative urine analysis. Patients with PIH (gestational hypertension) met the following criteria: systolic pressure of ≥140 mmHg and a diastolic pressure of ≥90 mmHg without proteinuria after 20 weeks of gestation with resolution to baseline by 12 weeks postpartum. Normal pregnant women met the following criteria: pregnancy with normal blood pressure (<140/90 mmHg), absence of proteinuria, without medical and obstetrical complications.

Exclusion criteria for all subjects were heart disease, use of antihypertensive medication, diabetes mellitus and renal disease. All subjects were asked to give informed consent to be included in the study after the nature of the research had been explained to them.

Sample preparation

Venous blood sample (10 ml) was collected from each subject. Blood was dispensed into 3.0 ml EDTA (1 mg/ml) containing vacutainer tubes and centrifuged at 7,000 r.p.m for 15 min. Plasma obtained was aliquoted under sterile conditions and stored at −80°C. Approximately 1.5 ml of sample was used to determine the renal profile.

Measurement of sFlt-1, VEGF and PIGF

Plasma concentration sFlt-1, VEGF and PIGF were measured in duplicate using commercially available ELISA kits from R&D System Inc. (Minneapolis). Plasma samples for sFlt-1 measurement were diluted to 20 fold with the calibrator diluent provided. 100 µl of diluent was added into each well of the 96 well plates precoated with antibodies against sFlt-1 or VEGF or PIGF. This was followed by the addition of 100 µl of each standard or diluted plasma samples into their respective wells. The plate was then incubated for 2 h on an orbital shaker. Washing was performed four times using wash buffer to remove any unbound compounds followed by incubation for 2 h with 200 µl polyclonal antibody. Another 4 cycles of washing with the wash buffer was performed. 200 µl of substrate solution were added to each well and plate was incubated in the dark for 30 min. 50 µl of stop solution was added to each well to stop further reaction.

The optical density was measured at 450 nm and 540 nm. The plasma levels of each factor were calculated using standard curves derived from a known concentration of the respective recombinant factors.

Statistical analysis

Experimental data obtained in triplicates were expressed as mean ± SD. Statistical analysis was performed by using the non-parametric Kruskal-Wallis one-way ANOVA followed by Dunn's test. A p value of less than 0.0001 was considered to show significant differences between the groups. Statistical calculations were carried out using the software Graphpad Prism, version 5.0 (Graph Pad Software Inc., Los Angeles).

Results

Clinical features of the subjects as wells as the basic demographic characteristics are tabulated (Table 1). There were no significant differences observed in maternal age between the three groups. Systolic and diastolic levels were found significantly increased in women with pregnancy induced hypertension and preeclampsia as compared to normal pregnant women.

Plasma concentrations of sFlt-1, PIGF and VEGF are shown in Fig. 1. In general, the concentration of sFlt-1 was found to be in the order of PE > PIH > Control. In contrast, the concentrations of pro-angiogenic factors, PIGF Fig. 1 (b) and VEGF Fig. 1 (c), were significantly lowered when compared to the control group.

Within the PE group, sFlt-1 values during LP (10058.79 ± 2678.684 pg/ml) is significantly higher as compared to EP (7220.141 ± 2148.333 pg/ml) and significantly

Table 1. Clinical features of subjects

Characteristic	Controls	PIH	PE
Age	29.6 ± 7.9	33.4 ± 5.5	29.7 ± 4.8
Races			
Malay	30 (88.2%)	31 (91.2%)	17 (50.0%)
Chinese	2 (5.9%)	3 (8.8%)	11 (32.4%)
Indian	2 (5.9%)	0	6 (0.18%)
Early Pregnancy (EP)			
24–28 weeks			
Systolic blood pressure (mmHg)	106 ± 5.9	132 ± 6.8*	156 ± 8.4*
Diastolic blood pressure (mmHg)	70 ± 2.54	85 ± 4.52*	98 ± 4.57*
Plasma Urea (mmol/L)	2.38 ± 0.5	2.26 ± 0.62*	3.0 ± 1.5*
Plasma Creatinine (μmol/L)	41.6 ± 8.0	41.6 ± 9.2	49.8 ± 10.1*
Late Pregnancy (LP)			
32–36 weeks			
Systolic blood pressure (mmHg)	115 ± 5.6	135 ± 5.2*	164 ± 6.2*
Diastolic blood pressure (mmHg)	78 ± 2.98	90 ± 5.4*	95 ± 6.35*
Plasma Urea (mmol/L)	2.06 ± 0.39	3.2 ± 0.73*	4.3 ± 2.2*
Plasma Creatinine (μmol/L)	50.5 ± 10.6	47.6 ± 13.1*	60.9 ± 12.5*
Postpartum (6 weeks after delivery)			
Systolic blood pressure (mmHg)	113 ± 5.87	122 ± 6.45*	131 ± 6.89*
Diastolic blood pressure (mmHg)	80 ± 4.52	76 ± 3.98*	91 ± 4.98*
Plasma Urea (mmol/L)	4.15 ± 1.2	3.5 ± 1.1*	4.2 ± 1.4*
Plasma Creatinine (μmol/L)	59.4 ± 14.02	59.4 ± 11.4	57.6 ± 11.4*

*Significant as compared to control (p<0.001).

lower compared to PN (5294.725 ± 1161.516 pg/ml). This pattern is similar for PIH and Controls. There were no significant differences on sFlt-1 concentration in PE, PIH and controls after delivery.

Fig. 1 (b) and (c) shows the concentration of PIGF and VEGF respectively in the three groups at three gestational ages. The levels of the pro-angiogenic factors (PIGF and VEGF) were inversely related to sFlt-1 levels in PE, PIH and Controls. Postpartum VEGF values were found to be approximately equivalent between PE, PIH and Controls. Imbalance in the levels of pro- and anti-angiogenic factors in the three groups at three gestational ages is summarized in Fig. 2 (a–c). In the control group (Fig. 2a), it is evident that PIGF levels are persistantly higher than sFlt-1 levels. However, in the PIH and PE groups, higher sFl-1 persisted throughout pregnancy. We also analyzed the ratio of log [sFlt-1/PIGF] in the maternal plasma of the preeclampsia patients and controls. The plasma log [sFlt-1/PIGF] ratio was significantly higher in the preeclampsia subjects than in controls (Fig. 3).

Discussion

Preeclampsia is a systemic syndrome featuring the development of hypertension and proteinuria at the second trimester of pregnancy that causes significant medical complications for both mother and fetus [*18*, *19*]. Previous studies have show that the levels of circulating sFlt-1 and other anti-angiogenic factors including endoglin are found to be elevated in preeclamptic women compared to normal pregnant women [*8*, *15*, *20–22*].

Inhibition of pro-angiogenic factors by increased levels of sFlt-1 leads to endothelial dysfunction. Endothelial function can be restored *in vitro* by exogenous PIGF and VEGF [*23*]. During pregnancy, circulating sFlt-1 was found to be higher in preeclamptic subjects when compared to levels in non–pregnant women [*24*].

Our investigation demonstrated a marked imbalance between circulating plasma levels of soluble fms like tyrosine kinase-1, (sFlt-1) and pro-angiogenic factors (PIGF and VEGF) in both pregnancy induced hypertension (PIH) and preeclamptic (PE) women compared to normal pregnant women (Fig. 2a, b and c) at all stages of pregnancy.

The role of VEGF in preeclamptic women has drawn huge attention. There have been some studies reporting decreased levels or even elevated levels of VEGF in PE [*25–28*]. We, however, observed significantly lower levels of VEGF in our study in PE subjects (Fig. 1c). Furthermore, lower levels of PIGF were observed in preeclamptic subjects (Fig. 1b). Our investigations suggest that elevated levels

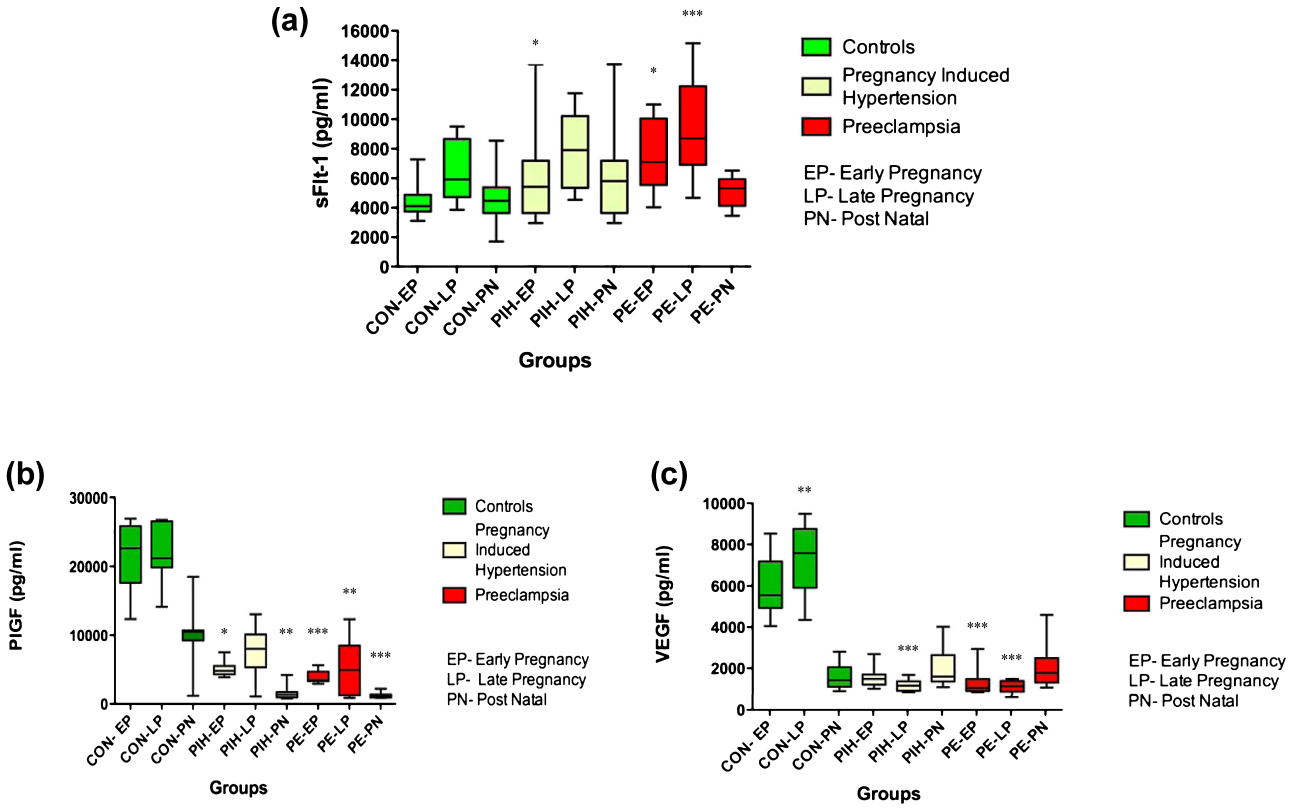

Fig. 1. Graph shows sFlt-1(a), PIGF (b), VEGF (c) concentrations in maternal plasma of control, hypertension (PIH) and preeclampsia (PE), at three different gestational ages [mid Pregnancy (EP), late pregnancy (LP) and Postpartum (PN)]. Top and bottom horizontal edges denote the 75th and 25th percentiles respectively. The middle line inside the box plots indicates median and the vertical whiskers above and below the boxes shows the largest and smallest values. *, **p<0.05 as compared to control groups, ***p<0.001 as compared to control groups.

of sFlt-1 precede the endothelial dysfunction seen in preeclampsia, since imbalances in pro and anti-angiogenic factors occur in the early stages of pregnancy in subjects who are preeclamptic.

Maynard and coworkers [*8*] showed an increased level of sFlt-1 followed by decreased levels of pro-angiogenic factor in maternal serum of preeclamptic women compared to controls. Similarly, Park and group [*29*] reported that levels of maternal serum sFlt-1 were higher in preeclampsia compared to controls. They indicated that the sFlt-1 levels in severe preeclampsia were elevated compared to mild preeclampsia and an elevated maternal sFlt-1 level is a risk factor in developing preeclampsia.

Several factors in our study clearly suggest that increased placental production of sFlt-1 may contribute to the pathogenesis of preeclampsia. Plasma levels of sFlt-1 were found to be elevated in preeclampsia, although it was not significantly increased in the PIH patients. This suggests that, sFlt-1 plays a major role in preeclampsia but not in pregnancy induced hypertension.

Inappropriate modification of maternal uterine spiral arteries [*30*] results in increased production of sFlt-1. This prevents cytotrophoblast cell invasion which leads to placental perfusion and subsequently develops into hypoxia [*7*]. The angiogenic proteins, sFlt-1, VEGF, PIGF seems to act as an essential regulatory factor in early development of placenta and pseudovasculogenesis [*8*]. Zhou and group suggested that exogenous sFlt-1 inhibits placental cytotrophoblast invasion *in vitro* [*23*]. Overproduction of sFlt-1 damages the endothelium in 28 – 36 weeks of gestational ages. Furthermore elevated sFlt-1 levels may be considered as the root cause in improper placental differentiation in preeclampsia [*8*].

Our results have important implications both therapeutically and diagnostically for the usage of pro-angiogenic proteins in preeclampsia and pregnancy induced hypertension as well as in other diseases such as cancer [*8*]. Our study shows that the over expression of sFlt-1 and significantly lower levels of proangiogenic factors in early pregnancy can be used as a biomarker for the onset of preeclampsia and PIH disease. Therapies and preventive strategies for preeclampsia must target the imbalance of these factors.

Differences in the production of circulating angiogenic factors, weeks prior to the onset of preeclampsia are

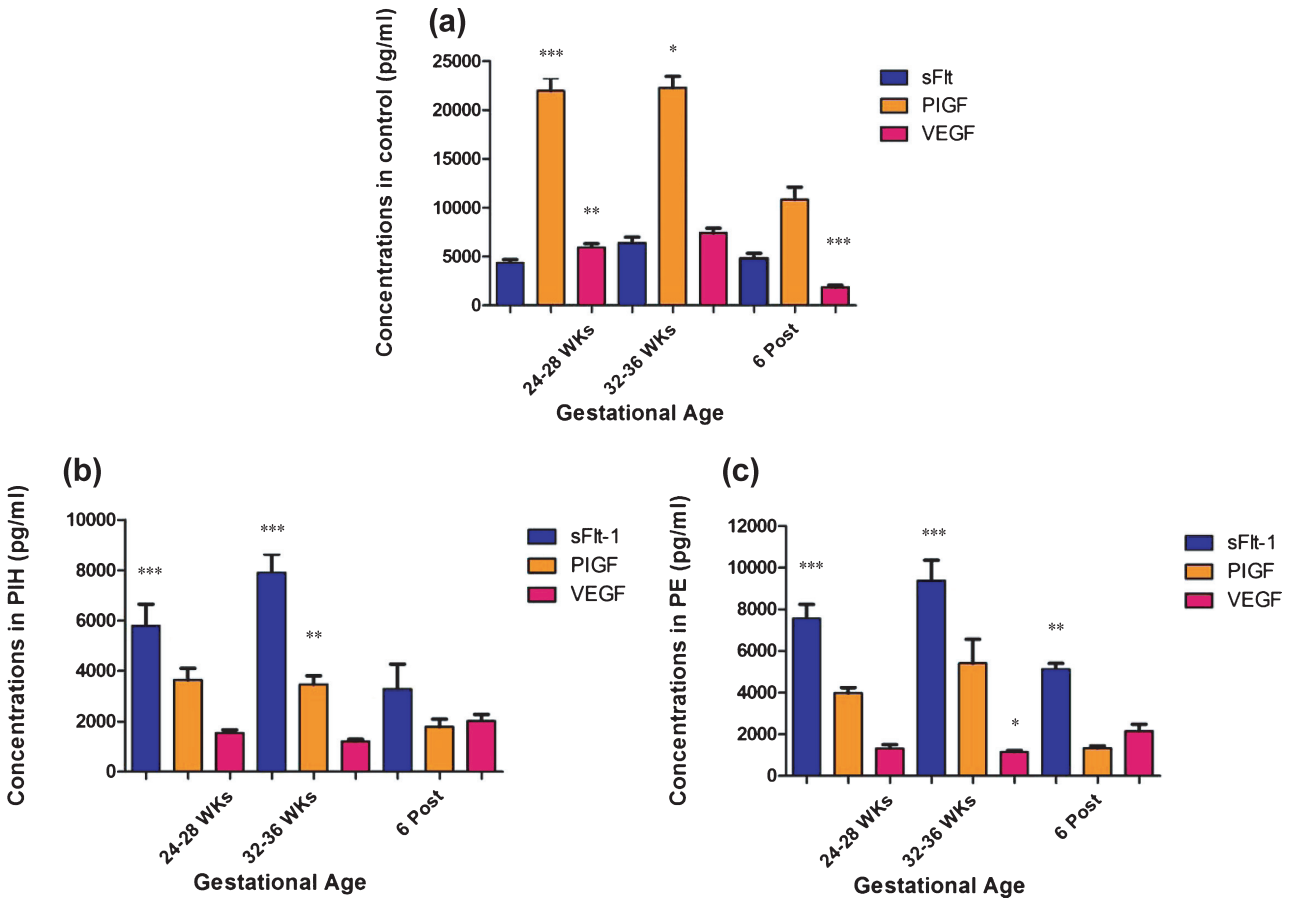

Fig. 2. Box plot represents angiogenic factors concentration in control (a), hypertension (PIH) (b) and preeclampsia (PE) (c), at three different gestational ages, [mid pregnancy (EP, 24–28 weeks), late pregnancy (LP, 32–36 weeks) and postpartum (PN, 6 weeks after delivery)]. *, **p<0.05 as compared to control groups, ***p<0.001 as compared to control groups.

potential biomarkers for screening and/or diagnosis. Significant increase in sFlt-1 and decrease in pro-angiogenic factors may be noticed from 2nd trimester onwards [*29*, *31*] and is evident 5–8 weeks prior to onset of the disease [*15*, *22*].

Maternal plasma sFlt-1 concentrations are specifically elevated in severe preeclampsia and early onset preeclampsia [*15*, *32*]. Serum levels of PIGF are decreased in patients who develop preeclampsia as early as 12 weeks of gestational age [*33*] although by 18–20 weeks of gestational age the decrease is marked [*15*, *18*, *19*].

Previous reports have shown that the sFlt-1: PIGF ratio can be used as an index of anti-angiogenic activity that reflects alteration in both biomarkers [*34*]. It serves as a better biomarker for identifying early onset of preeclampsia than either one. [*22*]. PIGF levels of the controls were positively correlated, while those of the preeclampsia patients were negatively correlated with sFlt-1 levels. Our study revealed that the sFlt-1/PIGF ratio in the PE patients was significantly higher compared to control.

If increased level of sFlt-1 is a feature of preeclampsia, inhibiting its action may be a strategy to prevent the onset

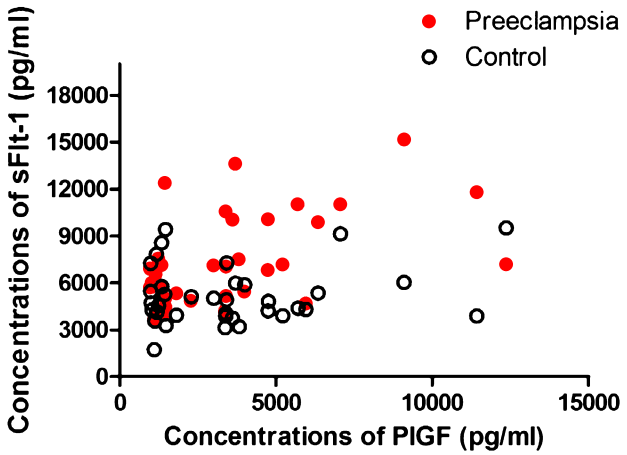

Fig. 3. Correlation between sFlt-1 and PIGF levels in maternal plasma of the PE (closed circle) and controls (open circle) (p<0.001).

of this disease. Maynard *et al.* [*8*] who used an *in vitro* angiogenesis assay, suggested that exogenous VEGF/PIGF therapy might restore endothelial function in preeclamptic patients. Therapy using pro-angiogenic molecules may be relevant here. In this context, nicotine may be considered since it has been shown to stimulate angiogenesis [*35*]. Furthermore, smoking individuals showed lower incidence of PE [*36*] by lowering sFlt-1 levels in human [*37*]. Hence, short term use of nicotine in cases of severe preeclampsia might be an effective treatment.

Alteration in the role of the placenta and oxidative stress level were seen in high risk preeclamptic women who consumed antioxidant complement [*38*]. Oral administration of supplementary antioxidants such as vitamin C and E has shown to diminish the risk of preeclampsia in pregnant women [*39*]. Report showed that the consumption of vitamin C and E influences the enzymatic activity of antioxidant and suppresses the development of preeclampsia [*40*]. Therefore, vitamin C and E can be used as a definite antioxidant for treating and preventing the risk of preeclampsia.

Conclusion

Overall, our findings showed overproduction of sFlt-1 in pregnancies that could contribute to proteinuria and abnormal blood pressure levels in preeclampsia and PIH subjects. We also found significant reduction in the levels of pro-angiogenic factors in these patients compared to controls. Excessive levels of sFlt-1 presenting in early pregnancy may be used as a biomarker for early detection of preeclampsia or any pregnancy hypertensive disorder.

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Abbreviations

Sflt-1, soluble fms tyrosine kinase 1; VEGF, vascular endothelial growth factor; PIGF, placental growth factor; PE, preeclampsia; PIH, pregnancy induced hypertension; PN, Postnatal.

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