Analysis of Vascular Endothelial Growth Factor (VEGF) -634C/G and +936C/T Polymorphisms and Serum VEGF Levels in Women Suffering From Preeclampsia

Sayed hojjat Hosseini
Ahvaz Jundishapur University of Medical Sciences: Ahvaz Jondishapour University of Medical Sciences
Ata Ghadiria
Ahvaz Jundishapur University of Medical Sciences: Ahvaz Jondishapour University of Medical Sciences
Abdolah Mousavi Salehi
Ahvaz Jundishapur University of Medical Sciences: Ahvaz Jondishapour University of Medical Sciences
Saber Rokhafrooz
Ahvaz Jundishapur University of Medical Sciences: Ahvaz Jondishapour University of Medical Sciences
Mahin Najafian
Ahvaz Jundishapur University of Medical Sciences: Ahvaz Jondishapour University of Medical Sciences
Mehri Ghafourian (ghafourian-m@ajums.ac.ir)
Ahvaz Jundishapur University of Medical Sciences: Ahvaz Jondishapour University of Medical Sciences

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Abstract

Preeclampsia (PE) is a syndrome related with pregnancy and characterized by hypertension and proteinuria, occurring in approximately 6-8% of pregnancies and accounting for approximately 40% of premature births. This study aimed to investigate the polymorphisms of -634C/G and +936C/T in VEGF gene and their relationship with serum VEGF levels in pregnant women with PE.

In this case-control study, peripheral blood samples were collected from 135 women with PE and 135 normal pregnant women as the control group. DNA was extracted using the phenol-chloroform method. Then, the polymorphisms of VEGF gene were detected by PCR-RFLP method using specific primers. Besides, VEGF concentrations were measured by ELISA method on serum samples and control subjects using ELISA kits.

In this research, maternal age, gestational week, maternal hemoglobin and BMI were significantly correlated with the likelihood of PE, while the occurrence season variable was not effective in PE among the pregnant women. There was no significant difference in the two polymorphisms of -634C/G and +936C/T in VEGF gene between the two groups. Also, the serum VEGF level in PE patients was significantly higher than the normal group ($P<0.001$).

Despite a significant increase in serum VEGF concentrations in women with PE, it seems that -634C/G and +936C/T polymorphisms of VEGF gene are not related with the onset of PE. Further studies are required to fully understand the risk factors related to preeclampsia syndrome.

Introduction

Severe disorders during pregnancy include a wide range of conditions causing abnormalities and mortality in the mother and her fetus. It has been estimated that these severe abnormalities occur in 6-8% of pregnancies (1, 2). A wide range of these disorders is related to hypertension-related abnormalities in pregnancy, among which preeclampsia (PE) is a common disorder and a specific syndrome of pregnancy. Evidence suggests that immune system activity plays a crucial role in the pathogenesis of patients with PE. In most cases, this disorder begins after thirty-seven weeks of gestation but may be detected at any time during the second half of pregnancy. PE can be mild or severe, its progress may be slow or rapid and delivery is the only way to improve the patient (1). The prevalence of PE in nulliparous and multiparous women is approximately 3-7% and 1-3%, respectively (3).

There is probably no single reason for the occurrence of PE (4). Genetic features, some underlying diseases, pregnancy history, chronic hypertension, coagulation disorders, kidney diseases, diabetes, autoimmune diseases such as lupus, familial history of PE, lack of family planning, twin or multiple pregnancies, age less than 18 or over 35, and other factors may be involved. However, the role of all these factors has not been fully demonstrated (4–7).

Vascular endothelial growth factor (VEGF) is known as an effective factor for proliferation, differentiation, migration, and invasion of endothelial cells (1). Preservation of endothelial cell function is important in the development and regulation of angiogenesis. VEGF receptor (VEGF-R) frequently performs biological functions through NEGF-R2 (2). The human VEGF gene is located on chromosome 6p21.3 with full length of 28 Kb and encoding gene length of 14 Kb, including eight exons and seven introns (3).

The permeability of capillaries and small vessels, especially in capillary veins, increases by biological function of VEGF (4). Above all, in the placenta, VEGF expression is essential for the function of endothelial cells and trophoblasts. The placental trophoblast cells' primary growth as well as proliferation, development, differentiation, migration, and infiltration of endothelial cells affect each other. Due to the necessity for fetal growth and angiogenesis, VEGF and its receptor system are strongly involved in fetal development. During fetal development, slight changes in VEGF expression can lead to abnormalities or death of the fetus (5).

According to a number of studies, VEGF gene polymorphisms play an essential role in regulating protein expression and increasing susceptibility to PE, although there are inadequate proofs in this regard (6–8). Considering the fact that the Iranian population is genetically different from other populations, the mutation of VEGF in Iranian pregnant women may vary with other communities. The study of these genes in relation to PE may give different results in the Iranian genetic pool. Moreover, the study of serum VEGF levels in women with PE as well as polymorphisms of SNPs (-634C/G and +936C/T) in these women is of high importance. Therefore, this study aimed to measure the serum levels of VEGF in women with PE and to investigate the relationship between -634C/G and +936C/T polymorphisms of VEGF gene with the onset of this disease in Ahvaz, Iran.

Materials And Methods

Study population and design:

In this case-control study, peripheral blood samples were collected from 135 women with PE and 135 normal pregnant women as the control group. The study population was in the age range of 18-37 years and randomly selected from women referring to the women's ward of two hospitals affiliated to Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Women with PE who had a history of high blood pressure (BP), pre-pregnancy bleeding, diabetes mellitus, history of PE and autoimmune diseases were excluded from the study. PE was diagnosed by a gynecologist using clinical examinations and laboratory tests. This project was approved by Medical Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1393.147). In this study, the samples were taken only from women who signed the informed consent form.

Requirements for the patient and control groups:

Women with PE, systolic BP≥140 mmHg and diastolic BP ≥90 mmHg, proteinuria of 300 mg or more in 24-hours urine or proteinuria of 30 mg/dl in two samples of urinary tract developing after 20th week of

pregnancy (9) were chosen as the case group. The control group included 135 pregnant healthy women living in Khuzestan Province with a history of at least one alive and healthy child and without a history of high BP during pregnancy. The age range of women was 18 to 37 years old. In this study, the control group included those women who had no history of chronic kidney disease, high BP, or bleeding before pregnancy.

Method:

Sampling

Patients were trained by a nurse. In sterile conditions, 7ml of blood was taken from the patient, 4 ml of which was poured into EDTA vials to carry out DNA extraction. After DNA extraction, the DNA samples were kept at -20°C until the test was performed. Also, 3 ml of the remaining blood was poured into a sterile clotting tube to determine the serum concentration of VEGF, and after separation of serum by centrifuging, it was stored at -70°C for further tests (10).

DNA extraction and genotyping

Samples of genomic DNA were collected from the leucocytes in whole blood samples of EDTA anticoagulated micro-tubes according to Miller et al. method (11). In this project, 5'-AAGGAAGAGGAGACTCTGCGCAGAGCA-3' (forward) and 5'-TAAATGTATGTATGTGGGGTGTGTCTACAGG-3' (reverse) primers were used in PCR-RFLP to examine the SNP +936C/T polymorphism. Also, for the investigation of SNP -634C/G polymorphism, 5'-TTGTTCCCCACTCARTGATCG-3' (forward) and 5'-CCGTCAGCGCGACTGGTCA-3' (reverse) were used (12). The PCR cycles for this reaction include the initial denaturation cycle, namely a 5 min cycle at 95°C, 50 s at 53°C and 30 s at 72°C, and after this step, 32 cycles in the second denaturation phase at 94°C for 40 s, the annealing phase at 62°C for 60 s and the extension phase for 60 s at 72°C. The final synthesis phase was performed for 10 min at 72°C. After the PCR time was elapsed, the PCR product was used for electrophoresis (13). After the PCR process, to check the +936C/T polymorphism, 1 µl of NlaIII (the restriction enzyme) was added to PCR product and incubated for 37 hours at 18°C. If there was a sequence of the effect point of this enzyme in DNA, the primary DNA, which is 208bp in length, was cut into two pieces of DNA at 122bp and 86bp lengths under the influence of this enzyme, and these two pieces were characterized by distance from each other after electrophoresis on the gel (10). Furthermore, to check the -634C/G polymorphism, 1 µl of FaqI restriction enzyme was added to the PCR product and incubated for 37 hours at 18°C. If there was a point of effect on this restriction enzyme, the primary DNA, which was 304bp in length, was broken down into two pieces of 193bp and 111bp (10).

Statistical Analysis:

Mann-Whitney test was used to compare the expression results of -634C/G and +936C/T polymorphisms in VEGF gene and their association with serum levels of VEGF in pregnant women with PE. To evaluate

the relationship between these two polymorphisms and serum VEGF levels, Pearson correlation coefficient and Spearman test were used. The confidence coefficient in all calculations was 55% and $p<0.05$ was considered as statistically significant.

Results

According to descriptive statistics results, there was a significant difference in demographic and laboratory variables between normal pregnant women and women with PE categorized as control and case groups, respectively (Table 1). The maternal age, mother height, mother, s BMI, mean weight in women with PE were higher than the control group. However, hemoglobin concentration, birth weight, and pregnancy week were lower in PE women compared to normal pregnant women ($P<0.05$).

Table 1
Demographic data of case and control groups

Variables	Case (n =135)	Control (n =135)	P-values
	Range (Mean ± SD)	Range (Mean ± SD)	
Mother's age (year)	18-37 (28.6 ± 4.29)	18-36 (26.3 ± 4.83)	**< 0.05**
Mother's height (cm)	1.5-1.7 (1.64 ± 0.034)	1.5-1.7 (1.62 ± 0.039)	**< 0.05**
Mother's weight (kg)	68-96 (80.8 ± 4.38)	60-81 (71.2 ± 4.06)	**< 0.05**
Mother's BMI (kg/m2)	24.4-37.5 (30.07 ± 1.97)	23.7-32.4 (27.25 ± 1.48)	**< 0.05**
Pregnancy week (week)	25-39 (34.35 ± 2.24)	34-38 (36.52 ± 1.03)	**< 0.05**
Birth weight (g)	2000-3750 (2586.6 ± 427.4)	3200-3540 (2934.8 ± 372.5)	**< 0.05**
Haemoglobin (g/dl)	9.1-13.3 (10.01 ± 0.75)	9.5-13.6 (11.71 ± 0.8)	**< 0.05**
Systolic BP (mm Hg)	141–157 (144 ± 5.4)	111–134 (120 ± 9)	**<0.05**
Diastolic BP (mm Hg)	92–106 (95 ± 7.2)	69–84 (77 ± 7)	**<0.05**
Proteinuria (g/24 hours)	1.08–1.42 (1.31 ± 0.6)	Absent	**-**

the relationship between type of sample and the occurrence season indicates that there is no relationship between the type of sample and the season of PE occurrence ($r=0.523$; $P=0.914$).

The results showed that there was no correlation between the incidence of PE and any of the alleles related to SNP -634C/G and SNP +936C/T ($P>0.05$). Considering the odds ratio, we conclude that the odds of carrying G and C alleles in both control and PE groups is the same and thus harboring any of the C and G alleles does not increase or decrease the risk of PE (Table2). Data analysis showed that there

was no relationship between PE with C and T alleles. The results indicated that there was no significant relationship between these mutations and the incidence of PE (P>0.05). Data on +936C/T genotype showed that the heterozygote CT genotype was higher in both groups than normal and PE women, but in general, there was no significant difference between these three genotypes and the incidence of PE (P>0.05).

Table 2
Evaluation of genotypes related to -634C/G and +936C/T polymorphisms

Genotypes	Control		PE		OR (CI)	P- value
	N	%	n	%		
SNP-634C/G						
CC	54	40.3	44	32.8	0.724 (0.440-1.193)	0.205
CG	54	40.3	59	44.0	1.165 (0.717-1.893)	0.536
GG	26	19.4	31	23.1	1.250 (0.695-2.248)	0.455
CG+GG	80	59.7	90	67.2	1.381 (0.838-2.274)	0.205
SNP+936C/T						
CC	52	38.8	52	38.8	1.105 (0.617-1.693)	1
CT	70	52.2	65	48.5	0.861 (0.533-1.391)	0.541
TT	12	9.0	17	12.7	1.477 (0.676-3.227)	0.326
CT+TT	82	61.2	82	61.2	1.000 (0.612-1.635)	1
Abbreviations: PE: preeclampsia; SNP: single nucleotide peptide						

Table 3 shows the association between +936C/T and -634C/G genotypes with serum VEGF levels in women with PE and control group. The mean concentration of VEGF in women with PE and the normal group was 141.9 and 61.5(pg/ml) respectively (Fig. 1), indicating a significant difference in the concentration of this factor in both groups, and as a result, revealing a significant difference in VEGF between the two groups (P≤0.001). In patient group, the level of VEGF marker was significantly greater than the normal group, and these results indicate that the VEGF marker is increased in these patients (P ≤0.05).

Table 3
Relationship between +936C/T and -634C/G genotypes with serum VEGF levels in women with PE and control group

VEGF with SNP +936	**CC**	**CT**	**TT**	**P- value**
All Subjects	141.4 ± 23.03	108.3 ± 17.59	126.4 ± 39.4	0.381
Control	104.2 ± 20.43	84.4 ± 17.7	20.6 ± 5.84	0.052
PE*	174.4 ± 39.02	135.3 ± 31.35	220.4 ± 59.11	0.124
VEGF with SNP-634	**CC**	**CG**	**GG**	
All Subjects	118.7 ± 21.56	122.2 ± 20.75	132.3 ± 29.56	0.841
Control	102.8 ± 23.44	85.9 ± 21.57	62.6 ± 14.17	0.838
PE	134.2 ± 36.06	155.5 ± 33.87	217.5 ± 58.09	0.232
PE*=Preeclampsia				

Discussion

This study, which aimed to investigate the polymorphisms of VEGF gene in pregnant women with PE, showed that despite a significant increase of serum VEGF concentrations in these women, it appears that -634C/G and +936C/T polymorphisms of VEGF gene are not associated with the onset of PE.

Screening for PE using maternal factors and genomic variations is preferred to other tools such as taking a medical history (14). In this study, maternal age and BMI, pregnancy week, infant weight, and hemoglobin concentration were risk factors for PE. In this study, as in another research, the mean age of the case group was significantly different from the control group, indicating that the risk of PE increases with age (15). In another research, the average age of >35 was associated with PE (16). A study found that the lower the gestational age at delivery in the previous pregnancy, the higher the risk of PE in the next pregnancy, which was a strong determinant of the disease (17). While in another study, no association was found between age and PE (18). Besides, a study of adverse pregnancy outcomes using a national multicenter perinatal database reported that advanced maternal age (≥45) is related with greater risk of adverse birth outcomes, especially for maternal complications such as PE (19). Since the association of PE with >35 age has been proven in numerous studies (16), our research indicates that the PE risk may increase with age. Similar to our results, Jean-Ju Sheen and colleagues showed that older women were more likely to develop PE (20) .Therefore, since only women aged 18-37 years were included in this study, it can be said that the risk of PE increases with age in any age range of the mother. In this research, it was observed that increasing BMI increases the risk of PE, which is consistent with some other studies (21, 22). In a two-year cohort of the impact of maternal pre-pregnancy BMI and gestational weight gain on the risk of PE, the association between overweight and disproportionate weight gain with a higher risk of PE was shown (23). Another study identified overweight pregnant women and obesity as

a risk factor for PE (24). Recently, another investigation assessed the effect of maternal weight on maternal and perinatal outcomes, suggesting that gestational weight gain affects the maternal and perinatal outcomes and that pre-gestational BMI is an indicator of PE (25). Moreover, another study found that hemoglobin levels in control and PE groups were not significantly different (26). A research found that the PE occurrence was more frequent in the summer (27); however, in the present study, there was no significant correlation between the occurrence season and PE. Recently, however, a study showed that elevated average temperature is a risk factor for pregnant women with PE, and this increase in temperature is more common in summer (28).

The hypothesis is that the imbalance of pro-angiogenic and anti-angiogenic factors in mother's bloodstream is a main regulator in the growth of normal endothelial function as one of the important characteristics of the disease. Early findings from a study showed that high levels of sFlt-1 expression by an adenovirus vector in rats can lead to gestational hypertension and renal proteinuria (27).

Recent studies on human genetics have revealed that genetic factors play a role in PE, but the exact genetic pattern is not clear. In this research, we tried to prove the relationship between VEGF gene and PE incidence. The role of VEGF in PE needs further attention. Several studies have reported the increase in serum levels of VEGF in women with PE (29, 30). Our study concluded that VEGF level increases in women with PE relative to the normal group, while a study concluded that the VEGF level is reduced in women with PE; this contradiction can be due to genetic variations in the studied populations or differences in the study population (31).

A study showed that in women with PE, an antagonist of VEGF known as Sflt-1 is increased in the blood of mothers with PE and the resulting rise in sflt-1 lowers serum VEGF levels, while the level of this VEGF antagonist was not changed in healthy pregnant women (31). In one study, it was found that the Sflt-1/ placental growth factor (PlGF) ratio is a valuable test in predicting adverse maternofetal outcomes (AMFO) in proven early-onset PE, which can improve the treatment process by predicting adverse effects(32). Considering the fact that most studies have been conducted on VEGF gene sequence in mothers, studying the level of VEGF in umbilical cord of infants as well as polymorphism of this gene in neonates is suggested.

The results of one study showed that there is an association between frequency of +936T allele and increased susceptibility to PE (33). But in our study, there was no significant correlation between the incidence of PE and this SNP, one of the most important reasons for which could be related to genetic differences. Since we did not investigate a single ethnic group in this research and our study population was a combination of different races, it is suggested that a study of the Iranian ethnic groups, including Lur, Kurd, Turk, and Arab, and so on to understand the role of race in this subject.

In our research, there was no significant correlation between PE incidence and -634G/C and +936C/T polymorphisms. Similar results were obtained in a study in which three SNPs of VEGF gene, including -2578C/A, -634G/C, and +366C/T were investigated using RFLP-PCR method. The obtained results showed that there is no relationship between these three SNPs and PE (12). Of course, one of the

limitations of these two studies was the use of RFLP-PCR technique. Therefore, it is suggested that Real-time PCR method should be used to better investigate SNPs.

In another study, no significant relationship was seen between the allele frequency of three VEGF gene SNPs, including -460C/T, +405C/CG, and +936C/T with the incidence of endometriosis (34). In contrast to the present study, it was observed that VEGF level increased in sera of patients with PE, however, there was no significant relationship between the incidence of PE and +936C/T polymorphism.

Conclusion

In conclusion, controlling weight and observance of an appropriate diet along with regular exercise are effective in preventing PE in pregnant women. The decrease in hemoglobin levels can also affect the exacerbation of the disease. Considering the fact that the serum VEGF levels increased in patient group relative to the normal group, this factor can be used as a predictive and diagnostic factor for the incidence of PE during pregnancy.

Declarations

Acknowledgments

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Conflicts of Interest

The authors report no conflicts of interest.

Compliance with Ethical Standards

This study has received ethics code from the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (*Ethics No:* IR.AJUMS.REC.1393.147*). Informed consent was obtained from all individual participants included in the study.*

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Figures

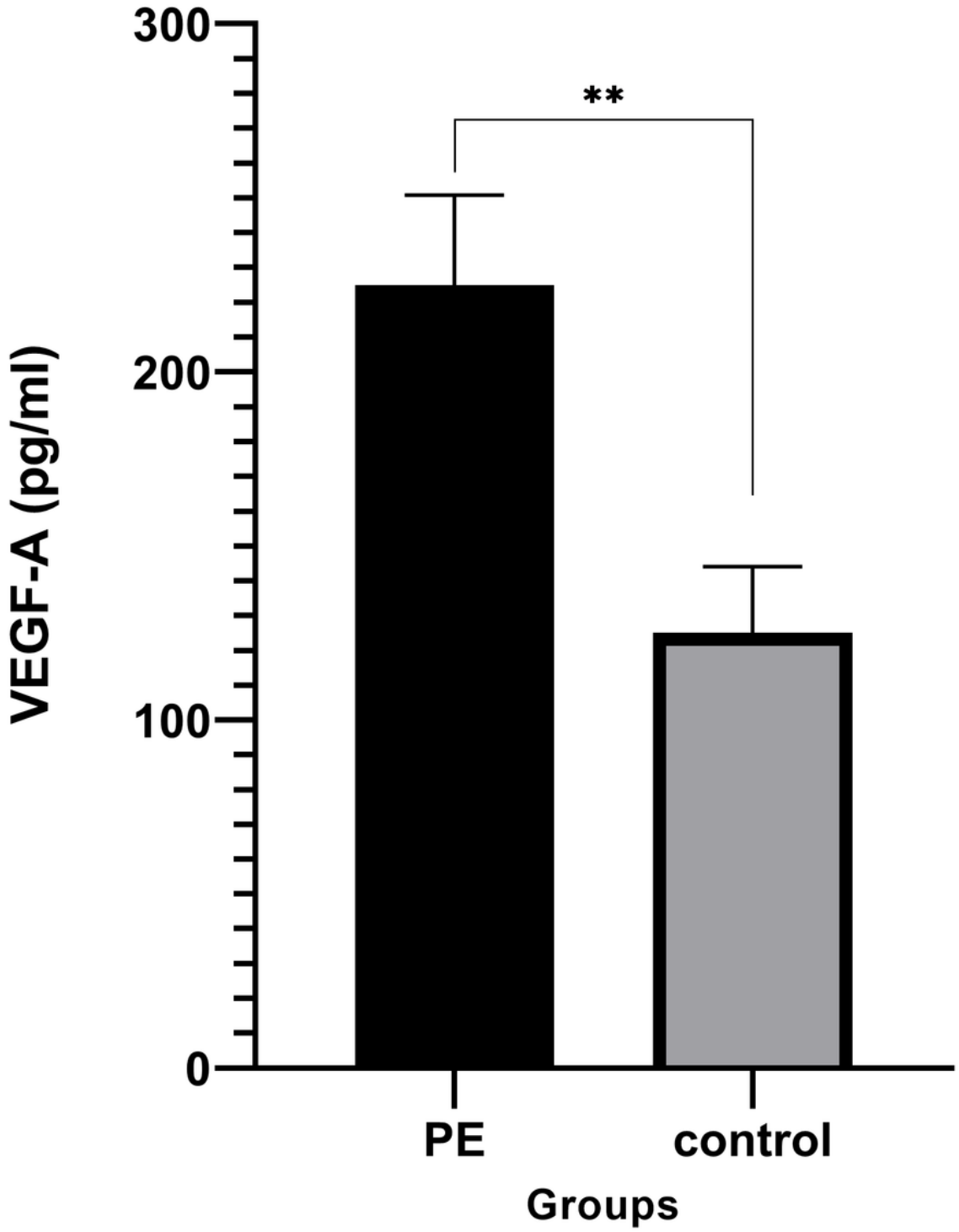

Figure 1

Comparison of serum VEGF-A levels between the two groups. (** p<0.001)