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Clinical significance of maternal serum vascular endothelial growth factor (VEGF) level in idiopathic recurrent pregnancy loss

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Abstract. – OBJECTIVE: The aim of this study was to determine whether maternal serum vascular endothelial growth factor (VEGF) levels are associated with unexplained recurrent pregnancy losses (RPLs).

PATIENTS AND METHODS: Twenty-one pregnant women with idiopathic RPLs who were selected from 47 cases with RPLs were compared with age-matched 24 control participants. Transvaginal obstetric ultrasonographies were performed and maternal serum samples were collected between 5th and 10th gestational weeks to evaluate serum VEGF and progesterone (P4) concentrations. Enzyme-linked immunosorbent assay technique was used in measurements of VEGF and P4.

RESULTS: Prevalence of idiopathic cases among all RPLs was 44.7%. Median serum VEGF value was found statistically higher in RPL group when compared to control group (210.33 ± 108.23 pg/ml *vs*. 123.91 ± 18.8 pg/ml, respectively). There was no statistical difference between the median values of serum P4 levels in idiopathic RPL group and the control group (19.53 ± 5.79 ng/ml and 20.08 ± 7.85 ng/ml, respectively). Serum VEGF levels did not differ significantly with regard to gestational age within the RPL and control groups ($p = 0.72$ and $p = 0.89$, respectively). A positive correlation was found between VEGF levels and the patients' age within RPL group (r = 0.515).

CONCLUSIONS: Serum VEGF levels are independent by the gestational age. Serum VEGF concentrations correlate positively to maternal age. Increased maternal age, especially maternal age over 35 years, is related to elevated serum VEGF concentration. Increased maternal serum VEGF concentration is related with recurrent pregnancy loss.

Key Words:
Vascular endothelial growth factor, Progesterone, Recurrent pregnancy loss, Recurrent miscarriage.

Introduction

Recurrent pregnancy loss (RPL), also called as recurrent miscarriage, is a critical medical problem of reproductive medicine. RPL is classically defined as the occurrence of three or more consecutive pregnancy losses[1,2]. A more recent definition by American Society for Reproductive Medicine qualifies presence of two or more pregnancy losses, not necessarily to be consecutive, as RPL[3]. It is observed in about 0.5-2% of the women in reproductive age[2,4,5]. RPL has been associated with maternal thrombophilic disorders[6], immune dysfunction[7], uterine anomalies[8], endocrine abnormalities[9], and parental chromosomal abnormalities[10]. However, etiological factors could be demonstrated in solely less than 50% of the couples[11-13]. There are still debates on many of the alleged reasons that are thought to cause recurrent pregnancy loss except genetic factors, anatomic factors and autoimmune disorders[14-19]. Recent studies investigated deterioration of vascular evolution, which is a basic mechanism in achieving decidualization of the endometrium, embryo implantation, and placental development, as one of the attributed causes of miscarriages[20-23]. It is supposed that an inappropriate angiogenesis results with ineffective implantation or inadequate development of placenta after a regular implantation[24,25].

A rather sophisticated angiogenesis is required for the implantation and further development of human embryo. Accordingly, angiogenic growth factors have a major role in embryonic and fetal development. Although there are many growth factors that induce angiogenesis, vascular endothelial growth factor (VEGF) is the most potent one[20,26]. Demonstration of embryonic losses in animal models in which

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VEGF gene was disrupted indicates the key role of VEGF[26].

In addition to its effects on stimulation and arrangement of angiogenesis, it is thought that VEGF controls growth and differentiation of cytotrophoblasts through VEGF receptors. VEGF receptors are found on cytoplasmic membranes of trophoblasts[27]. Therefore, it guides and orientates the implantation. All these influences show that VEGF has taken over one of the fundamental roles in placental and early embryonic development.

Maternal serum VEGF concentrations were shown to increase in early stages of first trimester in normal pregnancies[28]. Corpus luteum, endometrium and placenta were known as VEGF sources. However, endometrium and placenta were defined as major sources of VEGF, and corpus luteum was to have a little support[28]. For this reason, major source of VEGF was ascertained to be the maternal fetal interface, and this invention increased the importance of this glycoprotein molecule especially in placental disorders.

Progesterone (P4) has been known to protect pregnancy by various ways including inhibition of T lymphocyte-mediated tissue rejection, blockage of memory B cells to some extent and modulation of immune system[29,30]. Serum P4 levels above a particular level (20.58 ng/mL was given as the median value for P4) are widely accepted to demonstrate a favorable prognosis of the pregnancy[31]. Similarly, it has been shown that serum P4 could differentiate spontaneous miscarriages and ectopic pregnancies from that of healthy intrauterine pregnancies[31]. In this study, we measured serum P4 measurements to exclude non-viable intrauterine pregnancies.

Although there are studies concerning the significance of VEGF measurements in spontaneous miscarriages, ectopic pregnancies, and preeclampsia, studies investigating VEGF in patients with RPLs are substantially limited[32-34]. Moreover, the studies investigate a rather heterogeneous population of RPL. Therefore, we investigated serum VEGF levels in patients with RPL who solely have three or more consecutive miscarriages, and in whom any reason for RPL could not be demonstrated.

Patients and Methods

Eighty-four non-pregnant patients with history of three or more consecutive miscarriages were identified among the patients who admitted to our reproductive endocrinology and infertility outpatient clinic. Number, timing, and the sequence of miscarriages together with the number of live births if present were questioned to exclude late 1st trimester or 2nd trimester miscarriages, and sporadic spontaneous miscarriages. After a detailed anamnesis of their history, sporadic spontaneous pregnancy losses and late miscarriages were determined in 37 cases (Figure 1). Actual repetitive early pregnancy losses were established in 47 subjects. Physical examination, gynecologic examination, pelvic evaluation with transvaginal ultrasonography and hysterosalpingography were performed to all patients. Peripheral blood karyotyping was performed to all couples. A detailed survey including serum prolactin, free testosterone, 17-hydroxyprogesterone, dehydroepiandrosterone sulphate, fasting glucose, and insulin levels; thyroid function tests; mutations in factor V Leiden, methylenetetrahydrofolate reductase, and prothrombin genes; lupus anticoagulant, anticardiolipin Ig M and IgG antibodies; protein C, Protein S, activated protein C resistance, antithrombin III, homocysteine, folic acid, and vitamin B12 measurements were conducted for each patient. Patients with overt diabetes mellitus, chronic hypertension, connective tissue disorders, endometriosis, and polycystic ovary syndrome were excluded from

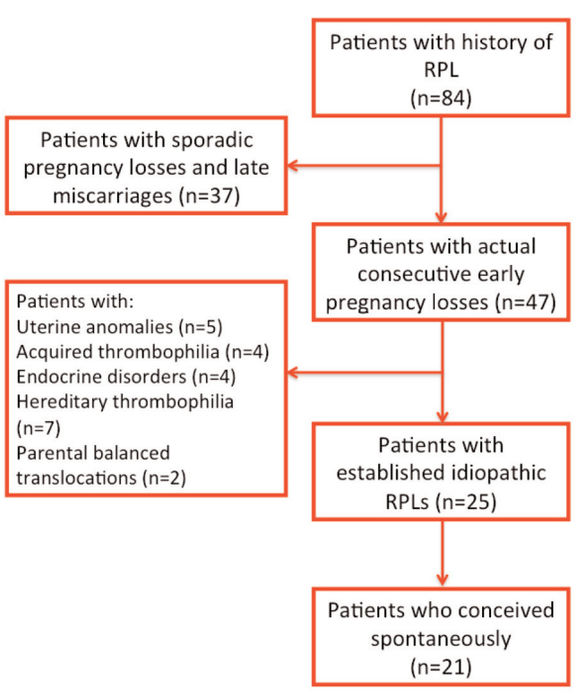

Figure 1. Flow chart for the enrollment of the participants.

the study. Of the 47 subjects, 25 subjects in whom RPL was not related to any etiological factor were further followed up. Twenty-one of the 25 patients who conceived spontaneously constructed the study group. Age-matched 24 control participants with healthy ongoing pregnancies were recruited from our antenatal outpatient clinic. Ethics approval for the study was obtained from Ethics Committee of Dr Zekai Tahir Burak Women's Health Hospital. Study participants were informed and gave written consent.

Peripheral venous blood samples of the participants were collected between 5th and 10th gestational weeks. Transvaginal obstetric ultrasonography for the evaluation of gestational age was conducted at the same day of sample collection. Samples were centrifuged in 1000 rpm for 10 min. Sera were separated and were preserved at -80°C until they were studied. Serum VEGF levels were assessed quantitatively by enzyme-linked immunosorbent assay (ELISA) technique. VEGF 165 kit (Biosource®, NY, USA) was used to detect VEGF 165 form, which is the most detected form in human serum. The sensitivity of the kit was 5 pg/ml. Serum P4 levels were measured by progesterone kit (Roche Diagnostics®, Mannheim, Germany) with a sensitivity of 0.03 ng/ml.

Statistical Analysis

Data were analyzed by using Statistical Program for Social Sciences 22.0 software (SPSS Inc., Chicago, IL, USA). Descriptive characteristics for continuous variables were demonstrated in mean ± standard deviations. *T*-test and nonparametric Mann-Whitney U test were used in analysis between groups, as appropriate. To verify that the both groups reflect the characteristics of their original population a randomness test for continuous variables (Wald-Wolfowitz runs test) was tested. Two-Sample Kolmogorov-Smirnov Test was checked to demonstrate whether groups are drawn from the same distribution. The possible effect of gestational age and age of the patients on VEGF levels were analyzed with one-way ANOVA test. Correlations between variables were investigated by using Pearson's correlation coefficient. Statistical significance level was accepted as < 0.05.

Results

Etiological factors that were established as the possible cause of RPL among our patients (n = 22) were uterine anomalies (n = 5, 23%), autoimmune causes (n = 4, 18%), endocrine disorders (n=4, 18%), hereditary thrombophilia (n = 7, 32%), and parental balanced translocations (n = 2, 9%). Prevalence of the idiopathic causes (n = 25) was 53%.

Table I represents the socio-demographic characteristics of the study participants. The difference between mean age of patients with idiopathic RPLs and controls was statistically insignificant (28.2 years and 27.5 years, respectively). There was not a statistically significant difference between mean values of BMI (kg/m^2) in RPL and control groups (26.4 and 25.1, respectively). There was not a statistically significant difference between means of gestational age in RPL and control groups (49.9 days and 50.7 days, respectively). The differences of gravidity, parity, number of live births, number of miscarriages, and curettages between both groups were statistically significant ($p < 0.01$).

The difference of median serum VEGF concentrations between patients with RPLs and con-

Table I. Socio-demographic characteristics of the study participants.

	Patients with RPL (n=21)	Controls (n=24)	*p*
Age (years)	28.2 ± 5.2	27.5 ± 4.4	0.819
Gestational ages (days)	49.9 ± 9.8	50.7 ± 7.9	0.406
Gravidity	4.7 ± 1.1	2.8 ± 1.3	**<0.01**
Parity	0.4 ± 0.6	1.7 ± 0.9	**<0.01**
Number of live births	0.3 ± 0.5	1.6 ± 0.9	**<0.01**
Number of abortions	3.3 ± 0.8	0	**<0.01**
Number of D&Cs	0	0.4 ± 0.2	0.210
Height (cm)	160.1 ± 6.5	161.1 ± 4.8	0.648
Weight (kg)	67.5 ± 15.4	62.2 ± 7.6	0.405
BMI	26.4 ± 5.1	25.1 ± 4.3	0.363

BMI: Body mass index, D&C: Dilatation and curettage, RPL: Recurrent pregnancy loss.

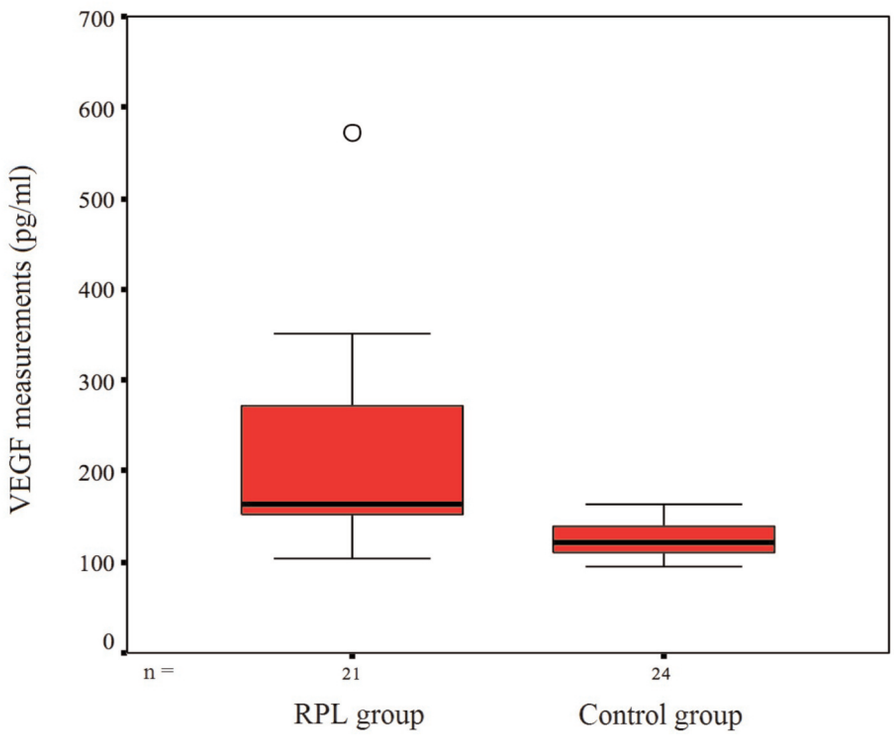

Figure 2. Distribution of serum VEGF levels in study participants.

trols was statistically significant ($p < 0.001$) (Table II). Figure 2 shows the distribution of VEGF levels with median values, 75th and 95th percentiles in both groups. The difference between medians of serum P4 concentrations in both groups was not statistically significant ($p = 0.767$). Figure 3 shows the distribution of P4 measurements in both groups with median values, 75th and 95th percentiles.

As the statistical significance is reached, participants were divided into subgroups according to gestational ages (Figure 4) and maternal ages (Figure 5). Adequate case counts were reached in subgroup analysis for the specified gestational age categories except 35-41 days and 49-55 days categories (Figure 4). The differences of VEGF levels between subgroups of RPL and control groups with regard to gestational age were statistically significant ($p < 0.01$). Serum VEGF levels did not differ significantly with regard to gestational age within the RPL and control groups ($p = 0.72$ and $p = 0.89$, respectively).

Although there was a null cluster of RPL patients in 31-34 years category, subgroup analysis was conducted for the rest of the specified maternal age categories (Figure 5). The differences of VEGF levels between subgroups of RPL and control groups with regard to maternal age were statistically significant except < 22 years category ($p < 0.01$). The difference between median serum VEGF levels in > 35 years category and the other categories was statistically significant within the

Table II. Progesterone and VEGF levels in both groups.

	Patients with RPL	Controls	p
Progesterone (ng/ml)	19,53 ± 5.79	20.08 ± 7.85	0.767
VEGF (pg/ml)	210.33 ± 108.23	123.91 ± 18.8	<0.001

RPL: Recurrent pregnancy loss.

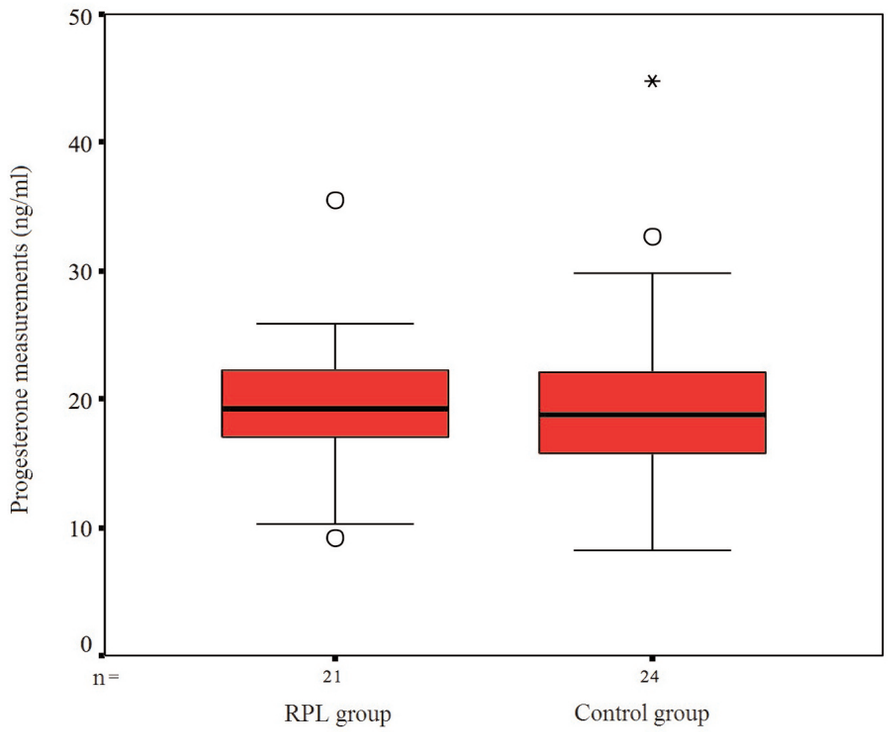

Figure 3. Distribution of serum progesterone levels in study participants.

RPL group ($p < 0.01$). A moderate to strong relationship was demonstrated between maternal age and VEGF levels within the RPL group ($r = 0.515$) (Figure 6). There was a weak correlation between maternal age and VEGF levels within healthy pregnant controls ($r = 0.171$) (Figure 6).

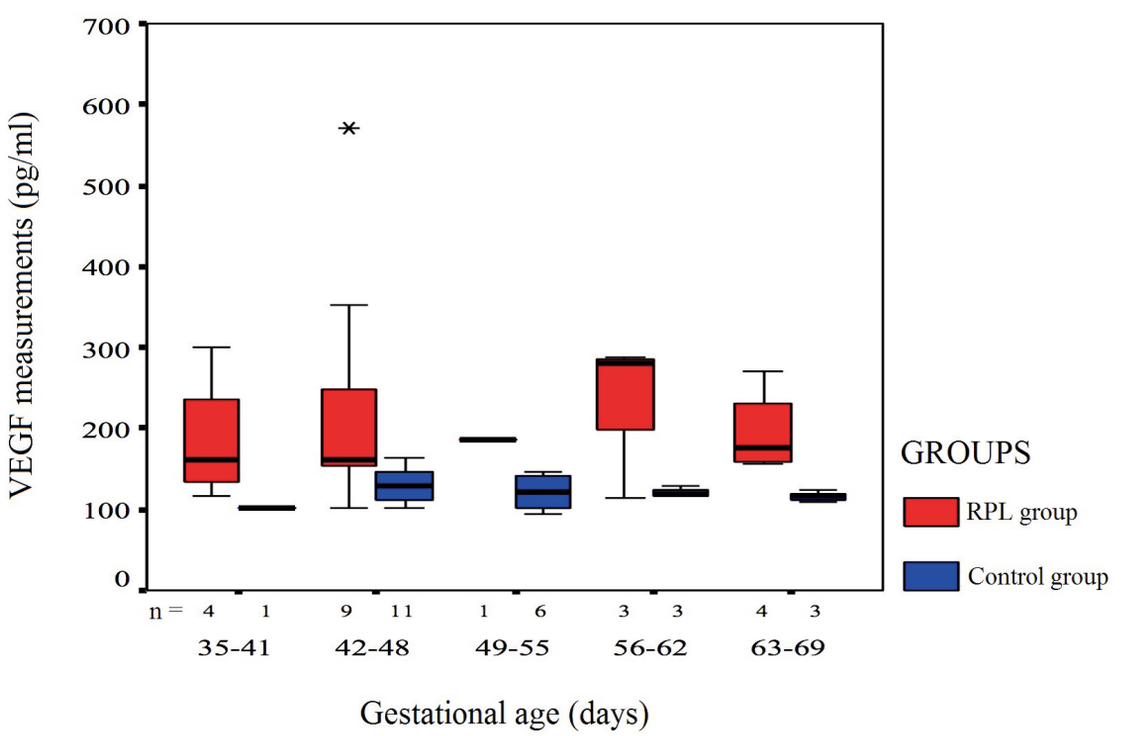

Figure 4. Relationship between serum VEGF levels and gestational age subgroups in both groups.

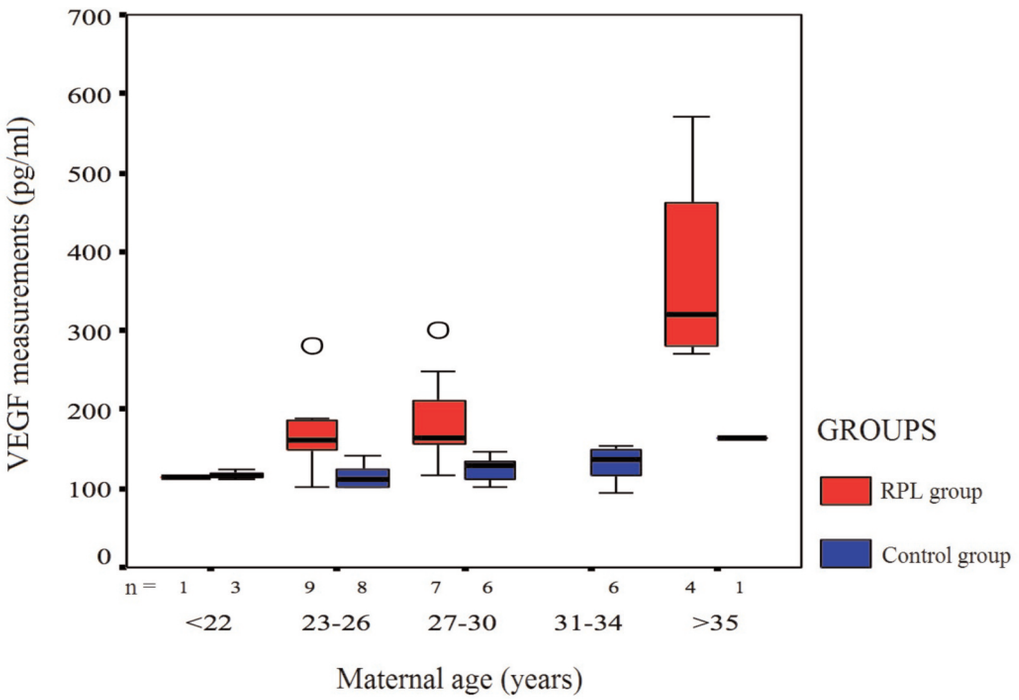

Figure 5. Relationship between serum VEGF levels and maternal age categories in both groups.

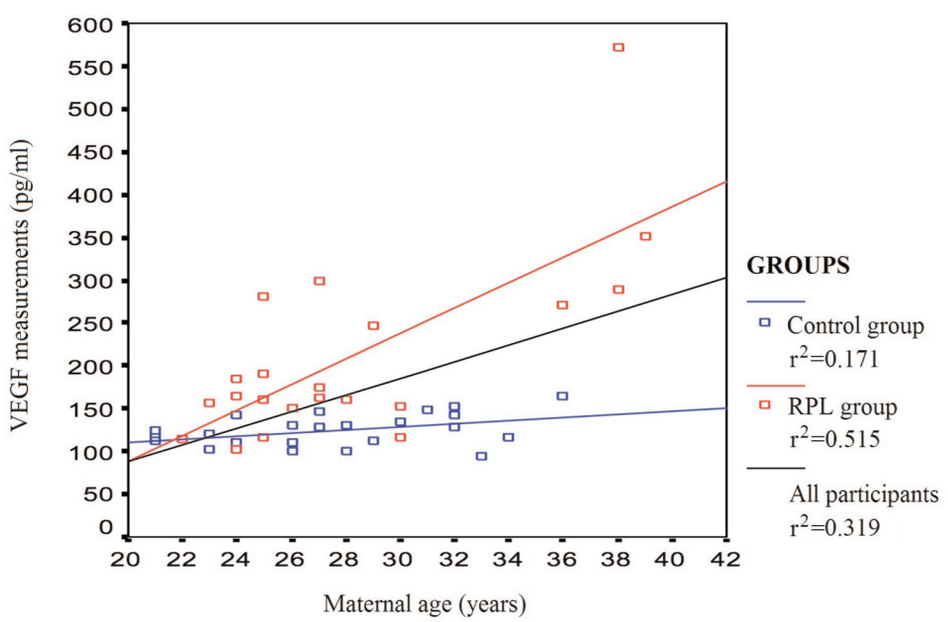

Figure 6. Relationship between serum VEGF levels and maternal age.

Discussion

In this study, we demonstrated that serum VEGF concentrations were increased in patients with RPL compared to healthy pregnant controls. This difference was observed in all gestational age categories. Although VEGF levels remained increased, they did not differ with gestational age within RPL group. VEGF levels differed with maternal age. A positive linear relationship was

found between VEGF levels and maternal age within RPL group. This relationship was most evident in over 35 years category. However, VEGF levels did not change significantly with maternal age among healthy pregnants.

Recent studies have pointed out possible roles of placental oxygenation and angiogenic mediators in the course of placental development of human embryos[9,13,20,22,23]. VEGF, which is demonstrated as the most important angiogenic growth factor, mediates the growth and arrangement of vascular endothelia within the placenta[20,22]. It has previously been shown that VEGF levels were increased during first trimester of normal pregnancies[28]. In addition, VEGF levels were also demonstrated to correlate positively with placental volume with increasing gestational age until 20th weeks[35]. When patients with history of RPLs were considered, serum VEGF levels were detected to be decreased with respect to normal pregnancies in some of the previous studies[35,36]. Conversely Pang et al[34] demonstrated an increase in serum VEGF levels in patients with RPL. The current study stands against the former studies, and is in favor of the latter study by showing that serum VEGF levels are markedly increased in patients with RPLs when compared to healthy pregnancies. Considering the role of VEGF as a promoter of angiogenesis, one would expect higher levels of VEGF to provide adequate placentation. Therefore, higher levels of VEGF were found in patients affected by idiopathic RPL could be explained by, predominance of placental autonomous production of VEGF. On the other hand, expression of VEGF is known to be upregulated as a response to hypoxia[34], because hypoxia is the main regulator of VEGF production. Eventually, our theory considering the hypoxia-related regulation of placental VEGF production could be affirmed as the main regulatory factor. In the mean time, a possible explanation to increased VEGF levels in RPL group could be the time of serum sampling. At the time of serum sampling, it is possible that the underlying pathophysiologic course has just begun or exaggerated, but yet, did not end up with placental deterioration, which subsequently results with the pregnancy loss.

We also demonstrated that VEGF levels were increased in patients with RPLs when compared to control cases in all of the subgroups divided according to gestational ages. The data obtained from all subgroups were in consistency with the main outcome of the study itself. In addition, we identified that VEGF levels remained unchanged among healthy pregnant controls with the varied gestational ages suggestive of independence from gestational week. Nevertheless, the latter result was not in accordance with the study of Wheeler et al, which concluded that VEGF was elevated by increasing gestational age[35]. Based on these results, the assumption can be speculated that circulating VEGF in patients with RPLs could vary due to functionality and reproducibility of the deteriorated placenta and permeability and integrity of maternal fetal interface.

When subjects were subgrouped into categories according to maternal ages, we identified a significant difference between subgroups of patients with RPL and controls. Increase in VEGF concentrations was most prominent in patients with RPL and age over 35. Similar to our results, study of Quenby et al concluded that increased maternal age above 35 years was an unfavorable prognostic factor in patients with recurrent miscarriage[37]. The results of current study and Quenby et al correspond to each other by pointing a distinguishable increase in VEGF levels by 35 years of age in ours, and increased amount of pregnancy losses by 35 years of age in their study population with the same hazard, respectively. In this study, we demonstrated that a potent direct linear relationship existed between VEGF and maternal age in RPL group, whereas similar relationship could not be established in controls. It is possible from the present data to say that increased maternal age, especially maternal age over 35 years, is related with elevated serum VEGF concentrations, which could possibly be related with the pregnancy prognosis. But, unfortunately in the presence of a missing subgroup (31-34 years subgroup), we could not calculate a true cut-off value for maternal age in relation to serum VEGF concentrations. These findings indicate the importance of maternal age in patients with RPLs. Additionally, our results emphasize a possible diagnostic role of elevated VEGF in estimating pregnancy outcome in patients with RPLs. Therefore, maternal serum VEGF levels might be a candidate marker in determining prognosis of pregnancies in patients with RPLs, and presumably in patients with threatened abortions with the accumulation of novel evidence.

In addition, we demonstrated that serum P4 levels did not differ significantly between RPL

patients and controls. As serum P4 concentrations could be used in discrimination of healthy intrauterine pregnancies from that of spontaneous miscarriages[31], favorable clinical conditions of the pregnancies of our RPL patients are ascertained. Therefore, the data implies that the sampling conducted for the measurement of serum VEGF on the study participants was appropriate, as it was demonstrated by P4 measurements indicating the good condition of the pregnancies of study participants. In parallel to that assumption, it could be speculated that the timing of sampling was prior to the events that could result with miscarriage, which is the major concern in patients with RPL.

Conclusions

This investigation pointed out the increased VEGF concentrations in patients with RPLs compared to healthy pregnants. We also demonstrated that there is a linear relationship between VEGF and maternal age in RPLs. There is a prominent increase in VEGF levels above 35 years of age in patients with RPL. VEGF can be used as a biochemical parameter in discrimination of RPL patients from that of healthy ones.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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