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Original article

Angiogenic factors measured in aspirated placental tissue between the 10 + 6 and 18 + 3 weeks of gestation

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ABSTRACT

This study was designed to determine the level of vascular endothelial growth factor-A (VEGF-A), basic fibroblast growth factor (bFGF) and endothelial nitric oxide synthase (eNOS) in chorionic villi during in first and second trimester, and their association with nuchal translucency (NT) measured by ultrasound. Seventy-five singleton healthy pregnancies with no detected congenital malformation were collected for NT measurements and chorionic villus sampling (CVS). Concentrations of angiogenic factors were assayed in chorionic villi sampled between 10 + 6 and 18 + 3 weeks of gestation. ENOS level was steady during this gestational period, while the concentrations of VEGF-A and bFGF significantly decreased. Placental concentrations of VEGF-A and bFGF correlated positively with each other (semi-partial correlation in multivariable linear regression (r): 0.90) and both correlated negatively with the eNOS level (r: -0.64 and r: -0.83, respectively). NT was positively correlated with eNOS concentration and negatively correlated with bFGF levels (r: 0.85 and r: -0.78, respectively). Inverse correlation was found between gestational age and VEGF-A and bFGF concentrations (r: -0.57 and r: 0.73, respectively). Alterations of angiogenic factors in chorionic villi might be an adjunct modality to NT and foetal growth as sonographic markers.

1. Introduction

Basic fibroblast growth factor (bFGF) is a potent inducer for vasculogenesis and it enhances branching angiogenesis in the placenta [1,2]. Vascular endothelial growth factors (VEGFs) are involved in the formation of primordial vessels [3,4]. Physiologic intraplacental hypoxia triggers expression of VEGF family which principally mediates branching and non-branching angiogenesis in the first trimester [5] leading to the development of capillaries of the villi until 10–12 weeks of gestation. From week 12 onwards, high VEGF levels boost richly branched capillary beds within mesenchymal and immature intermediate villi [5] and VEGF level in the placenta peaks at the end of the first trimester [6]. Different patterns of VEGF and bFGF expression were noticed at delivery in pregnancies complicated by foetal growth restriction (FGR) [7–9], type I diabetes [9] and hypertensive disorders [10] indicating a vascular remodelling of the placenta in these pathologic conditions. Endothelial nitric oxide synthase (eNOS) is found in the endothelium of vessels of stem villi [11]. High level of nitric oxide in villous (15.6 nmol L-citrulline/minute/g protein) and in non-villous trophoblast (9.3 pmol/mg protein/minute) may participate in maintenance of low vascular resistance in foetoplacental circulation in first and second trimester [12,13].

Nuchal translucency (NT) is a marker for different foetal malformations [14–18]. Possible mechanism of increased NT includes venous congestion [14] and the failure of lymphatic drainage [15,16]. Angiogenic factors may have a critical role in formation of various blood vessels and in lymphatic drainage in the placenta and in the embryo. One of the possible explanations of the correspondence between the examined angiogenic factors and higher NT could be the decreased drainage of the venous and lymphatic system in the embryonic neck [16,19–21]. Although, our previous findings suggest that VEGF-A level in the placenta obtained by means of chorionic villus sampling (CVS) at 10–13 weeks of gestation is not decisive in increased nuchal translucency (NT) [22].

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The aim of present study was to verify the relationship between VEGF-A, eNOS and b-FGF levels in the placenta and elevated NT. A complementary goal was to explore whether VEGF-A, eNOS and b-FGF levels are correlate with each other in placental tissue since these vascular factors are significantly interrelated in the formation of the vascular network of placental bed at 11–19 weeks of gestation.

2. Materials and methods

This was a prospective cross-sectional study, approved by the Institutional Review Board, on 131 pregnant women with spontaneous conception of singleton, non-smoking gestations, presenting for chorionic villus sampling (CVS) between 10 + 6 and 18 + 3 weeks at University of Szeged. All patients were dated by the sonographic measurement of the crown-rump length (CRL) below 13 + 6 weeks. At our centre, NT assessment and an anatomic scan is provided for all pregnancies between 11 + 0 and 13 + 6 weeks [23].

The indications of CVS of the 131 cases were increased NT (≥2MoM for gestational age) (n = 18), chromosome aberration or gene disorder in previous pregnancy (n = 65), and advanced maternal age (n = 48). Foetuses with aneuploidies (n = 41) or any major structural abnormalities (n = 14), and in utero foetal demise (n = 1) were excluded from the analysis. As a result, 75 non-pathologic pregnancies were included in the study.

For transabdominal CVS we used a steel needle with 1.1 mm outer diameter under ultrasonographic guidance applying free-hand technique. If the sample did not contain chorionic villi, then a second puncture was performed within a 3 cm range around umbilicus and the whole thickness of placental tissue was involved in sampling. Following CVS, anti-D immunoglobulin was administered to women without anti-D antigen.

VEGF-A, bFGF and eNOS concentrations were checked in 1−2 mg of samples of chorionic villi. Tissue samples were kept frozen at −20 °C until testing (up to 1 month). Samples were homogenized in phosphate-buffered saline by an ultrasonic disintegrator (Soniprep 150, MSE, London, United Kingdom) at 22-micron probe amplitude for 2 × 15 s. Homogenates were centrifuged at 10,000 x g and the supernatants were collected for analysis. The preparatory process was carried out at 4 C°. The protein contents of samples were determined with the help of Bradford method, using bovine serum albumin (Sigma, Budapest, Hungary) as standard [24]. VEGF-A was quantified by enzyme immunoassay (Human VEGF Quantikine ELISA Kit, R&D Systems, Wiesbaden-Nordenstadt, Germany). The kit was calibrated with a highly purified Sf21-expressed recombinant human $VEGF_{165}$ as stated by the manufacturer. The sensitivity of detection was 5 pg/mL. The intra- and interassay variabilities were characterized by 6.5 and 8.5 coefficients of variation (CV%), respectively. The bFGF kit (Quantikine HS ELISA Kit, R&D Systems, Wiesbaden-Nordenstadt, Germany) was calibrated using recombinant FGF. The sensitivity of FGF assay was 0.03 pg/mL. At concentration of 5 pg/mL, the intra- and interassay coefficients of variation were 4.3 and 4.7 CV%, respectively. eNOS (endothelial nitric oxide synthase/NOS3) was determined by ELISA (Cloud-Clone Corporation, Houston, TX, USA). The kit is validated to measure eNOS/NOS3 from tissue homogenates. The sensitivity of eNOS assay was <0.58 ng/mL. The intra- and interassay coefficients of variation were <10 and <12 CV%, respectively. The results were expressed as pg VEGF/mg protein, pg b-FGF/mg protein and pg eNOS/mg protein.

Data in respect of demographic details, medical history of pregnancy, maternal height and weight at booking, sonographic findings concerning NT between the 11 + 0 and 13 + 6 weeks of gestation, crown-rump length (CRL) or BPD (biparietal diameter) at the time of NT measurement as well as CVS were recorded. Data on body mass index (BMI) at booking was extracted from medical records. The number of attempts during the procedure was also registered. The gestational age at the time of CVS and the data regarding the pregnancy outcome (birth weight of the infant, gestational age at delivery) were acquired prospectively.

Data were evaluated using version 22 of SPSS statistical software (Armonk, New York, IBM). The non-parametric design of continuous variables was verified by Shapiro–Wilk test. The relationship between the level of angiogenic factors (VEGF-A, bFGF and eNOS) and other continuous variables was assessed using Spearman's rank correlation coefficients and regression analyses. For further statistical analyses, all dependent variables were transformed logarithmically (log10(x)) to be fitted for the Gaussian distribution and the regression model. Multiple linear regression was adjusted for maternal age, BMI at booking in pregnancy care, primiparity and gestational age at the time of CVS as these factors determine the actual placental volume and foetal weight. All constituted confounding factors. Correlation coefficients (B) were calculated for both the univariate and multiple linear regression, whereas standardized coefficients (ß) were given for univariate analyses and semipartial correlations (r) for multivariable regression. The significance level in two-tailed tests was set at 5%.

The signed informed consent was obtained in all cases and aseptic technique was applied. Our work was carried out in accordance with the Declaration of Helsinki and our study has been approved by the institutional research ethics committee (Reference:111/2009).

3. Results

Table 1 presents the patterns for maternal and sonographic features in the study groups. Women who participated in this study had a median age of 32 years, a median body mass index (BMI) of 24 kg/m^2 and approximately one third (32 %) of the participants were primiparous. The foetuses had a median CRL of 57 mm at the time of NT measurement.

Table 2 provides an overview of the levels of angiogenic factors in the placental samples derived from all study groups. The median concentrations of angiogenic factors were as follows: 11.10 pg/mg for VEGF-A, 1019.00 pg/mg for bFGF and 116.80 pg/mg for eNOS. eNOS concentration were steady in chorionic villi between 10 + 6 and 18 + 3 weeks of gestation ($p > 0.05$). By contrast, intrachorionic concentration of VEGF-A has been decreased from 1824 pg/mg to 201 pg/mg. In addition, bFGF concentration demonstrated a relatively large decrease from 1350 pg/mg to 510 pg/mg (Figs. 1, 2) ($p < 0.05$).

Table 3 demonstrates the correlations to the levels of angiogenic factors in the chorionic villi. Among maternal and sonographic factors, maternal age, CRL at the time of NT measurement and NT did not correlate with the VEGF-A levels. By contrast, inverse correlations emerged with high regression coefficients between the BMI and VEGF-A levels in univariate and

Table 1
Clinical and sonographic data (N = 75).

Maternal age (years)	32 [28–36]
Number of nulliparous women in the study (%)	24 (32.0)
BMI at booking (kg/m^2)	23.91 [21.5−26.7]
CRL at the time of measurement of NT (mm)	57 [49.8−63.0]
NT between 11+0 and 13+6 weeks of gestation (mm)	3.4 [1.7−4.3]
CRL at the time of CVS (mm)	72.8 [11.3]
GA at the time of CVS (weeks)	13.29 [12.7−13.8]
Number of insertion	1 [1,1]
Had only 1 insertion (%)	68 (90.7)

Data represent median and [interquartile range] or n (%).
SD = standard deviation, NT = nuchal translucency, BMI = body mass index, CRL = crown-rump length, CVS = chorionic villus sampling, GA: gestational age.

Table 2
Levels of angiogenic factors in samples of placental tissues (N = 75).

VEGF-A concentration in chorionic villi (pg/mg)	11.1 [5.8–25.2]
bFGF concentration in chorionic villi (pg/mg)	1019.0 [749.8–1251.1]
eNOS concentration in chorionic villi (pg/mg)	116.8 [69.0–192.6]

Data are displayed as median and [interquartile range].
VEGF-A = vascular endothelial growth factor-A, bFGF = basic fibroblast growth factor, eNOS = endothelial nitric oxide synthase, SD = standard deviation.

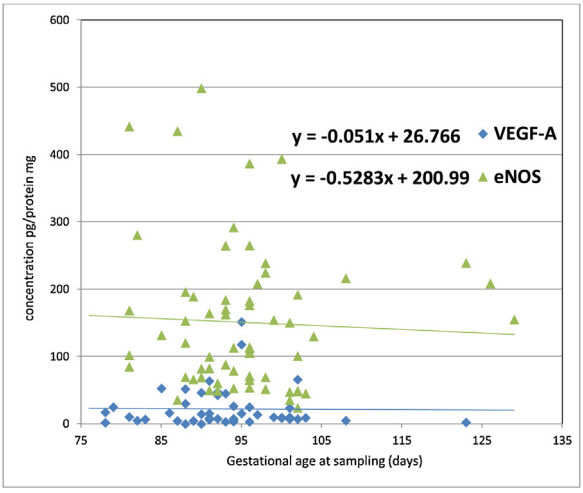

Fig. 1. VEGF-A and eNOS concentration in chorionic villous sample according to the gestational age.

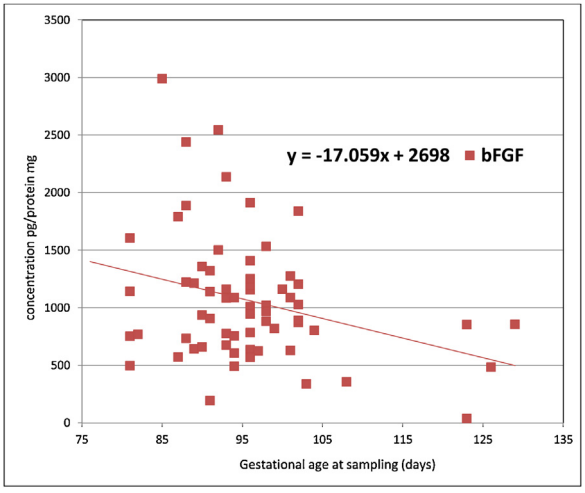

Fig. 2. bFGF concentration in chorionic villous sample according to the gestational age.

multivariable analyses (B = 3.20, ß^2 = 0.41 and B = 2.93, r^2 = 0.51, respectively). A similar trend was found between the gestational age and concentrations of VEGF-A (B = 7.80, ß^2 = 0.34 and B = 6.21, r^2 = 0.33, respectively).

There was a strong, direct correlation between the bFGF concentration and BMI (univariate analysis: B = 4.10, ß^2 = 0.51 and multivariable analysis: B = 4.32, r^2 = 0.45), whereas the bFGF concentrations tended to be higher with the lower NT thickness (univariate analysis: B = 4.91, ß^2 = 0.59 and multivariable analysis: B = 5.23, r^2 = 0.61). The bFGF concentration demonstrated a negative correlation with the gestational length (univariate analysis: B = 4.91, ß^2 = 0.59 and multivariable analysis: B = 5.23, r^2 = 0.61). The level of bFGF correlated directly with VEGF-A concentrations (univariate analysis: B = 11.31, ß^2 = 0.78 and multivariable analysis: B = 12.01, r^2 = 0.81).

ENOS levels correlated positively with the NT thickness (univariate analysis: B = 10.12, ß^2 = 0.69 and multivariable analysis: B = 12.45, r^2 = 0.72). Similarly, inverse correlations were observed for VEGF-A (univariate analysis: B = 7.29, ß^2 = 0.36 and multivariable analysis: B = 8.10, r^2 = 0.42), bFGF levels (univariate analysis: B = 15.21, ß^2 = 0.71 and multivariable analysis: B = 18.21, r^2 = 0.69) and eNOS concentrations.

4. Discussion

Present study reveals several interesting and important novel findings. VEGF-A concentration in the chorion is slightly, but significantly decreasing between the 11th and 19th weeks of gestation. This is somehow in contrast with the results reported by Geva and colleagues [25] who found that VEGF-A expression/peptide concentration remained unchanged in trophoblasts during an uncomplicated pregnancy in the second and third trimester. However, our results reflect that the VEGF-A concentration is decreasing after the late first trimester peak to the middle of the second trimester. These can be explained by the expansion of placental volume which may outweigh the increasing trends of capillary branching [5]. Similarly, bFGF expression decreased between the 10 + 6 and 18 + 3 weeks, which is consistent with previous studies showing that bFGF immunoreactivity in term placentas is lower than at the beginning of pregnancy [1,8]. Moreover, we found no change in eNOS concentrations in chorion during this period of gestation, which would be in contrast with a previous study [26], that demonstrates that chorionic eNOS activity decreases from first trimester to term in uncomplicated pregnancies. To the best of our knowledge, there is a paucity of data on changing in bFGF and eNOS concentrations in the chorion during the late first and early second trimester.

VEGF-A concentration correlates significantly and positively with bFGF level and negatively with eNOS level. In this paper we also report that bFGF correlates also negatively with eNOS level to a great extent. As foetal growth is exponential to a high degree and as the foetal/placental ratio increases markedly throughout gestation, it seems that eNOS concentrations must be constantly high in the chorion to comply with the increasing demand on angiogenesis and blood flow. We show here that the chorionic concentration of bFGF decreases steadily in the first and early second trimester indicating that vasculogenesis has a decreasing tendency in the placenta during this period which corresponds well to the literature findings [27–29]. bFGF is critical for de novo formation of blood vessels from precursor cells and promotes patterning of early branching events [2], but vasculogenesis and branching angiogenesis is less important. In addition, previous research has found that the levels of bFGF within maternal serum increasing in the late second and third trimester [2,30] indicating that branching angiogenesis is stimulated again with advancing gestation and increasing diameter of villi [27–29].

VEGF-A is essential to both vasculogenesis and angiogenesis [3,27–29]. VEGF-A mediates both collateral growth and capillary formation in the chorionic villi. Furthermore, the development of capillary network by prevalence of branching angiogenesis is intensive in the second trimester [27–29]. VEGF-A is an important regulator of blood flow and it is able to stimulate endothelial production of nitric oxide via its major signalling receptor, KDR [31]. It has been manifested that VEGF-A protein promotes an increase of eNOS via functional activation of VEGF receptor-2, leading to angiogenesis and hypotension in the target vessels [32].

Table 3
Correlation between maternal as well as sonographic data and the levels of angiogenic factors in chorionic villi (N = 75).

	VEGF-A concentration				
	Spearman's rho	Univariate linear regression		Multivariate linear regression	
		B	β^2	B	r^2
Maternal age	−0.09	0.16	0.01	0.18	0.02
BMI at booking	−0.27*	3.20*	0.41*	2.93*	0.51*
CRL at the time of measurement of NT	−0.02	0.21	0.03	0.19	0.03
NT between 11 + 0 and 13 + 6 weeks of gestation	−0.04	0.19	0.04	0.18	0.03
GA at the time of CVS	−0.30*	7.80*	0.34*	6.21*	0.33*
	bFGF concentration				
	Spearman's rho	Univariate linear regression		Multivariate linear regression	
		B	β^2	B	r^2
Maternal age	0.11	0.17	0.03	0.19	0.04
BMI at booking	0.31*	4.10*	0.51*	4.32*	0.45*
CRL at the time of measurement of NT	−0.03	0.20	0.03	0.19	0.03
NT between 11 + 0 and 13 + 6 weeks of gestation	−0.36*	4.91*	0.59*	5.23*	0.61*
GA at the time of CVS	−0.29*	3.19*	0.53*	3.23*	0.54*
VEGF-A concentration in chorionic villi	0.62*	11.31*	0.78*	12.01*	0.81*
	eNOS concentration				
	Spearman's rho	Univariate linear regression		Multivariate linear regression	
		B	β^2	B	r^2
Maternal age	0.01	0.18	0.02	0.06	0.01
BMI at booking	−0.09	0.21	0.01	0.11	0.01
CRL at the time of measurement of NT	−0.14	0.19	0.01	0.17	0.01
NT between 11 + 0 and 13 + 6 weeks of gestation	0.225*	10.12*	0.69*	12.45*	0.72*
GA at the time of CVS	−0.05	0.25	0.03	0.19	0.02
VEGF-A concentration in chorionic villi	−0.52*	7.29*	0.36*	8.10*	0.42*
bFGF concentration in chorionic villi	−0.24*	15.21*	0.71*	18.21*	0.69*

VEGF-A = vascular endothelial growth factor-A, bFGF = basic fibroblast growth factor, eNOS = endothelial nitric oxide synthase, BMI = body mass index, GA = gestational age, NT = nuchal translucency, CVS = chorionic villus sampling.
Multiple linear regression analyses were controlled for maternal age, BMI at booking, nulliparity and gestational age.
* Significant result ($p < 0.05$).

VEGF-A induces neovascularization in the uterine arteries and placental bed, and it is a powerful vasodilator of fetoplacental vasculature that precedes the release of nitric oxide via eNOS activation [33]. Based on our results, this might be explained by the fact that the intensity of eNOS stimulation is declining in the regulation of placental blood flow as gestation advances, while VEGF-A is expressed steadily in the second trimester resulting in a negative correlation between the concentrations of these two angiogenic factors. This corresponds well also with the ultrasound scanning of the placental flow using 3-D power Doppler indices, demonstrating that vascularization flow index that reflects volume flow in the placental bed increases only slightly in the course of second trimester [34].

It is of utmost importance that an inverse correlation can only be established between chorionic VEGF-A level and foetal size. This is somewhat controversial to the findings by Sundrani et al. [35] who demonstrated that maternal serum level of VEGF-A predicted well the birth weight of the infant. A possible explanation is that a sustained steady placental VEGF-A concentration is required for physiologic angiogenesis which does not reflect the plasma level of VEGF-A. Serum level of VEGF-A has a peak at 26–30 weeks of gestation in normal pregnancies, and in the second and third pregnancy it may prognosticate the foetal size [35]. Moreover, we did not find any correlation between the actual foetal size and chorionic level of bFGF and eNOS. This is in contrast with the fact that, serum concentrations of these angiogenic factors are positively correlated with foetal size during both in the second and third trimesters [2,36,37]. One can speculate that the actual intrauterine foetal size in the second trimester usually correlates very weakly with birth weight of the neonate.

The results of our previous study [22] are in accordance with our present findings, and placental VEGF-A does not correlate with NT. However, we found a negative correlation between the expression of bFGF and NT, whereas eNOS concentration in the chorionic villi is proved to be strongly correlated positively with NT. The precise role of eNOS and bFGF still needs to be elucidated and further studies are required to prove that these molecules are perinatal markers for genetic or structural abnormalities as well. Theoretically, it is logical that vasculogenic factors may also have an important role in the disturbed placental vascularization in the chorionic tissue and the pathological lymphoangiogenesis of elevated nuchal edema. Previous research in fetuses with increased NT showed abnormal lymphatic endothelial differentiation characteristics in the nuchal area, including increased VEGF-A expression in the lymphatic endothelial cells of the enlarged jugular lymphatic sacs [19]. VEGF-A is an important growth factor in placental angiogenesis as well-determining the level of vascularization [20]. Furthermore, free plasma VEGF-A circulates in the fetal circulations at a detectable level that may even potentiate lymphangiogenesis in various fetal organs [21,38]. Furthermore, eNOS is expressed in the vascular endothelium and is a key mediator during the pre-embryonic stage, early development of embryonic heart and vasculogenesis [39,40].

The study has some limitations. ENOS is an enzyme, with its properties of regulation of its activity. To determine the level of active eNOS could be more practical to determine downstream indicators, such as nitrates, or to detect the phosphorylated eNOS in serine 1177. More studies are recommended to clarify the precise implications of the angiogenic factors in the placental and foetal development during the mid-trimester.

We conclude that maternal VEGF-A and bFGF concentrations in the chorionic villi correlate positively with each other and negatively with eNOS concentration independently between 10 + 6 and 18 + 3 weeks of gestation. Furthermore, a lower VEGF-A concentration is related to higher fetal size at the time of CVS. VEGF-A decreases slightly and bFGF decreases rapidly in the chorionic villi, whereas eNOS is unchanged in the mid-pregnancy. Placental bFGF and eNOS might be used as placental markers for potential anomalies because they are correlated well with NT.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors report no declarations of interest.

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