Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 6 [2] January 2017: 31-40

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE **OPEN ACCESS**

Association of Angiogenic Cytokines (VEGF-A and VEGF-C) and clinical characteristic in women with unexplained recurrent Miscarriage

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ABSTRACT
Recurrent miscarriage (RM) defined as 2 or more spontaneous miscarriage before 20 weeks of gestational, affects at least 1% of couples trying to conceive. In over 50% of cases, the cause of the loss of pregnancy remains unexplained .Reduced expression of Angiogenic factors such as vascular endothelial growth factor (VEGF-A) and VEGF-C has been linked with spontaneous miscarriage, likely due to defective fetal and placental angiogenesis. To investigate the relationships between serum level of VEGF-A and VEGF-C with clinical Characteristic in women with URM and comparing with pregnant and healthy women. A case-control study, which was conducted between 90 non-pregnant women with history of RM, those with unexplained RM were eligible (cases) age-matched with 70 non-pregnant women without history of recurrent abortion with at least one child (controls) and 70 pregnant women without history of recurrent abortion with at least one child (controls).Those with unexplained RM were eligible. Demographic and Anthropometric data were retrieved by pre-test questionnaire and serum level of VEGF-A and VEGF-C measured by ELISA kit. This study shows that maternal levels of VEGF-A and VEGF-C are distinctly lower in RSA (189.87±88.1 vs 238.8±99.6) compared to healthy (239.1±99.7 vs 275.5±133.08) and pregnant (301.5±76.4 vs 402.5±128.6) women as control groups. Univariate analysis demonstrated that clinical characteristic factors were significantly associated with concentration of VEGF-A and VEGF-C in cases and controls. Our findings suggest that these molecules could be used as potential predictive markers of miscarriage in these women presenting with URM.
Keywords *: Recurrent Miscarriage,VEGF-A, VEGF-C, Age, BMI*

Received 21.06.2016 Revised 16.09.2016 Accepted 11.11.2016

INTRODUCTION

Angiogenesis is one of the essential molecular procedures in the female reproductive system [1]. The interaction between the endometrium and the trophectodermal cells of the blastocyst is one of the crucial moments for the beginning and maintenance of successful pregnancy[2].In cases of deregulation different pathologies in human reproduction could be presented such as recurrent implantation failures, recurrent miscarriages (RM) or endometriosis[3, 4].

The American Society of Reproductive Medicine (ASRM) defined recurrent miscarriage(RM) as two or more failed clinical pregnancies as documented by ultrasonography or histopathologic examination[5], affects 1–5% of all couples trying to conceive [6,7].The commonly accepted definition stipulates that the fetus or embryo should weigh 500 g or less, a stage that corresponds to a gestational age of up to 20 weeks[8]. Although many different factors related to RM, including chromosomal, anatomical and endocrine aberrations, and infection, have been investigated, .50%of RM cases are still without identifiable factors and labeled as unexplained (unexplained RM) [8]. It's been proposed that URM belongs to an autoimmune illness associated with the failure of fetal-maternal immunologic tolerance [9].

Vascular endothelial growth factor (VEGF)[10], also known as vascular permeability factor (VPF), is an important angiogenic cytokine. VEGF regulates proliferation, differentiation, and survival of endothelial cells and enhances vascular permeability[11] .VEGF-A consists of at least six isoforms through alternative

splicing in humans (121, 145,165, 183, 189, and 206 amino acids) which have different biological properties and bioavailability [12] VEGF functions are mediated via binding to its tyrosine kinase receptors ;VEGF receptor 1 (VEGFR1/Flt1) and VEGF receptor 2 (VEGFR2/Flk1/KDR). Expression of VEGF-A and VEGF-C has been demonstrated in the human endometrium throughout the menstrual cycle with an increase in the late proliferative and secretory phases[13, 14]. In addition, VEGF's expression was found in decidual cells of early pregnancy [15]On the other hand, expression of VEGF receptors was shown in human endometrium [13].

Dysregulation in VEGF-C production at the maternal-fetal interface could be a signal for poor angiogenesis and pregnancy complications. Reduced expression of VEGF-C has been reported in pregnancies experiencing intrauterine growth restriction and preeclampsia. Involvement of VEGF-C may thus explain the non-killer phenotype of uNK cells despite possessing toxic granules and expressing cytotoxicity receptors.Satyan S, in 2009,[16] prove that vascular endothelial growth factor (VEGF)C, a pro-angiogenic factor produced by uNK cells, is responsible for their noncytotoxic activity. Peripheral blood NK cells fail to produce VEGF-C and remain cytotoxic. This response can be reversed by exogenous VEGF-C and it deals with the growth of blood vessels and lymphatics [17].

On the other hand, in RM with increasing numbers of previous miscarriages comes an increasing risk of losing the next pregnancy. Kupka et al. [18] found a miscarriage rate of 21% in couples with no previous miscarriage, in comparison with 27% with a single previous loss, and 31% with three previous losses. In addition, the age of women with RM will influence the findings in studies of endocrinological and nongenetic immunological biomarkers[19]. Immune parameters such as production of autoantibodies and T helper 2 cytokines are affected both directly by improved expectant mothers age. Also, It has been reported that maternal obesity and BMI (Body Mass Index) is associated with an increased risk of recurrent miscarriage after spontaneous and assisted conception [20].

Given these data, in the present study, we purpose to discuss the association of angiogenic cytokines (VEGF-A and VEGF-C) and to correlate Demographic and Anthropometric factors in patients with the history of unexplained recurrent miscarriage (URM) compare with first trimester of pregnant and healthy women.

MATERIAL AND METHODS

Subjects

Were recruited after informed consent for this prospective cross sectional study .These were 90 non-pregnant women with history of URM and at least 2 pregnancy losses before 20 weeks of gestation (cases) age matched with 70 non-pregnant women without history of recurrent abortion with at least one child and 70 pregnant women without history of recurrent abortion with at least one child (controls). Women were excluded in case of positive for Antiphospholipid Antibody (APLA), anatomical factors for losses, endocrine abnormalities, couple with Karyotype abnormalities, Multiple Pregnancy, pathologic hyperlipidemia, Hepatic or pulmonary and renal diseases, Anemia and coagulopathies.

The cases were selected at the Obstetrics and Gynaecology Department and Manipal Assisted Reproduction Centre (MARC) of the Manipal University .Ethical approval was obtained from the University of Manipal, Ethical Board.

Collection of blood

5ml of venous blood sample was dispensed into ethylene diamine tetra-acetic acid (EDTA) tubes for the estimation of angiogenic cytokines (VEGF-A and VEGF-C) .The samples were then centrifuged at 500g for 5mins to obtain plasma and serum respectively, and stored at -20ºC until analysis.

Demographic Characteristics

Semi structured pre-test questionnaire was completed by each subject in order to obtain demographic data. The demographic characteristics tested were age and age at menarche (AM), length of menstrual cycle (LMC), number of previous miscarriage (NPM), number of previous pregnancies (NPP) and number of live birth(s) (NLB).

Anthropometric Characteristics

The anthropometric parameters measured were weight, height, body mass index (BMI) .BMI was used as an indicator of obesity, and subjects were stratified into five groups based on World Health Organization classification as underweight (< 18.5 kg/m2), normal (18.5–24.9 kg/m2), overweight (25–29.9 kg/m2), and obese (≥ 30 kg/m2).

Biochemical Characteristics

VEGF-A and VEGF-C were analyzed with enzyme-linked immunosorbent assay (ELISA) kits (all manufactured by R&D Systems, Minneapolis, MN).VEGF-A and VEGF-C quantified with an ELISA that measures the extracellular (soluble) domain of VEGFR-2 and VEGFR-1. No cross-reactivity or interference is detected between the two receptors in the ELISA assays. All ELISA assays were run under Good

Laboratory Practice conditions, and performance specifications of each ELISA were validated for their intended purpose, as per established guidelines and read at 450 nm immediately.

Statistical analysis

Where appropriate, the data are expressed as the mean ± SD. The statistical analysis was conducted using the SPSS Statistical Package version 23, (Chicago, IL, USA) were employed for the analyses of data. Student's t –test was used for the comparison of quantitative variables. Pearson's n correlation coefficient was used for the relationship between quantitative variables. $p<0.05$ was regarded as significant.

RESULT

These were 90 non-pregnant women with history of URM and at least 2 pregnancy losses before 20 weeks of gestation (cases) age matched with 70 non-pregnant women without history of recurrent abortion with at least one child and 70 pregnant women without history of recurrent abortion with at least one child (controls).The descriptive level of serum Angiogenic cytokine (VEGF-A and VEGF-C), Age and BMI in patient and control groups are shown in (Table-1).

The correlation of serum level of VEGF-A and VEGF-C with Demographic and Anthropometric Characteristics in 90 non-pregnant women with history of URM(Table-2), with 70 non-pregnant women (Table-3) and 70 pregnant women without history of recurrent abortion with at least one child (Table-4). There was significant negative correlate with angiogenic factors among the case and control groups with Age and BMI. Also, there was significant negative correlate with VEGF-A among URM with a number of previous miscarriage and length of menarche. There is positively strong correlation with concentration of VEGF-A and VEGF-C among all groups (Pearson correlation of VEGF-C and VEGF-C = 0.688, P-Value <0001).

We assessed whether VEGF-A and VEGF-C levels in women with a history of recurrent miscarriage comparing with healthy and pregnant women as differed in Age (Figur-1) and BMI groups (Figure 2) Shows the increase estimated level VEGF-A and VEGF-C in young age groups when compare with old age and decrees level of VEGF-A and VEGF-C in obese among of all groups.

Multiple regression and final equation for VEGF-A and VEGF-C among of patients and control groups show significantly linear regression(R-Esq. 52.7%, p-value<0.10) (Figure-3)

ROC analysis in unexplained recurrent miscarriage group shows the area under the curve for VEGF-A is near 0.807 and cut pint 200.17(pg/ml) and for VEGF-C is near 0.872 and cut point is 234.8(pg/ml) (Figure 4). Similarly, ROC analysis in pregnant group shows the area under the curve for VEGF-A is near 0.869 and cut pint 250.11(pg/ml) and for VEGF-C is near 0.897 and cut point is 289.57(pg/ml) (Figure 5).

Table-1 Descriptives of all parameters in cases and controls groups.

		N	Mean	Std. Deviation	95% Confidence Interval for Mean		Mini	Max
					Lower Bound	Upper Bound		
VEGF-A (pg/ml)	**URM**	90	189.875	88.1672	171.4092	208.3417	54.32	463.72
	HEALTHY	70	239.103	99.7780	215.3119	262.8944	106.32	670.43
	PREGNANT	70	301.572	76.4535	283.3424	319.8018	155.43	700.21
	Total	230	238.852	99.63577	225.9075	251.7974	54.32	700.21
VEGF-C (pg/ml)	**URM**	90	209.902	99.23208	189.1187	230.6862	87.32	670.43
	HEALTHY	70	275.557	133.0853	243.8243	307.2905	78.32	1001.32
	PREGNANT	70	402.506	128.6099	371.8402	433.1721	150.21	900.32
	Total	230	288.5029	143.5256	269.8557	307.1502	78.32	1001.32
BMI (kg/m^2)	**URM**	90	27.8207	7.28099	26.2957	29.3456	14.72	44.02
	HEALTHY	70	24.9033	6.17487	23.4309	26.3756	15.88	44.22
	PREGNANT	70	24.1539	5.98971	22.7257	25.5821	15.47	37.51
	Total	230	25.8168	6.75133	24.9396	26.6939	14.72	44.22
AGE (years)	**URM**	90	30.6667	6.34230	29.3383	31.9950	21.00	45.00
	HEALTHY	70	28.9571	5.86425	27.5589	30.3554	18.00	47.00
	PREGNANT	70	28.6429	6.04820	27.2007	30.0850	20.00	45.00
	Total	230	29.5304	6.15353	28.7310	30.3299	2.00	47.00

Table-2 Correlations of parameters in women with history of Unexplained Recurrent Miscarriage.

		VEGF-A (pg/ml)	VEGF-C (pg/ml)	BMI (kg/m²)	AGE (years)	Number Of Previse Miscarriage (NPM)	Age at Menarche (AM)	Length of Menstrual Cycle (LMC)
VEGF-A (pg/ml)	Pearson Correlation	1	.584**	-.208*	-.557**	-.451**	-.153	-.407**
	Sig. (2-tailed)		.000	.049	.000	.000	.150	.000
VEGF-C (pg/ml)	Pearson Correlation	.584**	1	-.270*	-.231*	-.079	-.061	-.137
	Sig. (2-tailed)	.000		.010	.028	.461	.570	.197
BMI(kg/m²)	Pearson Correlation	-.208*	-.270*	1	.165	.015	-.113	.081
	Sig. (2-tailed)	.049	.010		.120	.885	.287	.446
AGE(years)	Pearson Correlation	-.557**	-.231*	.165	1	.758**	.457**	.495**
	Sig. (2-tailed)	.000	.028	.120		.000	.000	.000
Number Of Previse Miscarriage (NPM)	Pearson Correlation	-.451**	-.079	.015	.758**	1	.607**	.642**
	Sig. (2-tailed)	.000	.461	.885	.000		.000	.000
Age at Menarche (AM)	Pearson Correlation	-.153	-.061	-.113	.457**	.607**	1	.422**
	Sig. (2-tailed)	.150	.570	.287	.000	.000		.000
Length of Menstrual Cycle (LMC)	Pearson Correlation	-.407**	-.137	.081	.495**	.642**	.422**	1
	Sig. (2-tailed)	.000	.197	.446	.000	.000	.000	
TOTAL	N	90	90	90	90	90	90	90

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table-3 Correlations of parameters in Healthy women

		VEGF-A(pg/ml)	VEGF-C(pg/ml)	BMI(kg/m²)	AGE(years)
VEGF-A(pg/ml)	Pearson Correlation	1	.785**	-.248*	-.413**
	Sig. (2-tailed)		.000	.039	.000
VEGF-C(pg/ml)	Pearson Correlation	.785**	1	-.312**	-.324**
	Sig. (2-tailed)	.000		.008	.006
BMI(kg/m²)	Pearson Correlation	-.248*	-.312**	1	.050
	Sig. (2-tailed)	.039	.008		.679
AGE(years)	Pearson Correlation	-.413**	-.324**	.050	1
	Sig. (2-tailed)	.000	.006	.679	
TOTAL	N	70	70	70	70

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION

The placenta is a unique pregnancy-related tissue and plays a key role in occurrence of unexplained recurrent pregnancy loss (URPL). Abnormal placentation might play a key role in occurrence of URPL. During placentation failures spiral artery remodeling and angiogenesis are implicated in the pathogenesis of these complications[21, 22]. The human placenta is rich in angiogenic growth factors including vascular endothelial growth factor (VEGF) which play an important molecules regulating of forming placental vessels. The role of VEGF family in embryonic development, trophoblast vascularization were reported [23, 24].

The VEGF-A plays an important role in angiogenesis with many effects including endothelial cell proliferation, migration, increase in vascular permeability and maintenance of vessel fragility [25].VEGF-C deals with growth of blood vessels and lymphatics [26]. VEGF-A and C binds with high affinity to two related receptor tyrosine kinases expressed on vascular endothelial cells [27]. the previous study shown the role of VEGF-A and VEGF-C in implantation and placentation [28-30]. Also, there is some evidence that the role of VEGF in recurrent miscarriage[31, 32].

This study shows that maternal levels of VEGF-A and VEGF-C are distinctly lower in URM compared to pregnant and healthy women as control groups. Univariate analysis demonstrated that clinical characteristic factors were significantly associated with concentration of VEGF-A and VEGF-C in cases and controls, and which are known to play a relevant role in angiogenesis regulation. In the current study, we observed a lower serum concentration of angiogenic factors and this reduction in concentration in some of the women with URM were 2 to 3 folds inferior to healthy women and 4 to 5 folds lower than pregnant women. Our data gave firm support to the association between 'low expression' VEGF-A and VEGF-C susceptibility to recurrent miscarriages.

It is proved that with increasing age, angiogenesis and biomarker change [32, 33]. Several studies shown the negative correlation with VEGF and maternal age [34]. Also, it is familiar that the risk of miscarriages increases with progressing maternal age in the general population, there should be tight age-matching of patients with [33]. However, in RM age seems only to display a significant impact on pregnancy outcome after age 30 [19]. So it may be sufficient to undertake stratification or adjustment in multivariate analyses according to age below and above 30 years.

Obesity with its associated metabolic complications has emerged as one of the most critical healthcare problems in the worldwide.[35] Angiogenesis is critical for adequate fat expansion and adipose tissue remodeling[36]. Clinical studies show that angiogenic factors are blunted in human obesity, and are associated with inflammation and metabolic dysfunction [37]. Tinahones, in 2009 reported, there is a positive correlation of VEGF-A and VEGF-C with fatty acid in the obese human, however, numerous studies suggest a possible correlation between VEGF levels and human obesity [38].

Being underweight or overweight has an adverse effect on reproduction. Overweight women have a higher incidence of pregnancy complication and miscarriage [39]. The prevalence of obesity in infertile women is high and some study shown BMI is associated with RPL [40].

By investigating the angiogenic factors in the serum of the women with URM, this study demonstrates the concentration of VEGF-A and VEGF-C significantly decrease with increasing of Age, BMI and number of previous miscarriage. This support the hypotheses and suggests VEGF-A and VEGF-C as a principle angiogenesis factors and highly investigated factors are under the clinical characteristic of patient, thereby it regulates embryo implantation and causes to success pregnancy. In additional, the VEGF-A level has shown the same pattern of decreased concentration as the VEGF-C.

Table-4 : Correlations of parameters in Pregnant women.

		VEGF-A(pg/ml)	VEGF-C(pg/ml)	BMI(kg/m²)	AGE(years)
VEGF-A(pg/ml)	Pearson Correlation	1	.344**	-.167	-.304*
	Sig. (2-tailed)		.004	.168	.010
VEGF-C(pg/ml)	Pearson Correlation	.344**	1	-.359**	-.374**
	Sig. (2-tailed)	.004		.002	.001
BMI(kg/m²)	Pearson Correlation	-.167	-.359**	1	.287*
	Sig. (2-tailed)	.168	.002		.016
AGE(years)	Pearson Correlation	-.304*	-.374**	.287*	1
	Sig. (2-tailed)	.010	.001	.016	
TOTAL	N	70	70	70	70

**. Correlation is significant at the 0.01 level (2-tailed).

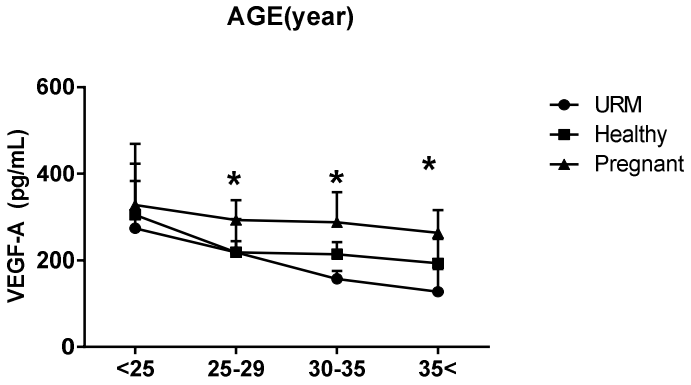
*. Correlation is significant at the 0.05 level (2-tailed).

In close, the results of the present study showed that alteration in the expression of proteins involved in proliferation and migration of endothelial cells as well as control of coagulation by these cells might play an important role in the pathogenesis of URPL. This study confirms that the cycling endometrium is a highly angiogenic tissue and that this process is likely to be altered in women with a history of URM and may contribute to the etiology of this condition.

Our data gave firm support to the association between 'low expression' VEGF-A and VEGF-C susceptibility to recurrent miscarriages. Our findings suggest that these molecules could be used as potential predictive markers of miscarriage in these women presenting with URM. Thus in URM, diagnostic and therapeutic

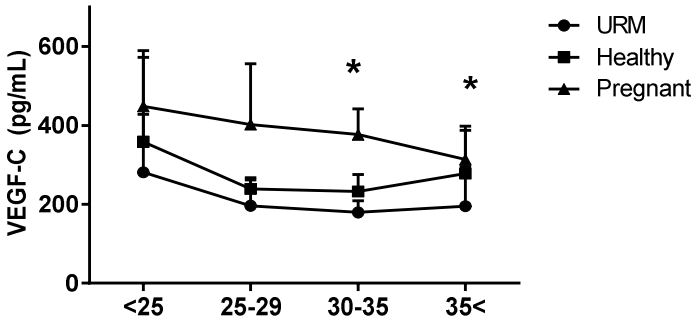
measures aimed at characterizing and modulating other angiogenic factors appear promising in the future.

A



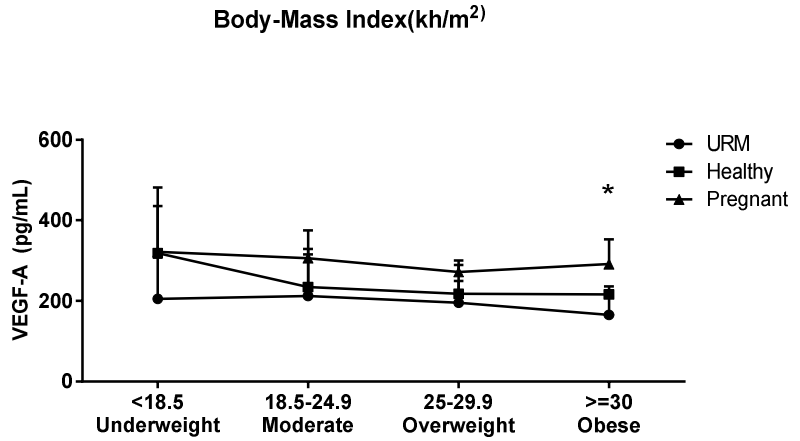
	URM			Healthy			Pregnant		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
<25	274.018	109.836	20	305.081	163.950	20	327.931	95.220	27
25-29	218.747	76.378	21	218.873	25.237	21	293.314	45.583	17
30-35	157.114	18.366	26	214.041	27.695	21	288.339	68.958	17
35<	127.381	52.519	23	193.051	56.491	8	263.089	52.465	9

B

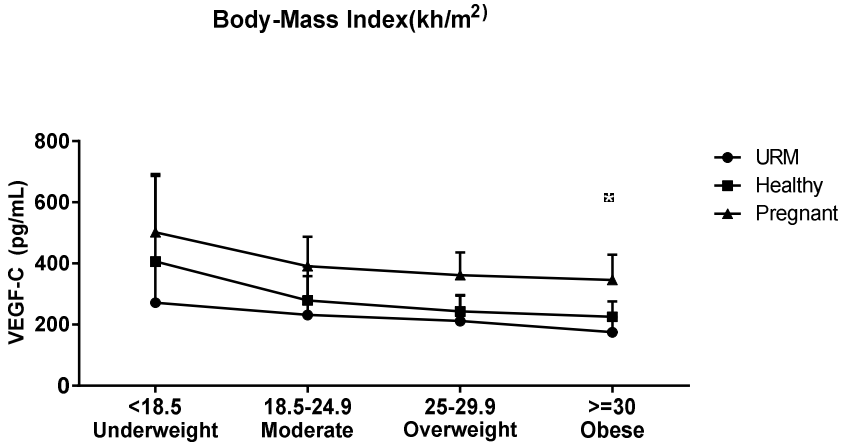


	URM			Healthy			Pregnant		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
<25	280.679	147.396	20	358.672	213.200	20	448.464	140.331	27
25-29	196.523	65.311	21	238.791	27.963	21	402.295	153.760	17
30-35	179.181	29.606	26	232.208	43.062	21	376.680	65.238	17
35<	195.302	100.676	23	278.074	119.338	8	313.814	73.948	9

Figure 1 .evaluation of angiogenic factors (VEGF-A and VEGF-C) and comparing with Age groups in women with Unexplained recurrent miscarriage (* p <0.05).



	URM			Healthy			Pregnant		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
<18.5 Underweight	205.144	102.357	9	318.910	162.540	8	321.689	113.353	16
18.5-24.9 Moderate	212.241	103.729	25	234.182	94.710	33	306.121	68.811	29
25-29.9 Overweight	195.277	93.922	23	217.894	31.414	17	271.993	28.061	10
>=30 Obese	165.003	61.245	33	216.141	19.946	12	291.039	61.776	15



	URM			Healthy			Pregnant		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
<18.5 Underweight	271.861	145.309	9	406.231	286.392	8	501.427	184.957	16
18.5-24.9 Moderate	231.763	127.022	25	278.679	110.929	33	391.284	96.071	29
25-29.9 Overweight	211.706	84.620	23	242.996	51.145	17	361.390	75.168	10
>=30 Obese	175.187	48.850	33	225.985	50.371	12	346.097	81.953	15

Figure 2 .evaluation of angiogenic factors (VEGF-A and VEGF-C) and comparing with BMI groups in women with Unexplained recurrent miscarriage (* p <0.05).

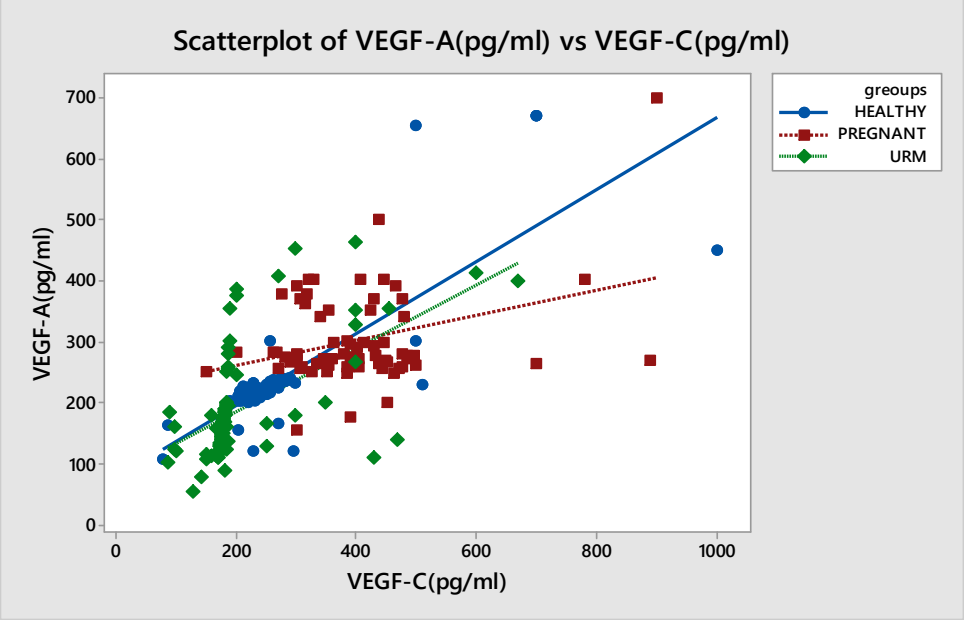
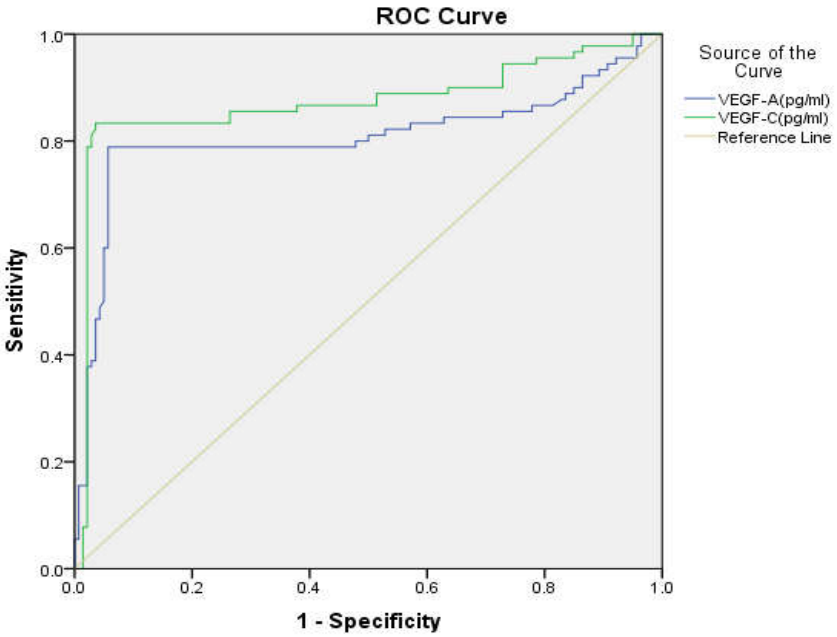


Figure 3 .Regression of VEGF-A and VEGF-C in patients and comparing with control groups.

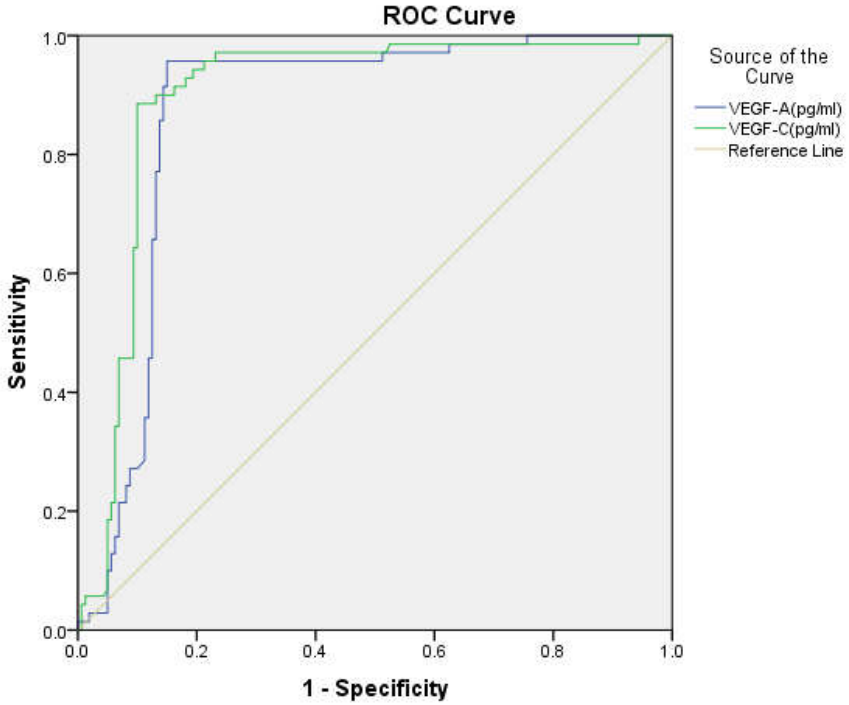


Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
VEGF-A(pg/ml)	.807	.036	.000	.738	.877
VEGF-C(pg/ml)	.872	.029	.000	.815	.930

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

Figure 3 .ROC analysis of evaluation of serum VEGF-A and VEGF-C in unexplained recurrent miscarriage.



Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
VEGF-A(pg/ml)	.869	.026	.000	.819	.919
VEGF-C(pg/ml)	.897	.024	.000	.851	.943

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

Figure 5.ROC analysis of evaluation of serum VEGF-A and VEGF-C in pregnant women.

CONCLUSION

The results of the present study showed that alteration in the expression of VEGF-A and VEGF-C the involved in proliferation of placenta and implantation and as well as clinical characteristic such as age, BMI, and pervious miscarriage might play an important role in the pathogenesis of URM. Though the present study introduces new proteins that might be used as diagnostic markers and might also be beneficial in therapy of URM, repeating this study with a larger sample size is recommended.

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CITATION OF THIS ARTICLE

A Bagheri, P Kumar, A Kamath, P Rao. Association of Angiogenic Cytokines (VEGF-A and VEGF-C) and clinical characteristic in women with unexplained recurrent Miscarriage. Bull. Env. Pharmacol. Life Sci., Vol 6[2] January 2017: 31-40