Original article:

Detection of Serum Vascular Endothelial Growth Factors (VEGF) And Soluble Vascular Endothelial Growth Factors (sVEGF / sflt-1) of Pregnancy Induced Hypertension Mothers (PIH) With pathological changes of Placenta In a Tertiary Care Hospital of West Bengal.

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Abstract:

Introduction:Pregnancy Induced Hypertension (PIH) results from imbalance between pro-angiogenic factors (VEGF & PIGF) and antiangiogenic factors (sVEGFR-1/sflt-1).***Subjects and methodology***: A mixed random study comprising of random cases of different gestational ages 28-36 wks of PIH mothers along with control cases till completion of pregnancy after delivery. Age of enrolled mothers and their gestational age, blood pressure, serum free VEGF and sVEGFR-1(sflt1) were compared in both groups (control n=36, PIH n=36). Blood pressure of both control and PIH mothers just before and after delivery showed significant correlation ($p<0.0001$). Serum levels of free VEGF were lower among PIH mothers at 28-36 wks ($p <0.0001$) and just before delivery (JBD) ($p <0.0001$) than normal control antenatal mothers and more or less similar in both groups at just after delivery ($p <0.390$). Serum sflt1 level (6459.81 ±1811.07 pg/ml) of PIH mothers showed higher value than control mothers (1062.19 ± 165.98 pg/ml) at the time of presentation and also just before delivery(JBD) & after delivery(JAD) and was highly significant ($p < 0.0001$). Serum free VEGF level of PIH mothers was negatively correlated with systolic $r= - 0.247$, $p = 0.147$) and diastolic ($r =-0.220$, $p =0.197$) blood pressure. The increased serum sflt1 level of PIH mothers was positively correlated with systolic ($r = 0.299$, $p = 0.07$) and diastolic ($r = 0.309$, $p = 0.067$) blood pressure. Semi quantitative expression of VEGF R1 and PCNA LI of placenta showed increased (3+) expression of VEGF R1 of 13 (43.33%) and > 50% PCNA LI expression of 12 (40%) cases than control. ***Discussion &Conclusion*:**The elevated levels of systolic and diastolic blood pressure along with alteration of proangiogenic and angiogenic growth factors among PIH mothers than normotensive control may help to identify PIH mothers as early as possible and referring them to higher/tertiary centers for better management and prevention of its grave complications of PIH.

Keywords: Pregnancy induced hypertension; vascular endothelial growth factors; soluble vascular endothelial growth factors.

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Introduction:

Vascular growth during implantation and placentation is critical for successful gestation. It has been thought that vascular insufficiencies during placentation may play a critical role in various obstetrical complications particularly in pregnancy induced hypertension.

Pregnancy induced hypertension are responsible for various obstetric complications. The exact etiology of this condition is unknown. Different

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research worker stated that various factors like genetic, immunological, environmental and other factors are playing an important role for the development of this disease. Extensive angiogenesis and invasion of maternal deciduas by trophoblasts are essential for the development and function of placenta. The cytotrophoblasts invade the uterine spiral arteries and switching their adhesion molecules similar to that of vascular cells and converting them to high resistant to low resistant vessels. Failure of such transformation leads to defective functional vasculogenesis and angiogenesis of placenta. In pregnancies complicated by PIH this trophoblastic cell invasion is inadequate, resulting in poor placental perfusion and fetal hypoxia (Lim et al 1997[1], Zhou et al 1998[2]). Vascular endothelial growth factors family (VEGF) and their receptors ligands are recently known as potent angiogenic factors secreted by endometrium, decidua and placenta. Trophoblastic cell proliferation and differentiation varies throughout pregnancy and alters with placental hypoxia. Functional alterations of these factors are seemed to be associated with reduced placental vascular development and vascular resistance which fails to provide the developing conceptus with an optimum uterine environment to meet metabolic demand, resulting increase fetal hypoxia affecting 6-8% of all pregnancies and increase maternal & fetal morbidity & mortality (Lim et al 1997[1], Zhou et al 19982 Kumar SG et al 2010[13]). These have been thought to be one of the possible causes of PIH. The purpose of present study is to find out placental expression of angiogenic factor vascular endothelial growth receptor (VEGFR-1) by immunohistochemistry and measurement of serum level of soluble VEGF & soluble form of VEGFR-1 from serum by ELISA in pregnancy induced hypertensive women in different gestational ages and histopathological changes in comparison to normal pregnancy.

The term Pregnancy Induced Hypertension is used to describe any new onset pregnancy related hypertension of which include: Gestational Hypertension, Preeclampsia, Eclampsia and chronic hypertension. Preeclampsia is a life threatening complication of pregnancy characterized by hypertension, proteinuria which occurs about 6-8 % (Kumar SG et al 2010[13]) of all pregnancies and increases the pregnancy-associated deaths. It is one of the major causes of premature delivery. Eclampsia is the occurrence of seizures in preeclampsia. Pathological changes associated with pregnancy induced hypertension (PIH) are due to generalized vasospasm and ischaemia, resulting end organ derangements of regional blood flow and endothelial dysfunction. The major cause of fetal compromise occurs as a consequence of reduced uteroplacental perfusion. The state of hypoxia and ischaemia of placenta thought to be associated with release of placental factors into the maternal circulation that cause clinical feature of PIH. Soluble form of vascular endothelial growth factor receptor-1 (sVEGFR-1/ sflt-1) a splice variant of VEGFR-1 and placental growth factor (PIGF) are such some factors responsible for development of PIH.

A number of vascular endothelial growth factors family (VEGF) and their receptor ligands (VEGFR) have been shown to be expressed in placenta throughout pregnancy (Ahmed et al 1995[3], Clark et al 1996[4], and Vuorela et al 1997[5]) and are essential for embryonic vascular development as loss of even a single allele results in embryonic death (Ferrara et al 1996[6]). Expression of vascular endothelial growth factor receptors (VEGFR-1 / Flt-1, VEGFR-2 /KDR, VEGFR-3 / Flt4) proteins can be studied by immunohistochemistry on placenta by using corresponding monoclonal antibody (Helske S. et al 2001) [7], (Tsatsaris V .et al 2003),[8]. Soluble form of VEGF can be measured by ELISA from patient's serum (Levine RJ et al 2004)[9]. The receptors for vascular endothelial growth factor (VEGF) and related ligands include VEGFR1 (Flt-1), VEGFR-2 (KDR/ Flk-1), and VEGFR-3 (Flt-4).The interaction may facilitate the presentation and binding of VEGF to its receptor. PlGF is localized to the placenta and binds only to VEGFR-1. Evidences from different works in this field proposed that imbalance between pro-angiogenic factors (VEGF & PIGF) and antiangiogenic factors (sVEGFR-1/ sflt-1) are responsible for pathophysiology of development of PIH. Circulating level of such factors of antenatal mothers may be one of the some important parameter to identify PIH mothers during antenatal checkup. Very little studies have been done in this field in our country of a huge population in spite of large number of occurrence causing about 8%[14] of maternal mortality.

Aims & Objectives:

1. To find out serum level of free vascular endothelial growth factor (VEGF) and soluble form of vascular endothelial growth factor (sVEGF) / sflt1 of PIH patients comparing with serum of normal term pregnancy as control.

2. To find out placental expressions of VEGFR-1 in pregnancy induced hypertension at different gestational ages taking normotensive term placenta as control along with Histopathological changes.

Materials & Methods:

The study was conducted from June 2005 to November 2010 in the department of Pathology and Obstetrics & Gynaecology, Burdwan Medical College & Hospital and further study in the department of Pathology Bankura Sammilani Medical College from August 2014 to December 2015, West Bengal, India after taking permission from Institutional ethical committee.

Study design: It is a mixed random study comprising of random cases of different gestational ages of PIH mothers along with control cases and also a group of patients longitudinally till completion of pregnancy after delivery.

Study proper: 36 control and 36 pregnancy induced hypertensive (PIH) mothers were selected by standard methods and serum sample were collected for estimation of serum free VEGF and soluble VEGFR-1 (sflt-1) at three gestational period (Random 28-36 wks, just before delivery and just after delivery) by standard ELISA (Sandwich type) method by corresponding antibody. Serum free VEGF and soluble VEGFR-1 (sflt1) level were estimated (human VEGF-A ELISA kit BMS277 and human sVEGF-R1 ELISA kit, BMS268/2) from Bender Medsystems GmbH Vienna Biocenter 2, Vienna, Austria, Europe with sensitive values 13.5 pg/ml (ranged 20.00-13.3 pg/ml)and 0.06ng/ml (ranged 0.16-10ng/ml) respectively. The intra- assay and inter- assay coefficient of variation of VEGF-A and sflt1 were 6.8% & 8.3% and 5.1% & 5.4% respectively. All the blood samples (10 ml each) collected kept in aliquot with proper labeling without anticoagulant and placed vertically in a rack for serum separation at room temperature. Then serum was collected after centrifuge (2500 g, 15 minutes) in two different aliquots and stored in – 70^0c as early as possible until estimation of serum VEGF and sflt1by standard ELISA method. Serum creatinin, liver function tests, blood coagulation profile and platelet count were done routinely and when needed.

Total 95 Placentae from different gestational ages were examined (MTP-25, PIH-40 and 30 controls) for routine histological changes and immunolocalization of vascular endothelial receptor-1 (VEGFR-1) and proliferating cell nuclear antigen labeling index (PCNA L1) were done by using corresponding primary antibody. Out of Total 25 MTP samples 6-12 weeks were 6 cases and 13-19 weeks were19 cases. Out of total 40 PIH Placentas samples 24-32 weeks-were 10 cases and 33 weeks to term were 30 cases. Control term placentas were 30 cases. Haematoxylin & Eosin Stain & other special stains were done from histopathological sections from routinely processed paraffin embedded tissue blocks. Immunolocalisation of VEGFR-1 protein & proliferate cell nuclear antigen (PCNA) were done by using proper primary monoclonal VEGFR-1 antibody (CAT –Mob 457 Clone FLT-11 from Diagnostic Biosynthesis Australia) & PC10 (DACO) on 5 µm thick tissue sections were taken on slides coated with superior tissue adhesive Tissue Bond ™ Reagent of (Catalog No K 013 by Diagnostic Biosynthesis) placental tissue at different gestational ages including normal control mother and PIH mothers.

Selection of 36 normotensive control antenatal mothers: Having regular antenatal checkup with 22-36 years of ages had normal body mass index may be Primigravida or multigravida. Gestational ages were 28-38weeks and Normotensive as recorded from first antenatal visit. Regular followed up with Mean ± SD systolic Blood Pressure 114.72 ±9.49 mm Hg, Mean ± SD diastolic Blood Pressure 75.22±9.19 mm Hg. Urinary protein—nil, Mean ± SD serum creatinine, platelet count were—0.9 ± 0.15 mg/dl and 1.9 ± 0.2 x 10^9/l respectively. No obvious clinical feature and past and family history in favor of PIH. All of them were singleton pregnancy. All of them show no evidences of intrauterine growth retardation (IUGR) and any past significant abnormal pregnancy.

Selection of 36 Pregnancy Induced Hypertensive (PIH) antenatal mothers:

Having regular antenatal checkup, 22-40 years of ages may be primigravida or multigravida of 28-36weeks of gestation. Blood pressure—as recorded from first antenatal visit follows up were > 140/90 mm Hg. Their urinary protein was 1+ / more. All of them showed features of PIH, singleton pregnancy, no evidences of intrauterine growth retardation (IUGR) as from history sheet and no history of any risk factors for PIH.

Statistical Analysis: Statistical analysis of parametric tests has been use for comparing two groups of tests. Quantitative variables of all clinical data were compared using pair sample t-test and correlation of blood pressure (systolic

and diastolic) with serum VEGF and sVEGFR-1 in PIH mothers was done by Pearson correlation/ Spearman correlation using SPSS 17 package. Different analysis of data and graphs presentations was done from Mean ± SD and SD Error Mean values.

Results:

Age of enrolled mothers and their gestational age, blood pressure, serum free VEGF and sVEGFR-1(sflt1) were compared in both groups (control n=36, PIH n=36) (Table I). Age of PIH (20.94±2.29) mothers was younger than control group (21.80 ±2.27) and no correlation was found with gestational age. Systolic blood pressure (156.83 ±83 mm of Hg) and diastolic blood pressure (107.38±22.17 mm of Hg) of PIH mothers at presentation were significantly compared more ($p < 0.0001$) with control mothers (systolic BP 114.72 ± 9.49 mm of Hg, diastolic 75.22 ± 9.10 mm of Hg). Blood pressure of both control and PIH mothers just before and after delivery showed significant correlation ($p<0.0001$). Serum levels of free VEGF (Fig I) were lower among PIH mothers at 28-36 wks ($p <0.0001$) and just before delivery (JBD) ($p <0.0001$) than normal control antenatal mothers and levels were increased and more or less similar in both groups at just after delivery ($p <0.390$) (Fig I). Serum sflt1 level (6459.81 ±1811.07 pg/ml) of PIH mothers showed (Fig II) higher value than control mothers (1062.19 ± 165.98 pg/ml) at the time of presentation and also just before delivery(JBD) & after delivery(JAD) and was highly significant ($p < 0.0001$). Serum free VEGF level of PIH mothers was negatively correlated with systolic $r= - 0.247$, $p = 0.147$) and diastolic ($r =-0.220$, $p =0.197$) blood pressure (Table II). The increased serum sflt1 level of PIH mothers was positively correlated with systolic ($r = 0.299$, $p = 0.07$) and diastolic ($r = 0.309$, $p = 0.067$) blood pressure (Table II).

Total 85 placentae were examined from different gestational ages, out of which 25(8.25%) were MTP (Medical termination of pregnancy cases (6-12 wks, n=6 and 13-19 wks, n=19), 40(47.05%) placentae were from PIH mothers (24-32 wks, n=10 and 33-term, n=30) and 30(35.29%) were control placenta (Table III). Examination of placenta on routine H&E stain showed increased numbers of areas of syncytial knot formation ($p <0.0001$), cytotrophoblastic cell proliferation ($p <0.0001$) , fibrinoid necrosis ($p <0.0001$) per low power field along with increased stromal fibrosis, hyalinization and calcification ($p <0.0001$) in PIH placenta than control placenta (Table III) & (fig III a-c). Semi quantitative expression of VEGF R1 and PCNA LI (Proliferating cell nuclear antigen labeling index) (Table VI) were analyzed on placentas at different gestational ages and showed increased (3+) (Fig IV a-c) expression of VEGF R1 of 13 (43.33%) and > 50% PCNA LI expression of 12 (40%) placenta of pregnancy induced hypertensive placenta than control group and placenta of lower gestational ages (Fig V a-c).

Discussion and conclusion:

PIH is a complex multifactor disorder thought to be wide spread endothelial dysfunction caused by imbalance of one of the maternal serum proangiogenic (VEGF) and antiangiogenic (sflt1) growth factors play a pathogenic role. The present study observed decreased level of serum VEGF in PIH mothers than normotensive control group by using sandwich type of ELISA which estimates free form of serum VEGF. Continuous maintenance level of serum VEGF is essential for endothelial integrity of maternal vasculature and fetoplacental development. Reduced level of free VEGF in the serum of PIH mothers indicates endothelial dysfunction and its clinical manifestation. Several workers observed increased serum level of sflt-1 and up regulation of mRNA in placenta associated with PIH and levels decrease within 48 hours after delivery of placenta indicates sflt-1of placental origin. The present study observed approximately > five times increase sflt-1 levels in PIH mothers than control mother. Thus, when maternal serum sflt-1 rises, it binds with the VEGF, thereby reduces the free functional VEGF for abnormal placentation in PIH mothers results increase level of serum sflt 1 was proposed by Maynars SE 2003 [10]&Kumar SG 2010[13] and histological examination showed elevation of syncytial knots, fibrinoid necrosis and regenerating trophoblastic cell hyperplasia as a result of villous injuries showed higher expression of proliferating cell nuclear antigen (PCNA) as observed by Gulsum Ozlem ELPEK et al 2000 [11] justify the present study. Immunolocalisation of VEGF R1 (3+) expression of 13 PIH placenta and (> 50%) PCNA LI expression 12 placenta indicates state of hypoxia in placenta supports the hypothesis as observed by Kurumanchi et al. Data from different observation showed serum level of sflt-1 increased 5-10 weeks before the onset of clinical onset of PIH. Screening of early and late onset PIH, significant elevated level of sflt-1 along with other routine antenatal checkup

(hypertension and proteinuria) at 23-27 weeks and 32-35 weeks of gestation thought to be one of the important laboratory parameters to identify PIH. The elevated levels of systolic and diastolic blood pressure along with alteration of proangiogenic and angiogenic growth factors were observed by several workers among PIH mothers than normotensive control mothers. The present study showed positive and significant correlation of systolic($r=0.299$, $p < 0.07$) and diastolic(r-0.309, $p < 0.069$) blood pressure with serum sflt 1 level and negative correlation with serum VEGF level (systolic BP $r= -0.249$, $p < 0.146$ and diastolic BP $r= -0.220$, $p < 0.197$) of PIH mothers. In regular antenatal clinic all mothers with and without risk factors for PIH must be screened to find out PIH before clinical development of PIH. Mothers who develop clinical PIH showed rise sflt1starts 5-10wks before development of clinical feature of PIH as observed by some investigators[12]. Further study of these parameters in different gestational ages may help to identify PIH mother occurrence causing about 8%[14] of maternal mortality. In Indian population less study has been done in this field and no cut off value of these parameters were specified to identify PIH. The results as varies from study to study, people from different ethnic populations & different parts of the world are to be studied. Proper storage of collected serum, measuring kits from different commercial preparation, thorough investigation on a larger sample size may help to standardize the cut off base line level to identify PIH mothers preclinically as early as possible by referring them to higher/tertiary centers for better management and prevention of its grave complications and fulfilling the goal of getting healthy mother with healthy baby.

Serial No	Parameters	Control pregnant mothers(Mean ±SD) n=36	SD Error Mean	PIH pregnant mothers (Mean±SD) N=36	SD Error Mean	p value
1	Age of mothers(years)	21.80± 2.27 (17-27, Median-22)	-	20.94 ±2.29 (17-27, Median-21)		NS
2	Gestational ages in wks	33.60±3.11 (28-36)	-	20.94±2.29 (28-38)		NS
3	**Systolic Blood pressure mm of Hg**					
	28-36 wks	114.72 ±9.49 (100-130)	1.58	156 ±14.77 (140-200)	2.46	<0.0001
	Just before delivery	112.30 ±9.94 (100-130)	1.65	163.72 ±20.94 (140-210)	3.49	<0.0001
	Just after delivery	111.94±11.35 (100-135)	1.89	154.72±24.28 (110-210)	4.04	<0.0001
4	**Diastolic Blood Pressure mm of Hg**					
	28-36 wks	75.22±9.19 (60-90)	1.53	107.38±22.17 (100-130)	3.69	<0.0001
	Just before delivery	70.13±9.44 (60-90)	1.57	106.30±9.00 (90-130)	1.50	<0.0001
	Just after delivery	69.02±8.76 (69-90)	1.46	106.58±8.63 (100-130)	1.4	<0.0001
5	**Serum VEGF(pg/ml)**					
	28-36 wks	65.90±13.31 (38.48-102.32)	2.21	28.12±11.52 (21.71-92.94)	1.92	<0.0001
	Just before delivery	61.84±9.92 (39.09-80.05)	1.65	15.37±1.7 (13.12-18.66)	0.29	<0.0001
	Just after delivery	70.94±12.20 (47.03-94.50)	2.03	72.57±12.44 (49.56-94.50)	2.07	<0.390
6	**Serum sflt-1(pg/ml)(sVEGFR-1)**					
	28-36 wks	1062.19±265.98 (635.70-1925.30)	44.33	6459.81±1811.07 (2116.80-9406.20)	301.84	<0.0001
	Just before delivery	1602.63±415.37 (1012.80-2813.60)	69.22	7578.99±1485.34 (3011.60-9935.10)	247.55	<0.0001
	Just after delivery	774.74±222.64 (465.30-1504.00)	37.10	4075.79±1526.11 (1053.30-7123.60)	254.35	<0.0001

Table I. Clinical parameters and serum levels of VEGF & sVEGFR-1 of both control and PIH mothers.

Parameters	Systolic Blood pressure (r value)	*p* value	Diastolic Blood pressure (r value)	*p* value	Remarks
Serum level of VEGF	-0.247	0.146	-0.220	0.197	Negatively Correlated
Serum level of sVEGFR-1 (sflt-1)	0.299	0.07	0.309	0.067	Positively correlated and significant

Table II study showing correlation of Blood Pressure with serum VEGF & sVEGFR-1 in PIH mothers

Serial No	Histopathology of Placental Villi	Control group	PIH group	Statistical Significance
1	No of areas of Syncytial knot formation / lpf*	7.67± 1.38	26.97± 2.25	<0.0001 Significant
2	No of areas of Cytotrophoblastic cell proliferation/lpf*	6.06±.96	15.46±1.72	<0.0001 Significant
3	No of areas of fibrinoid necrosis/lpf	3.45± .90	11.38± 1.16	<0.0001 Significant
4	No of areas of stromal calcification /lpf	2.79±.85	12.51±1.2	<0.0001 Significant

Table III Comparison of histological changes of Control and PIH placenta lpf—Low power field

Placenta of diff. gestational ages	VEGFR-1 Light (+) intensity	VEGFR-1 Moderate(++) intensity	VEGFR-1 Severe(+++) intensity	PCNA LI <30%	PCNA LI 30-50%	PCNA LI >50%
MTP placenta n = 25 (8.25%)						
6-12 wks n= 6	6	_	_	4	1	1
13-19 wks n= 19	19	_	_	13	1	5
PIH placenta n=40 (47.05%)						
24-32 wks n = 10	2	3	5	2	6	2
33-term n = 30	8	9	13	7	11	12
Control placenta n = 30 (35.29%)	21	9	_	13	5	3

Table IV Semi quantitative analysis of expression of VEGFR-1 & PCNA LI of placenta of different gestational stage.

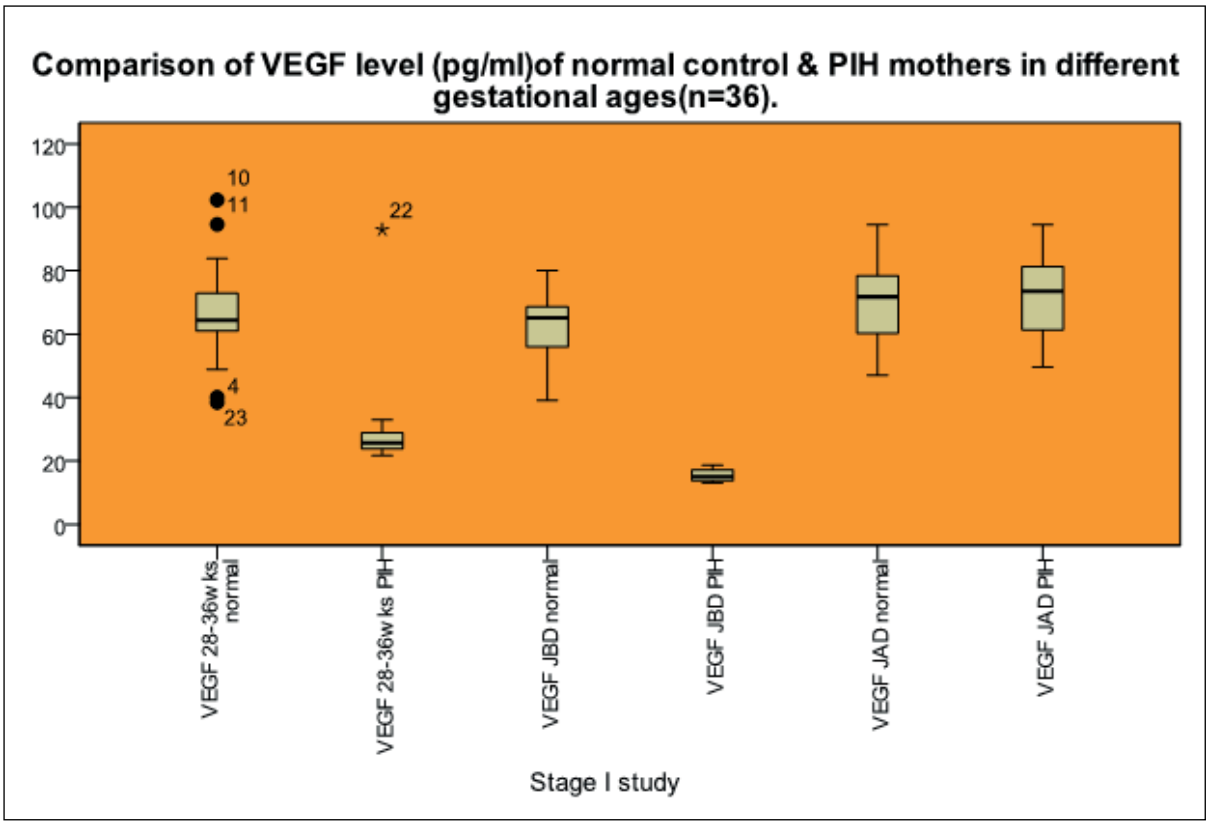

Fig I. Box plot showing levels of VEGF of normal control &PIH mother in study I
n = 36 control. n =36 PIH. X-axis-3 antenatal visits, Y- axis VEGF pg/ml
at 28-38 wks*, JBD* &JAD*. (* p= <0.000.1)

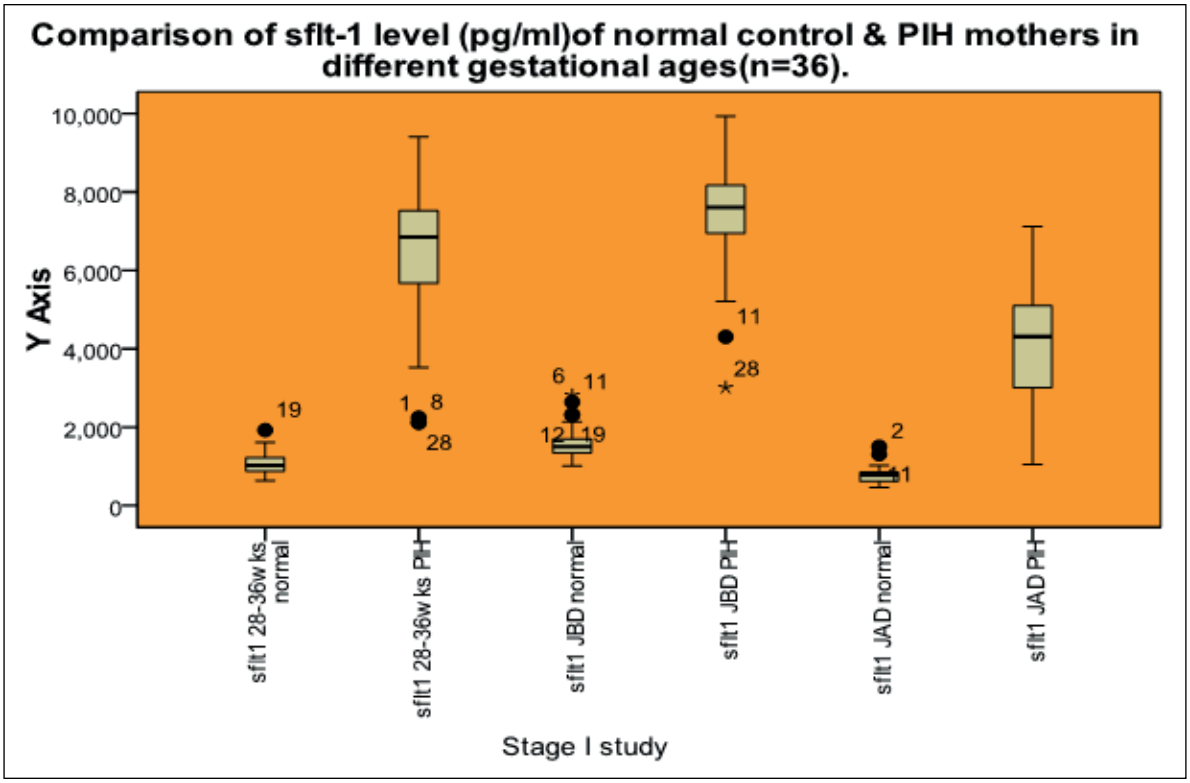

Fig II .Box plot showing levels of VEGFR-1(sflt-1) of normal control &PIH mother in study I. n = 36 control. n =36 PIH. X-axis-3 antenatal visits, Y- axis VEGFR-1 pg/mlat 28-38 wks*, JBD* &JAD*. (* p= <0.000.1)

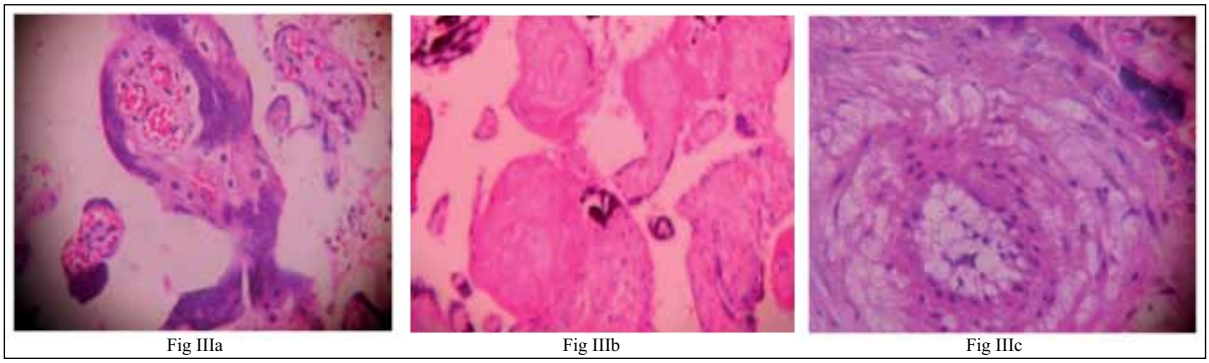

Fig IIIa Fig IIIb Fig IIIc

Fig III Photomicrograph showing histological changes of placenta of PIH placenta, a = ↑ syncytial knots of villi, b = fibrin deposition & calcification and c = atherosis. (H&E stain)

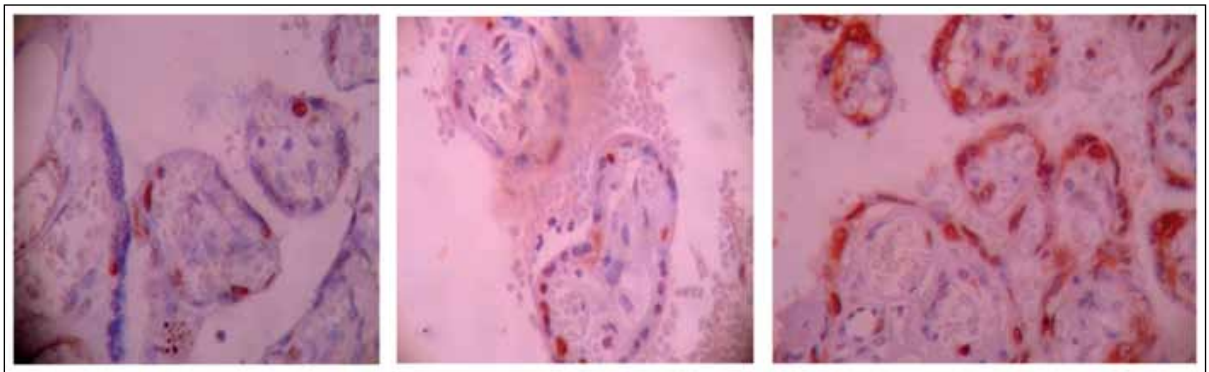

Fig IVa Fig IVb Fig IVc

Fig IV Photomicrograph Showing nuclear positivity of expression of PCNA LI of PIH placental villi, a=<30%, b= 30-50 % and c = >50%.

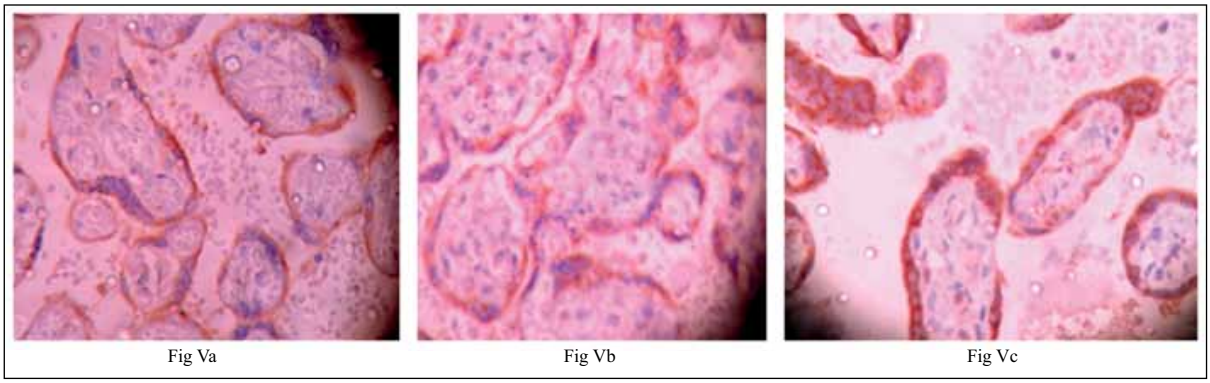

Fig Va Fig Vb Fig Vc

Fig V Photomicrograph showing cytoplasmic positivity of Vascular Endothelial Growth Receptor-1(VEGFR-1) of PIH placental villi, a = (+), b = (++) and c = (+++).

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