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Study on the Effect of Severity of Maternal Iron Deficiency Anemia on Regulators of Angiogenesis in Placenta

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ABSTRACT

Aims: In this study, we hypothesized that maternal anemia leads to altered expression of angiogenic proteins vascular endothelial growth factor (VEGF), placental growth factor (PLGF), nitrotyrosine (NT) residues, and endothelial nitric oxide synthase (e-NOS) in the placenta. Hence, we study the expression of the abovementioned proteins in the placentas of mothers with different grades of anemia.
Materials and methods: Our study was conducted in 48 pregnant women (36–40 weeks of gestation), who were divided into four groups—normal, mild, moderate, and severe anemia. After delivery, the expression of the angiogenic proteins was studied in their placentas by immunohistochemistry.
Results: In our study, 58.3% of the pregnant women were anemic, among which 20.83% had mild anemia, 18.75% had moderate anemia, and 18.75% had severe anemia. Immunohistochemical staining intensity for VEGF, PLGF, NT residues, and e-NOS proteins was observed to be higher in the placentas of anemic women when compared with the non-anemic women.
Conclusion: Our study showed that there is an increased expression of angiogenic proteins in the placentas of anemic mothers, which probably is an adaptive response leading to changes in placental vessels.

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anemia; endothelial nitric oxide synthase (e-NOS); iron deficiency; NT residues; pregnancy; placental growth factor (PLGF); vascular endothelial growth factor (VEGF)

Impact statement

What is already known on this subject?

Studies have shown that iron deficiency and anemia during pregnancy results in long-term problems for the offspring, such as development of hypertension in adulthood. A link between intrauterine environment and adult onset disease was pioneered by Barker and coworkers, who had reported associations between low birth weight and the risk of developing cardiovascular disease in adulthood (fetal/developmental programing).

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However, the mechanisms underlying developmental programing remain elusive, precluding the identification of potential avenues for clinical therapy.

What do the results of this study add?

Anemia causes hypoxia, and hypoxia leads to increased levels of vascular endothelial growth factor (VEGF) and placental growth factor (PLGF), which in turn promote production of nitric oxide (NO) and nitrotyrosine residues leading to vasculogenesis and angiogenesis. Although there are many studies on these growth factors in pre-eclampsia, there are none performed in the condition of maternal anemia. Hence, this study might show changes in expression levels of the above mentioned angiogenic growth factors in the placenta, in the condition of maternal anemia, which may be responsible for histologic changes in placental vessels.

What are the implications of these findings for clinical practice and/or further research?

The findings from our study may help plan further studies on the long term affect of maternal anemia on the offspring, with the goal of developing early intervention to prevent these long term complications.

Introduction

Anemia is one of the most common nutritional deficiency disorders in the world [1], among which iron deficiency anemia (IDA) is the most prevalent problem afflicting pregnant women and is also one of the most important preventable causes of perinatal complications, like premature delivery, intrauterine growth restriction, neonatal and perinatal death [2].

Studies have shown that IDA during pregnancy results in long-term problems for the offspring such as development of increased blood pressure in adulthood. This could be due to "fetal or developmental origins/programming of disease," the concept of which is now well accepted but the "programming" mechanisms remain poorly understood [3,4]. Barker and coworkers [5], in their studies, had earlier indicated that development of hypertension in later life may be the result of events that had occurred *in utero* and that there is an association between large placental weight, low birth weight, and high blood pressure in later life [6].

In pregnant women, anemia causes a state of hypoxia which promotes an increase in fetal cardiac afterload [7]. It has been shown that hypoxia leads to increased expression of vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) by the placenta, [8] which in turn promotes vasculogenesis and angiogenesis in the placenta. Endothelium-derived nitric oxide (NO) is another mediator of angiogenesis and has a role in modulating vascular resistance. Trophoblast and placental vascular endothelium are also the sites of production of NO, which upon interaction with locally produced superoxide ions leads to production of peroxynitrite which nitrates amino acids such as tyrosine which can be localized by measurement of nitrotyrosine (NT) residues. These NT residues were observed to be increased in placentas of pregnancies

complicated by pre-eclampsia or diabetes [9]. A hypoxic placenta may release these placental factors into maternal circulation causing systemic endothelial cell dysfunction thereby contributing to renal, cardiovascular, and neurological problems associated with preeclampsia. [10].

In this study, we hypothesized that maternal anemia leads to changes in the expression of the proteins (VEGF, PLGF, NT residues, and e-NOS) in the placentas, which may probably lead to alteration in the vasculature of the placentas. Hence, we planned to study the expression of the above mentioned proteins in the placentas of women with maternal anemia.

Materials and methods

Pregnant women (between 36 and 40 weeks of gestation), reporting to the Gandhi Government Hospital, Hyderabad, for their delivery, were enrolled in the study between the years 2013 to 2014. Institutional ethical committee clearance was obtained before the start of the study. After signing an informed consent, the women were enrolled in the study.

Pregnant women aged $\geq$18 years and $\leq$45 years, between 36 and 40 weeks of gestation, with singleton pregnancy were included in the study. Women who were diagnosed with hemolytic anemia, hypertension, diabetes mellitus, HIV, Hepatitis C, Hepatitis B, and those with multiple gestations were excluded from the study. Obstetric and medical history was obtained using a pretested questionnaire.

About 5 ml of venous blood was drawn from the women in labor. After delivery, 5 ml of blood was collected from the umbilical cord of the newborns and was transported immediately to the Pathology and Microbiology Division of National Institute of Nutrition (NIN). Blood collected in ethylene diamine tetraacetic acid (EDTA) was analyzed for complete hemogram on an ADVIA 120 automatic hematology analyzer. Blood collected in plain vials was centrifuged after clotting and the serum was stored at –80 °C until further analysis for serum ferritin (SFr) and serum transferrin receptors (sTfR). After birth, anthropometric and biochemical assessments of the newborns were performed and noted.

Anemia in pregnancy was defined as Hb <11.0 g/dl [11]. Furthermore, anemia was categorized by the WHO criteria as: mild anemia (Hb 10–11 g/dl), moderate anemia (Hb 7–9.9 g/dl), and severe anemia (Hb <7 g/dl). The following cut off values for red blood cell (RBC) indices as suggested by WHO and Centers for Disease Control and prevention [12] on pregnant women were used: RBC $<3.8 \times 10^{12}$ cells/l, hematocrit <32%, mean corpuscular volume (MCV) <76 fl, mean corpuscular hemoglobin concentration (MCHC) <26 pg, MCHC <32 g/dl, red cell distribution width (RDW) >14.5%, SFr <12 ng/ml, and hemoglobin distribution width (HDW) >3.2 g/dl (CDC 1998). SFr was estimated using ferritin SA ELISA kit (Calbiotech), while sTfR was estimated using human transferrin receptors ELISA kit (Qayee-Bio Ltd). After delivery, the placentas were collected in 10% buffered formalin and transported to the histopathology laboratory of NIN. After overnight fixation in 10% buffered formalin, four sections were taken of about 2–4 cm from either side of the center of the placenta, away from the margins and close to the maternal surface. The tissues were then processed in an automatic

tissue processor, embedded in paraffin, and 5 μm thick sections were stained with hematoxylin and eosin (H&E). The stained sections were then studied under a light microscope (Nikon Eclipse E800) by 2 histopathologists and relevant images were captured by a digital camera attached to the microscope.

The immunohistochemical expression for VEGF, PLGF, NT residues, and endothelial NO synthase (e-NOS) was studied in the placental tissues. The primary antibody used for VEGF immunostain was monoclonal mouse anti-human VEGF, clone VG1, from DakoCytomation and the one used for PLGF immunostain was PLGF (Mouse) antibody by Abcam. The NT antibody used was Nitrotyrosine (39B6):sc-32757 mouse monoclonal antibody by Santa Cruz Biotechnology and the e-NOS primary antibody used was NOS3 (A-9):sc-376751, a mouse monoclonal antibody by Santa Cruz Biotechnology. The secondary antibodies used were from Dako Real Flex Mini Enivision.

Evaluation of the immunohistochemical staining (intensity and localization) in villous capillaries, cytotrophoblasts, and syncytiotrophoblasts was carried out in 10 fields for each section. Immunoreactivity was scored using a semi-quantitative scale: 0 negative/no staining; 1+ weak positive; 2+ moderately positive; 3+ strongly positive staining.

Statistical analysis

Statistical evaluation was carried out with SPSS (Statistical package for Social Science, version 19, SPSS, Chicago, IL). Descriptive statistics mean, standard deviation (SD), and prevalence were calculated for all the variables. Mean values for all the variables were compared by unpaired "*t*" test across both groups (anemic and non-anemic). Relationships between Hb, MCV, MCH, MCHC, RBC, RDW-CV%, HDW, SFr, and sTfR were calculated by correlation coefficients and Chi square test was performed for associations. Non-parametric tests were performed wherever required. The level of significance was considered as $p < .05$.

Results

Among the 48 pregnant women recruited in our study, 20 (41.7%) had normal Hb levels and the remaining 28 (58.3%) had anemia of varying grades with 10 women (20.83%) presenting with mild anemia, 9 women (18.75%) with moderate anemia, and 9 women (18.75%) with severe anemia.

Table 1 shows that the Hb, RBC count, packed cell volume (PCV), MCV, MCH, MCHC, RDW, and HDW values are significantly lower in anemic mothers when compared with non-anemic mothers. In contrast, cord blood from babies born of anemic mothers have higher but statistically insignificant mean Hb levels when compared with the Hb levels of babies born to normal mothers. In the cord blood apart from PCV and SFr levels, which are significantly lower in anemic cases, remaining parameters are almost similar in those from both anemic and normal mothers.

In Table 2, significant differences are observed between the maternal and cord blood values in most hematological parameters. Moreover, a lower placental weight is

Table 1. Comparison of blood parameters values including SFr and sTfR between anemic and normal controls of maternal and cord blood.

Blood parameters		Hb (g/dl)	*p* value	RBC × 10^6 cells/μl	*p* value	PCV (%)	*p* value	MCV (fl)	*p* value	MCH (pg)	*p* value	MCHC (g/dl)	*p* value
Mother	Normal	12.22 ± 0.8 (*n* = 16)	.0***	4.19 ± 0.53 (*n* = 16)	.01*	37.23 ± 2.29 (*n* = 16)	.0***	89.58 ± 8.6 (*n* = 16)	.01*	29.53 ± 3.5 (*n* = 16)	.0***	32.88 ± 1.6 (*n* = 16)	.0***
	Anemic	8.57 ± 2.16 (*n* = 25)		3.62 ± 0.78 (*n* = 25)		28.94 ± 6.06 (*n* = 25)		80.61 ± 11.9 (*n* = 25)		23.65 ± 3.8 (*n* = 25)		28.94 ± 3.0 (*n* = 25)	
Cord blood	Normal	15.43 ± 1.8 (*n* = 16)	.95	4.20 ± 0.58 (*n* = 16)	.21	48.48 ± 6.50 (*n* = 16)	.0**	113.48 ± 7.6 (*n* = 16)	.45	36.10 ± 1.5 (*n* = 16)	.85	32.28 ± 1.8 (*n* = 16)	.35
	Anemic	15.48 ± 2.7 (*n* = 23)		4.40 ± 0.39 (*n* = 23)		50.69 ± 4.61 (*n* = 23)		115.46 ± 8.4 (*n* = 23)		36.20 ± 1.7 (*n* = 23)		31.70 ± 1.8 (*n* = 23)	

Blood parameters		RDW (%)	*p* value	HDW (g/dl)	*p* value	PLTS × 10^3 cells/μl	*p* value	Ferritin (ng/ml)	*p* value	TfR (ng/ml)	*p* value
Mother	Normal	16.33 ± 2.88 (*n* = 16)	.0***	3.69 ± 0.71 (*n* = 16)	.02*	2.37 ± 0.64 (*n* = 16)	.80	35.01 ± 21.42 (*n* = 11)	.85	255.41 ± 18.92 (*n* = 9)	.20
	Anemic	20.98 ± 3.86 (*n* = 25)		4.45 ± 1.17 (*n* = 25)		2.30 ± 0.96 (*n* = 25)		33.09 ± 30.83 (*n* = 21)		304.53 ± 111.55 (*n* = 23)	
Cord blood	Normal[a]	17.92 ± 1.11 (*n* = 16)	.68	3.83 ± 0.60 (*n* = 16)	.82	2.22 ± 1.07 (*n* = 16)	.41	110.23 ± 31.4 (*n* = 11)	.0***	194.11 ± 9.18 (*n* = 10)	.17
	Anemic[b]	18.07 ± 1.12 (*n* = 23)		3.78 ± 0.53 (*n* = 23)		2.43 ± 1.16 (*n* = 23)		59.93 ± 23.97 (*n* = 21)		281.65 ± 197.16 (*n* = 23)	

All values are in mean ± SD and $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$

[a]Cord blood of newborns of normal mothers. [b]Cord blood from newborns of anemic mothers

Abbreviations: Hb: hemoglobin; RBC: red blood cells; PCV: packed cell volume/hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; fl: fentolitre; pg: picogram; RDW: red cell distribution width; HDW: hemoglobin distribution width; PLTS: platelets; TfR: transferrin receptors; SD: standard deviation.

Table 2. Comparison of blood parameter values including SFr, sTfR, placental weight, and newborn anthropometric parameters between different grades of maternal anemia and cord blood.

	Hb (g/dl)		RBC × 10^6 cells/μl		PCV (%)		MCV (fl)		MCH (pg)		MCHC (g/dl)		RDW (%)	
Maternal Hb	M ($n=41$)	C ($n=39$)	M ($n=41$)	C ($n=39$)	M ($n=41$)	C ($n=39$)	M ($n=41$)	C ($n=39$)	M ($n=41$)	C ($n=39$)	M ($n=41$)	C ($n=39$)	M ($n=41$)	C ($n=39$)
Normal (Hb > 11 g/dl)	$12.22^{a}\pm0.78$ ($n=16$)	15.43 ± 1.83 ($n=16$)	$4.20^{a}\pm0.54$ ($n=16$)	4.20 ± 0.59 ($n=16$)	$37.23^{a}\pm2.30$ ($n=16$)	48.50 ± 60.50 ($n=16$)	$89.58^{a}\pm8.68$ ($n=16$)	113.5 ± 7.63 ($n=16$)	$29.53^{a}\pm3.54$ ($n=16$)	36.10 ± 1.53 ($n=16$)	$32.89^{a}\pm1.65$ ($n=16$)	32.28 ± 1.89 ($n=16$)	$16.33^{a}\pm2.89$ ($n=16$)	17.92 ± 1.11 ($n=16$)
Mild (10–11 g/dl)	$10.52^{b}\pm0.31$ ($n=10$)	14.84 ± 3.84 ($n=10$)	$4.18^{a}\pm0.387$ ($n=10$)	4.47 ± 0.416 ($n=10$)	$34.02^{b}\pm1.03$ ($n=10$)	50.30 ± 4.54 ($n=10$)	$81.97^{ab}\pm8.66$ ($n=10$)	112.45 ± 6.58 ($n=10$)	$25.33^{b}\pm2.72$ ($n=10$)	35.24 ± 1.40 ($n=10$)	$30.91^{b}\pm1.23$ ($n=10$)	32.01 ± 2.11 ($n=10$)	$19.17^{b}\pm2.53$ ($n=10$)	18.06 ± 0.76 ($n=10$)
Mod (7–9.9 g/dl)	$8.557^{c}\pm0.85$ ($n=8$)	15.55 ± 1.47 ($n=7$)	$3.574^{b}\pm0.68$ ($n=8$)	4.24 ± 0.386 ($n=7$)	$29.48^{c}\pm2.88$ ($n=8$)	49.70 ± 4.13 ($n=7$)	$85.02^{ab}\pm16.95$ ($n=8$)	117.57 ± 9.45 ($n=7$)	$24.63^{b}\pm4.57$ ($n=8$)	36.70 ± 1.100 ($n=7$)	$29.13^{c}\pm2.54$ ($n=8$)	31.32 ± 1.88 ($n=7$)	$20.88^{bc}\pm5.44$ ($n=8$)	17.71 ± 0.85 ($n=7$)
Severe (<7 g/dl)	$5.64^{d}\pm1.25$ ($n=7$)	16.48 ± 1.13 ($n=6$)	$2.80^{c}\pm0.68$ ($n=7$)	4.45 ± 0.37 ($n=6$)	$20.70^{d}\pm3.97$ ($n=7$)	52.51 ± 5.53 ($n=6$)	$74.77^{b}\pm10.95$ ($n=7$)	118.03 ± 9.67 ($n=6$)	$20.32^{c}\pm3.47$ ($n=7$)	37.21 ± 2.187 ($n=6$)	$27.18^{c}\pm2.38$ ($n=7$)	31.65 ± 1.72 ($n=6$)	$23.77^{c}\pm2.60$ ($n=7$)	18.53 ± 1.79 ($n=6$)
p value	.00***	.62	.00***	.46	.00***	.49	.02*	.39	.00***	.08	.00***	.72	.00***	.59

	HDW (g/dl)		Ferritin (ng/ml)		TfR (ng/ml)		Placental weight (g)	Neonatal birth weight (kg)	Newborn length (cm)
Maternal Hb	M ($n=41$)	C ($n=39$)	M ($n=32$)	C ($n=32$)	M ($n=34$)	C ($n=33$)	($n=38$)	($n=17$)	($n=17$)
Normal (Hb > 11 g/dl)	3.70 ± 0.71 ($n=16$)	3.83 ± 0.60 ($n=16$)	35.01 ± 21.42 ($n=11$)	$110.23^{a}\pm31.40$ ($n=11$)	255.41 ± 18.92 ($n=10$)	194.11 ± 9.2 ($n=10$)	445.84 ± 85.35 ($n=13$)	7.72 ± 15.11 ($n=9$)	47.88 ± 1.80 ($n=8$)
Mild (10–11 g/dl)	4.61 ± 1.59 ($n=10$)	3.96 ± 0.49 ($n=10$)	30.97 ± 13.18 ($n=8$)	$62.02^{b}\pm19.45$ ($n=8$)	328.38 ± 166.78 ($n=10$)	290.07 ± 207.92 ($n=9$)	461.90 ± 142.62 ($n=10$)	3.08 ± 0.36 ($n=6$)	49.86 ± 1.34 ($n=7$)
Mod (7–9. g/dl)	4.121 ± 0.88 ($n=8$)	3.64 ± 0.63 ($n=7$)	30.46 ± 28.51 ($n=7$)	$62.48^{b}\pm31.44$ ($n=7$)	282.66 ± 46.89 ($n=8$)	303.42 ± 262.89 ($n=8$)	420.87 ± 87.12 ($n=8$)	2.95 ± 0.07 ($n=2$)	49.50 ± 0.70 ($n=2$)
Severe (<7 g/dl)	4.49 ± 0.84 ($n=7$)	3.67 ± 0.47 ($n=6$)	39.00 ± 55.64 ($n=6$)	$54.19^{b}\pm25.49$ ($n=6$)	309.540 ± 75.10 ($n=6$)	247.00 ± 47.44 ($n=6$)	428.00 ± 171.65 ($n=7$)	Not available	Not available
p value	.14	.64	.94	.00***	.47	.51	.89	.70	.07

Values in column having different superscript letters are significantly different by one way ANOVA and LSD test ($p<.05$). All values are in mean ± SD and $^{*}p<.05$; $^{**}p<.01$; $^{***}p<.001$.

Abbreviations: M: mother's blood; C: cord blood; Hb: Hemoglobin; RBC: red blood cells; PCV: packed cell volume/hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular heamoglobin concentration; fl: fentolitre; pg: pictogram; RDW: red cell distribution width; HDW: hemoglobin distribution width; TfR: transferrin receptors; SD: standard deviation.

Table 3. Pearson's correlations between maternal and cord blood parameters including SFr and sTfR.

Parameters	Hb (M)	RBC (M)	PCV (M)	MCV (M)	MCH (M)	MCHC (M)	RDW (M)	HDW (M)	SFr (M)
Hb (C)	0.46	0.99	0.54	0.34	0.24	0.35	0.43	0.82	0.66
RBC(C)	0.59	0.45	0.79	0.14	0.11	0.35	0.19	0.63	0.81
PCV(C)	0.14	0.52	0.35	0.68	0.13	0.02*	0.09	0.96	0.43
MCV (C)	0.05*	0.03	0.17	0.22	0.61	0.00**	0.15	0.87	0.33
MCH(C)	0.03*	0.07	0.03	0.38	0.14	0.09	0.07	0.48	0.04*
MCHC (C)	0.31	0.55	0.86	0.43	0.46	0.00**	0.53	0.57	0.57
RDW(C)	0.28	0.88	0.35	0.05	0.04*	0.26	0.03*	0.22	0.00**
HDW(C)	0.44	0.36	0.20	0.80	0.96	0.65	0.46	0.01*	0.04*
S.Ferritin (C)	0.00**	0.34	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**	0.42
sTfR(C)	0.46	0.94	0.41	0.22	0.28	0.98	0.478	0.71	0.78

*Correlation is significant at the .05 level (two-tailed); **Correlation is significant at the .001 level (two-tailed).
Abbreviations: M: mothers blood; C: cord blood; Hb: hemoglobin; RBC: red blood cell; PCV: packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; HDW: hemoglobin distribution width; SFr: serum ferritin; sTfR: serum transferrin receptors.

Table 4. Placental morphology in different grades of maternal anemia.

Placental morphology	Hb range (g/dl)	Number of cases	Mean value	Standard deviation (SD)	*p* value
No. of Capillaries/villus	>11	15	3.87	1.246	.000*
	10–10.99	10	5.00	0.816	
	7–9.9	10	6.70	0.949	
	<7	7	7.71	0.951	
No. of syncytial knots/Villus	>11	15	3.72	1.20	.000*
	10–10.99	10	4.56	1.28	
	7–9.9	10	6.21	1.50	
	< 7	7	7.02	1.62	
Intravillous fibrin in % of tissue section	>11	15	10.33	4.806	.890
	10–10.99	10	10.00	5.270	
	7–9.9	10	8.50	7.835	
	< 7	7	10.00	5.774	
Intervillous fibrin in % of tissue section	>11	15	14.33	7.761	.999
	10–10.99	10	14.50	6.433	
	7–9.9	10	14.00	6.992	
	< 7	7	14.29	13.363	

*$p < .001$.
Abbreviation: Hb: hemoglobin.

observed in the anemic women when compared with the controls, which is however not statistically significant.

Table 3 shows significant correlation between the maternal MCHC and the cord blood PCV, MCV, and MCHC values and also between the cord blood ferritin and maternal Hb, PCV, MCV, MCH, MCHC, RDW, and HDW values.

Table 4 shows that the number of capillaries/villus as well as the number of syncytical knots/villus significantly ($p < .05$) increase with increasing severity of anemia.

Table 5 shows varied intensity of expression of VEGF in different villus trophoblast cells and capillaries. A statistically significant ($p = .001$) increase in the expression of VEGF in the capillaries of placentas from the anemic women is observed, while no statistical difference in the expression is observed in the syncytiotrophoblasts and cytotrophoblasts. All the abovementioned cells show negative staining in the non-anemic cases (Figure 1a), weak staining in the mild anemia cases, and a strong positive staining in the severe anemia cases, especially in the capillaries (Figure 1b).

Table 6 shows varied intensity of expression of PLGF in the villus trophoblast cells and the capillaries. A statistically significant ($p < .05$) increase in the expression of PLGF in all the three cell types-cytotrophoblasts ($p = .000$), syncytiotrophoblasts

Table 5. Localization and immunostaining intensity of VEGF expression in placental villous tissues.

$n = 41$	Staining intensity score	ST, n(%)	CT, n(%)	Wall of capillaries, n(%)	Mean ± SD
Normal Hb ($n = 17$)	0	15(88.2%)	15(88.2%)	9(53%)	0.23 ± 0.33
	1+	2(11.8%)	2(11.8%)	8(47%)	
	2+	0	0	0	
	3+	0	0	0	
Mild anemia ($n = 9$)	0	8(88.9%)	9(100%)	0	0.48 ± 0.18
	1+	1(11.1%)	0	7(77.8%)	
	2+	0	0	2(22.2%)	
	3+	0	0	0	
Moderate anemia ($n = 9$)	0	8(88.9%)	8(88.9%)	1(11.1%)	0.55 ± 0.30
	1+	1(11.1%)	1(11.1%)	2(22.2%)	
	2+	0	0	5(55.6%)	
	3+	0	0	1(11.1%)	
Severe anemia ($n = 6$)	0	5(83.3%)	5(83.3%)	0	0.92 ± 0.42
	1+	1(16.7%)	1(16.7%)	1(16.7%)	
	2+	0	0	2(33.3%)	
	3+	0	0	3(50%)	
Statistical comparison p value		.602	.379	.001*	.003*

Values are mean ± SD * $p < .01$. [Staining intensity scored as follows: negative (0), weak (1+), moderate (2+), and strong (3+)]
Abbreviations: ST: syncytiotrophoblasts; CT; cytotrophoblasts; Hb: hemoglobin.

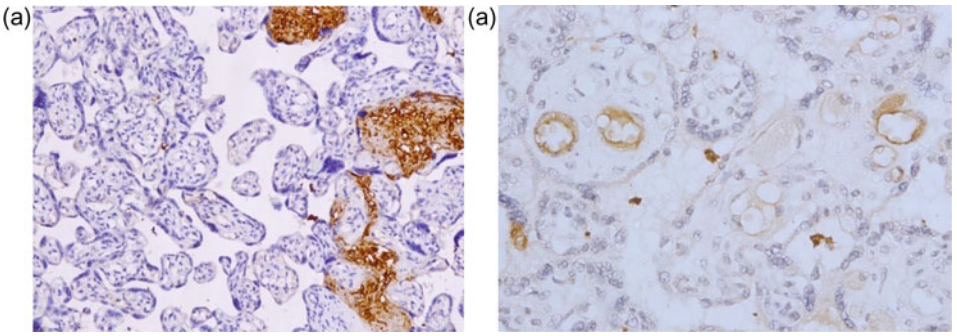

Figure 1. (a) Microphotograph shows immunostaining of VEGF in placentas from normal mothers. Intense staining is observed only in intervillous fibrin while syncytiotrophoblasts, cytotrophoblasts, and capillaries do not show any immunostain. VEGF immunostain; original magnification,×4. (b) Microphotograph shows immunostaining of VEGF in placentas from anemic mothers. Moderate to marked immunostaining is observed along the lining and endothelial cells of capillaries while syncytiotrophoblasts and cytotrophoblasts do not show any immunostain. VEGF immunostain; original magnification,×10.

($p = .005$), and capillaries ($p = .000$) is observed in the placentas from the anemic women when compared with the non-anemic women (Figure 2a). Similar to VEGF, all the above mentioned cells in the placentas of non-anemic women show no staining in all the cell types (Figure 2a). The severely anemic cases show a higher staining intensity of 3+ (Figure 2b), while the mild anemia cases show a weak staining intensity of 1+.

Similarly, Table 7 reveals a statistically significant ($p < .05$) increase in the expression of e-NOS in all the three cell types: cytotrophoblasts ($p = .002$), syncytiotrophoblasts ($p = .006$), and capillaries ($p = .000$) in the placentas from anemic women. All the above

Table 6. Localization and immunostaining intensity of PLGF expression in placental villous tissues.

$n = 39$	Staining intensity score	ST, n(%)	CT, n(%)	Wall of capillaries, n(%)	Mean ± SD
Normal Hb ($n = 16$)	0	6(37.5%)	6(37.5%)	7(43.75%)	0.67 ± 0.60
	1+	8(50%)	9(56.25%)	8(50%)	
	2+	2(12.5%)	1(6.25%)	1(6.25%)	
	3+	0	0	0	
Mild anemia ($n = 10$)	0	0	0	1(10%)	1.1 ± 0.27
	1+	7(70%)	9(90%)	9(90%)	
	2+	3(30%)	1(10%)	0	
	3+	0	0	0	
Moderate anemia ($n = 9$)	0	1(11.1%)	1(11.1%)	1(11.1%)	1.6 ± 0.74
	1+	2(22.2%)	2(22.2%)	2(22.2%)	
	2+	5(55.55%)	6(66.7%)	6(66.7%)	
	3+	1(11.1%)	0	0	
Severe anemia ($n = 4$)	0	0	0	0	2.1 ± 0.5
	1+	0	0	0	
	2+	2(50%)	3(75%)	3(75%)	
	3+	2(50%)	1(25%)	1(25%)	
Statistical comparison p value		.005*	.000**	.000**	.000**

* $p < .01$; ** $p < .001$. [Staining intensity of PLGF were scored as follows: negative (0), weak (1+), moderate (2+), and strong (3+)]

Abbreviations: ST: syncytiotrophoblasts; CT:; cytotrophoblasts; Hb: hemoglobin.

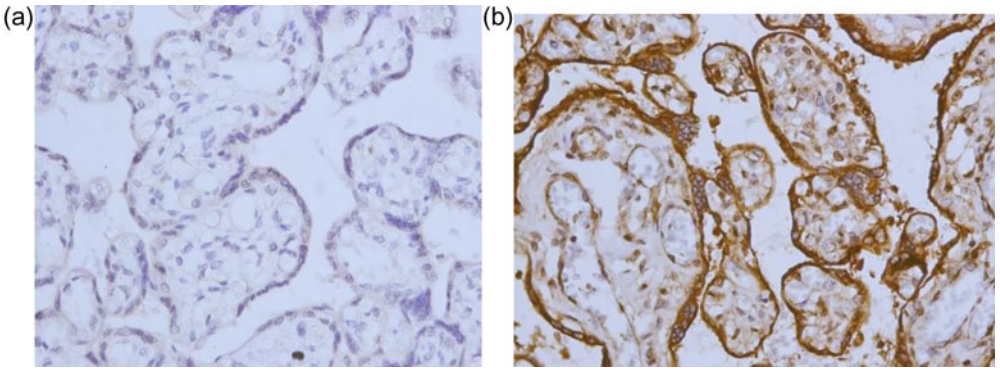

Figure 2. (a) Microphotograph shows immunostaining of PLGF in placentas from normal mothers. Syncytiotrophoblasts, cytotrophoblasts, and capillaries are all negative for immunostain. PLGF immunostain; original magnification,×10. (b) Microphotograph shows immunostaining of PLGF in placentas from anemic mothers. Intense staining is observed in the cytoplasm of syncytiotrophoblasts, cytotrophoblasts, and capillaries. PLGF immunostain; original magnification,×10.

mentioned placental cells in the non-anemic show no staining in any of the three cell types (Figure 3a), a weak to moderately positive staining intensity (1+ to 2+) in the mild and moderate anemia cases and an intense staining intensity of 3+ in the severe anemia cases (Figure 3b).

A statistically significant ($p < .05$) increase in the expression of NT residues is observed only in the syncytiotrophoblasts ($p = .001$) and the capillaries ($p = .001$), while it is statistically insignificant in the cytotrophoblasts from the anemic women (Table 8). Most of the mild anemia cases show a 1+ staining intensity (Figure 4a), those of the moderate anemia group show an intensity of 2+, while the severe anemia group shows a 3+ intense immunopositivity (Figure 4b).

Table 7. Localization and immunostaining intensity of e-NOS expression in placental villous tissues.

$n = 27$	Staining intensity score	ST, n (%)	CT, n(%)	Wall of capillaries, n(%)	Mean ± SD
Normal Hb ($n = 11$)	0	0	4(36.4%)	1(9.1%)	1.11 ± 0.64
	1+	8(72.7%)	7(63.6%)	10(90.9%)	
	2+	3(27.3%)	0	0	
	3+	0	0	0	
Mild anemia ($n = 6$)	0	0	0	0	1.17 ± 0.18
	1+	3(50%)	6(100%)	6(100%)	
	2+	3(50%)	0	0	
	3+	0	0	0	
Moderate anemia ($n = 6$)	0	0	0	0	1.5 ± 0.18
	1+	4(66.7%)	5(83.3%)	0	
	2+	2(33.3%)	1(16.7%)	6(100%)	
	3+	0	0	0	
Severe anemia ($n = 4$)	0	0	0	0	2.89 ± 0.19
	1+	0	0	0	
	2+	0	1(25%)	0	
	3+	4(100%)	3(75%)	4(100%)	
Statistical comparison p value		.002*	.006*	.000**	.000**

* $p < .01$; ** $p < .001$. [Staining intensity of e-NOS were scored as follows: negative (0), weak (1+), moderate (2+), and strong (3+)].
Abbreviations: ST: syncytiotrophoblasts; CT: cytotrophoblasts; Hb: hemoglobin.

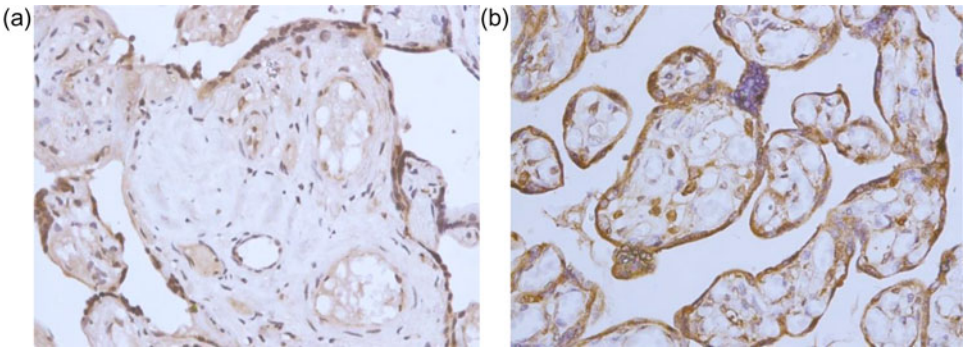

Figure 3. (a) Microphotograph shows immunostaining of e-NOS in placentas from normal mothers. Faint staining is observed in the cytoplasm of syncytiotrophoblasts, cytotrophoblasts, and capillaries .e-NOS immunostain; original magnification,×10. (b) Microphotograph shows immunostaining of e-NOS in placentas from anemic mothers. Increased staining intensity is observed in the cytoplasm of syncytiotrophoblasts, cytotrophoblasts, and capillaries .e-NOS immunostain; original magnification,×10.

Thus, in our study, the placentas from the anemic women exhibited a stronger/more intense immunostaining of the angiogenic factors VEGF, PLGF, e-NOS, and NT residues.

Discussion

In our study, 58.3% of the pregnant women had anemia of different grades, among which 20.83% had mild anemia and 18.75% each had moderate and severe grades of anemia, respectively. This finding is similar to that by Hamzullah et al. [13].

In our study, both the RDW and HDW values were raised in a significant (87.5%) number of the subjects with low Hb levels which is similar to the study by Sultana et al. [14].

Table 8. Localization and immunostaining intensity of NT residues expression in placental villous tissues.

$n = 29$	Staining intensity score	ST, n(%)	CT, n(%)	Wall of capillaries, n(%)	Mean ± SD
Normal Hb ($n = 12$)	0	0	1(8.3%)	4(33.3%)	0.89 ± 0.26
	1+	11(91.7%)	11(91.7%)	8(66.7%)	
	2+	1(8.3%)	0	0	
	3+	0	0	0	
Mild anemia ($n = 7$)	0	0	0	0	1.23 ± 0.25
	1+	6(85.7%)	6(85.7%)	4(57.1%)	
	2+	1(14.3%)	1(14.3%)	3(42.9%)	
	3+	0	0	0	
Moderate anemia ($n = 7$)	0	0	0	0	1.67 ± 0.30
	1+	2(28.6%)	4(57.1%)	0	
	2+	5(71.4%)	3(42.9%)	7(100%)	
	3+	0	0	0	
Severe anemia ($n = 3$)	0	0	0	0	2.1 ± 0.69
	1+	1(33.3%)	2(66.7%)	0	
	2+	0	0	2(66.7%)	
	3+	2(66.7%)	1(33.3%)	1(33.3%)	
Statistical comparison p value		.001*	.204	.001*	.000**

* $p < .01$; ** $p < .001$. [Staining intensity of NT residues were scored as follows: negative (0), weak (1+), moderate (2+), and strong (3+)].
Abbreviations: ST: syncytiotrophoblasts; CT: cytotrophoblasts; Hb: hemoglobin.

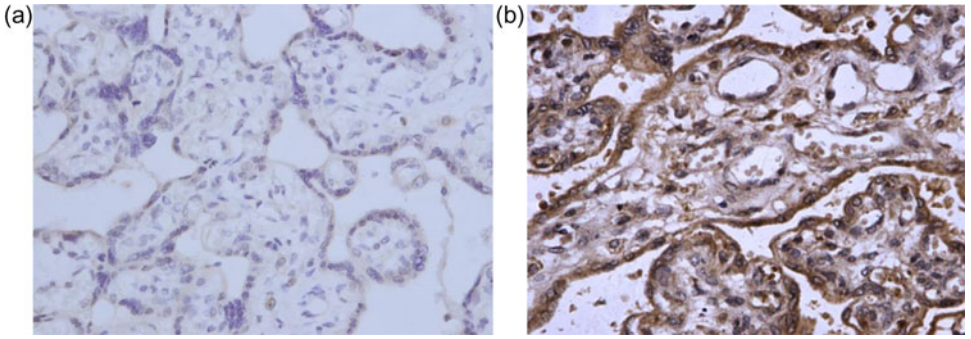

Figure 4. (a) Microphotograph shows immunostaining of NT residues in placentas from normal mothers. Syncytiotrophoblasts, cytotrophoblasts, and capillaries are all negative for immunostain. NT residues immunostain; original magnification,×10. (b) Microphotograph shows immunostaining of NT residues in placentas from anemic mothers. Syncytiotrophoblasts and cytotrophoblasts show intense staining for the immunostain while capillaries show mild staining. NT residues immunostain; original magnification,×4.

Comparison of the red cell parameters and the SFr levels between the maternal and cord blood in our study showed that despite the presence of maternal anemia, the corresponding cord blood parameters levels were either normal or increased. These findings are in contrast with those of Dallman [15], in whose study maternal iron deficiency adversely affected the neonatal red cell parameters. Although several studies have been carried out in maternal and cord blood, some have reported a negative impact of maternal IDA on the iron stores of the newborns [16], while others could not find any relationship [17]. Similar to our study, Laila et al. [18] observed higher red cell values in the cord blood in their study. This may be due to greater iron transfer from

the mother to the fetus and maximally stimulated erythropoiesis (explaining higher values for sTfR) at the end of gestation.

Correlations between the maternal and the cord blood parameters in our study show that apart from the SFr and MCHC values, most of the other parameters had a weak to insignificant correlation. Rios et al. [19] and Kelly et al. [20] also, in their studies, did not find any correlation between maternal and umbilical cord ferritin levels. These results suggest that the fetus is able to maintain adequate iron levels despite the presence of IDA in the mothers.

Similar to our study, Mongia et al. [21] too reported an increase in the number of syncytial knots and capillaries per villus with the increasing severity of anemia.

An association between low birth weight and early onset of essential arterial hypertension had first been postulated by Barker in the "fetal origins of adult disease hypothesis" [5,22,23]. Anemia causes a state of chronic hypoxia and two studies have further reported that this vasoconstrictor phenotype triggered by developmental hypoxia is not only present in fetal life but that chronic prenatal hypoxia can program permanent endothelial dysfunction in the resistance circulations of the adult offspring [24,25]. In the rat fetal offspring, on the day 20 of gestation, the aortic wall thickness was significantly increased in hypoxic pregnancy [26]. Angiogenesis plays a role in the development of villous vasculature in human placenta. VEGF, PLGF, and e-NOS have been identified as positive regulators of angiogenesis [27]. The VEGF protein has potent angiogenic, mitogenic, and vascular permeability-enhancing activities specific to the endothelial cells [28]. In our study, a statistically significant increase in the expression of VEGF was observed in the capillaries of placentas from the anemic women. Benirschke [29] stated that hypoxic stimuli may lead to excessive proliferation of villous capillaries and connective tissue via growth factors such as VEGF [29]. VEGF is involved in angiogenesis and anemia causes a state of hypoxia, this probably explains the increased proliferation of capillaries as well as the increased staining intensity for VEGF seen in the severe anemia cases in our study.

PLGF is another factor which shares 53% homology with VEGF [30]. In our study, a statistically significant increase in the expression of PLGF was observed in all the three cell types-cytotrophoblasts, syncytiotrophoblasts, and capillaries in the placentas from the anemic women. However, Kumazaki et al. in their study reported that a strong PLGF immunoreactivity was localized to the degenerative trophoblasts around infarctions [31]. In our study too, the placental villi of severely anemic women showed a higher staining intensity for PLGF thus indicating that PLGF is increasingly expressed in severe anemia and thus seems to play a role in the placental angiogenesis in anemia.

Endothelial NOS (e-NOS) is an enzyme encoded by the NOS3 gene. It is responsible for the generation of NO in the vascular endothelium which plays a crucial role in regulating vascular tone. Similar to VEGF and PLGF, e-NOS staining too, in our study, was observed to be localized to the cytotrophoblasts, syncytiotrophoblasts, and capillaries. The intensity of staining or the protein expression was observed to be statistically significantly higher in the anemic women when compared with the non-anemic women. Our study results are similar to those of Ancheva et al. [32] whose analysis of immunohistochemical expression of e-NOS in the placental tissue of the pregnant women revealed increased expression of *e-NOS* gene to 1.4-fold in the patients with IDA.

Trophoblast and placental vascular endothelium are the sites for the production of NO which upon interaction with locally produced superoxide ions leads to the production of peroxynitrite which nitrates amino acids such as tyrosine and whose action can be localized by measurement of NT residues. It was demonstrated that NT residues are increased in the placentas of pregnancies complicated by pre-eclampsia or diabetes [9]. As anemia in the mothers causes hypoxia which may lead to increased production of VEGF and which in turn produces NO (indicated by increased expression of NT residues). Hence, we wanted to study the expression of NT residues in the placenta in the condition of maternal anemia. A statistically significant increase in the expression of NT residues was observed in the syncytiotrophoblasts and capillaries of the placentas from the anemic women in our study.

Increased expression of VEGF, PLGF, and e-NOS found in the anemic cases in our study was similar to the Intrauterine Growth Restriction (IUGR) placentas [33], which may promote increased endothelial cell proliferation, migration, and pathological angiogenesis.

Ours is a preliminary study in which we have studied the effects of hypoxia caused by maternal anemia on the expression of angiogenic proteins in the placenta by using immunohistochemistry. The small sample size was a limitation of our study. The finding of increased expression of the proteins (VEGF, PLGF, e-NOS, and NT residues) in our study calls for further studies on m-RNA expression of the above mentioned proteins in the placenta which might suggest that alteration of these proteins in maternal anemia probably leads to changes in the placental vasculature with further effects on the fetus and with a probable potential to bring therapeutically useful solutions.

Disclosure statement

No potential conflict of interest was reported by the authors.

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