ORIGINAL ARTICLE

Differential Expression of Placental Growth Factor, Transforming Growth Factor-β and Soluble Endoglin in Peripheral Mononuclear Cells in Preeclampsia

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ABSTRACT

Objective: To measure the peripheral blood mononuclear cells (PBMCs) mRNA expression of placental growth factor (PlGF), Transforming growth factor beta (TGF-β), and soluble Endoglin (sEng) in the blood of preeclamptic and normotensive pregnant women.

Study Design: Cross-sectional analytical study.

Place and Duration of Study: Department of Physiology and Cell Biology, University of Health Sciences, Lahore, from November 2016 to April 2018.

Methodology: The study included 50 normotensive and 57 preeclamptic patients (18-40 years of age), all in the third trimester of pregnancy. The preeclamptic group was further divided into early-onset preeclampsia (EOP) and late-onset preeclampsia (LOP). Blood samples from patients and healthy controls were collected and mRNA expression was measured (18 patients and 18 controls) by real time PCR. Statistical analyses were done using SPSS (version 22). The values were considered significant at 0.05 level of significance.

Results: The PBMCs mRNA expression of PlGF, TGF-β and sEng were significantly different between the preeclampsia and control group ($p<0.001$). A significant decrease in expression of TGF-β was observed in LOP group compared to controls ($p<0.001$); whereas, the difference in the expression of EOP compared to controls was not significant ($p=0.12$). Similar to TGF-β, the expression of PlGF was significantly decreased among EOP and LOP compared to controls. Detailed analysis of sEng showed significantly increased expression in both EOP and LOP as compared to healthy group ($p<0.001$).

Conclusion: There is a significant difference in extra-placental expression of PlGF, and sEng in preeclampsia.

Key Words: *Preeclampsia, Placental growth factor (PlGF), Transforming growth factor-beta (TGF-β), Soluble endoglin (sEng), Early onset preeclampsia.*

INTRODUCTION

Preeclampsia (PE) is defined as the onset of systolic blood pressure >140 mmHg or diastolic blood pressure ≥90 mmHg at >20 weeks of gestation accompanied by 24-hour proteinuria ≥300 mg (≥1+ on dipstick), in atleast two random urine samples collected 4-6 hours apart.[1] The incidence of disease is reported to be 2-10%,[2] with a seven times higher risk in women in developing countries, 10-25% of these cases results in maternal death. Abnormal placental development due to disturbance in angiogenesis and impaired trophoblast invasion leads to perfusion disorder in the utero-placental compartment with resultant hypoxia and endothelial dysfunction.[3] Placental growth factor and TGF-β are proangiogenic proteins with an important role in the process of angiogenesis and embryogenesis. PlGF binds to vascular endothelial growth factor receptor 1 and displaces VEGF to bind to vascular endothelial growth factor receptor 2 (VEGFR-2), the major receptor of signaling cascades in angiogenesis, expressed in cytotrophoblast.[4,5] Increased Soluble Fms like tyrosine kinase -1 (sFlt) in preeclampsia, a spliced variant of VEGFR-1 binds PlGF and decreases its bioavailability.[6] Transforming growth factor-β regulates the vascular changes during implantation. Along with endoglin, a transmembrane glycoprotein co-receptor it enhances its angiogenic effect through activin receptor like kinase 1 (ALK1) signaling.[7] Similar to sFlt, increased circulating levels of a soluble form of endoglin, soluble endoglin (sEng) in preeclampsia has been reported by a number of researchers.[8-10] The sEng binds with circulating TGF-β, decreasing its bioavailability and interfering with Nitric oxide (NO) mediated vasodilatation with resultant endothelial dysfunction.[11] A number of researchers have studied the mRNA expression of multiple genes in placental tissue,[12-14] but a few have done so in the blood of preeclamptic and normotensive group.[15-17] We hypothesized that mRNA transcript of

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Received: July 04, 2018; Accepted: October 29, 2018

PlGF, TGF-β and sEng, highly expressed in placental tissue, is also changed in peripheral blood in preeclampsia. This non-invasive technique can be used to better understand the role of angiogenesis in preeclampsia.

The aim of the study was to measure and compare the mRNA expression of the above, by using real time PCR in preeclamptic and normotensive pregnancies.

METHODOLOGY

After acceptance from Ethical Review Board, this cross-sectional analytical study was conducted according to the guidelines of the Declaration of Helsinki in the Department of Physiology and Cell Biology, University of Health Sciences, Lahore, from November 2016 to April 2018.

Cases included 57 diagnosed preeclamptic women (18-40 years) in third trimester (28-40 weeks), divided into two groups, early-onset preeclampsia (EOP, 28-32 weeks) and late-onset preeclampsia (LOP, 32.1-40 weeks). Fifty normal pregnancies with same maternal and gestational age were selected as controls. Cases with history of smoking, diabetes, renal disease, arthritis, inflammatory bowel disease, chronic hypertension, cardiovascular illness (e.g. ischemic heart disease), or other chronic inflammatory diseases were excluded. Demographic data was compiled along with complete medical, obstetric, and family history.

Five ml whole blood was collected in EDTA coated vacutainer. After centrifugation at 4000 rpm for 5 minutes, buffy coat was stored at -20°C within an hour of sample collection. FavorPrep total RNA Isolation Kit (Favorgen, Taiwan) was used to extract total RNA following the manufacturer's instructions. Cell-free RNA was quantified using Nano drop and stored at -80°C in RNase-free water.

cDNA was reverse transcribed by using revert aid First Strand cDNA Synthesis kit (Thermo Scientific) following the manufacturer's instructions. Amplification of the cDNA was done by PCR and product confirmed on 2% agarose gel, using ethidium bromide staining. Genes expression of 18 cases and 18 controls was measured by using specific-primers for Real Time PCR, CFX 96 by using SYBR Green mix (Fermentas, USA), according to the manufacturer's instructions. One µl of cDNA with 8 µl of 2 X SYBR Green Real Time PCR Master Mix and 0.5 µl of forward and reverse primers was used along with RNase-free water to perform all reactions in a total 10 µl mixture (Fermentas, USA). Real time PCR conditions were set at 94°C for 4 minutes, followed by 30 cycles of 94°C for 30 s, annealing at 60°C for 30 seconds, and extension at 70°C for 42 s in a thermal cycle. Primer sequence and PCR product length detail is given in Table I. Samples were assayed in duplicate along with three housekeeping genes.

The statistical analysis was performed using SPSS version 22.0. Clinical parameters were expressed as Means ±SD (standard deviation). Three housekeeping genes, *i.e*. GAPDH, β-actin, and 18srRNA were studied and their mean used to normalize the expression of target. The PBMCs mRNA expression was compared between two groups by students t-test and one-way ANOVA with post-hoc Tukey's test for comparison among the multiple groups (EOP, LOP, and normotensive groups). Differences in gene expressions were reported as fold change with standard deviation (SD). A p-value of <0.05 was considered statistically significant.

RESULTS

Details of clinical characteristics of study population are given in Table II. Blood samples from both groups were collected from maternal and gestational age matched females. Based on the recruitment criteria, cases had higher systolic and diastolic blood pressures as compared to controls (p<0.001). There was also a significant difference in mean BMI in both groups (p<0.001).

Gene expression was reported as fold change between the different groups (Figures 1 and 2). The PBMCs

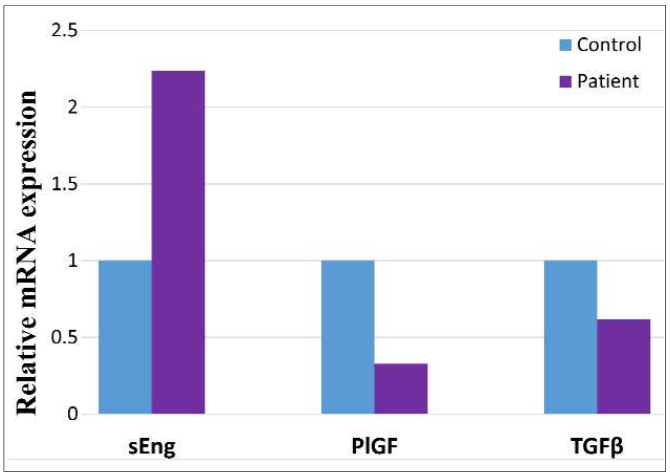

Figure 1: Comparison of PMBCs mRNA expression (fold change) between Normotensive group and Preeclampsia.

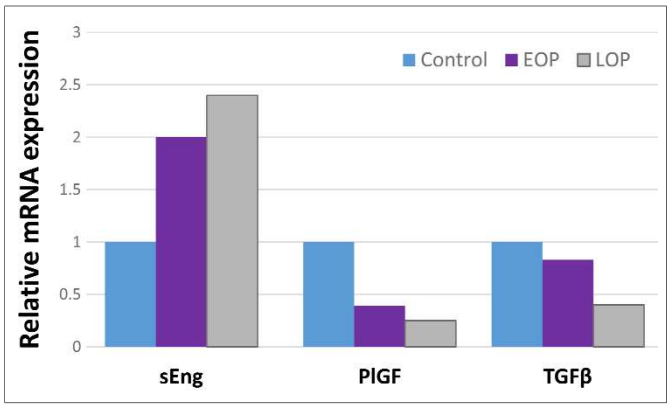

Figure 2: Comparison of PMBCs mRNA expression (fold change) between Normotensive group with EOP and LOP.

mRNA expression of PlGF and TGF-β was significantly different between the preeclampsia and control groups with a decrease in the diseased group (p<0.001 for both factors). sEng showed a significant increase (2.24 fold) in PBMCs mRNA expression in the cases (p<0.001) (Table III).

For a detailed knowledge of the differential expression in preeclampsia patients, the group was further divided into EOP and LOP, depending upon the time of onset of the disease. There was a significant difference in the PlGF expression in EOP and LOP with a more profound decrease in LOP. The expression of TGF-β was significantly different between EOP and LOP and showed a significant decrease in LOP compared to controls (p<0.001). However, the difference was not significant in EOP and normotensive group (p-value 0.12). Detailed analysis of sEng showed significantly increased expression in both early onset and late onset preeclampsia with a more profound increase of 2.4 fold in LOP as compared to healthy group (p<0.001). The difference was significant in EOP and LOP (p<0.01, Table III).

Table I: Sequence of primers used for qPCR.

Gene	Sequence	PCR product length (bp)
PlGF-FP	5'- ACG TGG AGC TGA CGT TCT CT - 3'	241bp
PlGF-RP	5'- CAG CAG GAG TCA CTG AAG AG - 3'	
TGF β-FP	5'-AGA GCA ACA CGG GTT CAG GTA - 3'	84bp
TGF β-RP	5-AGT TCA AGC AGA GTA CAC ACA GCA T-3'	
sEng- FP	5'- AAG TGT GGG CTG AGG TAG- 3'	109bp
sEng- RP	5' -AGG CGG TGG TCA ATA TCC - 3'	
GAPDH-FP	5' -CGC TCC TGG AAG ATG GTG AT -3'	214bp
GAPDH-RP	5' -ACG GAT TTG GTC GTA TTG GG- 3'	
β-actin-FP	5' -GCA TTT GCG GTG GAC GAT -3'	75bp
β-actin-RP	5' -TCC ACC TTC CAG CAG ATG TG -3'	
18s rRNA-FP	5' -CCT GTA TTG TTA TTT GTC ACT ACC T -3'	91bp
18s rRNA-RP	5'-AGA AAC GGC TAC CAC ATC CAA -3'	

FP = Forward primer; RP = Reverse primer; bp = base pair.

Table II: Clinical characteristics of PE patients and control group.

	Preeclampsia		Normotensive n = 50	p-value
	Early onset n = 25	Late onset n = 32		
Maternal age (years)	27.12 ±4.8	27.37 ±6.1	25.7 ±4.6	0.30
BMI (kg/m^2)	29.08 ±3.1	28.87 ±3.1	25.8 ±3.5	<0.001
Parity	1.92 ±1.9	1.53 ±1.7	1.42 ±1.3	0.46
Gestational age at sampling (weeks)	29.58 ±1.7	35.51 ±2.1	31.97±3.1	<0.001
Systolic BP	149.6 ±15	151.8 ±15	105.2 ±8.8	<0.001
Diastolic BP	97.2 ±7.3	97.1 ±5.8	67.6 ±7.7	<0.001

Data presented as Mean ± SD, p-value <0.05 was considered significant, analysed by ANOVA.

Table III: Comparison of mRNA expression between different groups.

Gene	Normotensive	Preeclampsia (a)	EOP (b)	LOP (c)	p-value (d)
PlGF	1.00	0.32 ±0.16	0.39 ±0.2	0.25 ±0.06	a-b <0.001* c-d =0.02** a-c <0.001** a-d <0.001**
TGF β	1.00	0.61 ±0.36	0.83 ±0.27	0.40 ±0.3	a-b <0.001* c-d <0.001** a-c =0.12** a-d <0.001**
sEng	1.00	2.2 ±0.45	2.0 ±0.32	2.4 ±0.44	a-b <0.001* c-d <0.01** a-c <0.001** a-d <0.001**

*Data is presented as Mean ± SD, *p-value analyzed by students t-test for preeclamptic and normotensive groups, **p-value analyzed by ANOVA with post-hoc Tukey's test. p-value <0.05 was considered significant.*

DISCUSSION

Abnormal placentation with reduced feto-placental perfusion is central to preeclampsia. Defective angiogenesis, being one of the key pathophysiological processes, has been probed in by a number of researchers but the results are controversial. Most of them have reported the serum levels of pro- and anti-angiogenic-factors along with mRNA expression in placental tissue. In 2011, Paiva and colleagues worked on both maternal blood and placental tissue in preeclampsia and found correlation of gene expression between the two.[17] mRNA expressions of genes, highly expressed in preeclampsia, have been studied in maternal blood samples by a few others.[15,16,18] The present study has analyzed and compared mRNA expression of two proangiogenic factors PlGF and TGF-β and an antiangiogenic factor sEng in blood of normotensive and preeclamptic cases. We found a significant decrease in PlGF expression (p<0.001) in the preeclamptic group. The difference was significant within the two subgroups of preeclampsia. The present finding supports the results of a number of studies which have reported a decrease in this proangiogenic factor, providing considerable evidence that disrupted angiogenesis is fundamental to the pathogenesis of preeclampsia.[12,15,19] In contrast, Toft *et al*. worked on placental tissue and found no significant difference in mRNA expression of PlGF between normal and preeclamptic pregnancies.[20]

TGF-β has been proposed to have a role in angio-genesis through ALK 1 receptors and maintain vascular health by NO production from (eNOS). A number of studies have reported the expression of TGF-β in preeclampsia with mass diversity in results. A significant decrease was found in the mRNA expression of TGF-β in preeclampsia (p<0.001). Detailed analysis between the normotensive and two subgroups of preeclampsia revealed significant decrease in TGF-β expression in the LOP as compared to the normal pregnancies (p <0.001). However, the difference was not significant between EOP and normotensive controls (p=0.12). The difference within the two subgroups of preeclampsia, *i.e*. EOP and LOP was significant (p <0.001). Decrease in TGF-β expression in preeclampsia, because of its role in

implantation and angiogenesis, supports the two-stage model of preeclampsia pathology where defective implantation and vasculogenesis lead to a hypoxic state in placenta.[3] The more profound decrease in the later weeks of gestation in LOP is supported by Singh *et al.* who reported gradual but significant decrease in expression of TGF-β mRNA as the normal gestation proceeds.[21] Moreover, Martinez-Fierro and colleagues reported significantly lower expression of TGF-β in PBMCs as a hallmark of preeclampsia.[22] In contrast, recently in 2017, Wang *et al.* worked on multiple soluble TGF-β receptors in addition to sEng. They reported a significant increase in the levels of these soluble receptors in the serum of EOP with resultant disruption of TGF-β signaling and vasculogenesis.[23]

Endoglin is a transmembrane co-receptor for TGF-β with an important role in endothelial health and relaxation. sEng, a soluble form of the receptor has antiangiogenic properties as it binds the TGF-β ligand making it unavailable to initiate intracellular signaling. Resultant decreased production of NO from endothelial NO synthase (eNOS) results in failure of endothelial relaxation and oxidative stress.[11] sEng is expressed by syncytiotrophoblast and secreted from placenta in normal pregnancy with gradual increase in the plasma levels as the gestation advances.[9,24] In this study, we found a significant 2.24 fold ($p<0.001$) increase in sEng mRNA in the preeclamptic group as compared to the normal pregnancies. The increase was even more profound in the LOP with a significant difference as compared to normotensive group ($p<0.001$). This may be attributed to the difference in the gestational age at the time of sampling, as mentioned earlier the levels increase with advancing gestational age. These results support a number of studies which reported increased sEng expression in preeclampsia, both in placental tissue and maternal blood.[15,20,25]

CONCLUSION

Angiogenesis is disrupted in preeclampsia due to an imbalance between pro- and anti-angiogenic factors with a decrease in PlGF, TGF-β and increase in sEng mRNA expression in cellular compartment of blood. Easy accessibility of blood sample makes it a non-invasive tool to diagnose and study the course of preeclampsia. Differences in the early and late onset preeclampsia need further investigation with serial blood sampling during all the three trimesters of gestation.

Disclosure: This research was partially funded from Research Development Fund by University of Health Sciences, Lahore.

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