Original Article

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Spectrum of Factors Triggering Endothelial Dysfunction in PIH

Biochemistry Section

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ABSTRACT

Introduction: Pre-eclampsia (PE) is a major cause of maternal and fetal/neonatal mortality and morbidity. The aetiology and pathogenesis of PE is yet to be completely understood. Evidence shows that, Endothelial Dysfunction (ED) plays a pivotal role in the genesis of this multi-system disorder that develops in PE and eclampsia.

Aim: To determine the circulating levels of factors Malondialdehyde (MDA), Ferric Reducing Ability of Plasm-α (FRAP), Tumour Necrosis Factor. (TNF-α), sFlt-1, VEGF, PlGF, Nitric Oxide (NO) that influence the ED.

Materials and Methods: Study groups consisted of Normo-tensive pregnant women (N) preclamptic women (PE) and eclamptic women (E) with 100 subjects in each group in the 3rd trimester of pregnancy. They were investigated for MDA, FRAP, TNF-α, sFlt-1, VEGF, PlGF, NO. Statistical analysis was done using Analysis of Variance (ANOVA).

Results: When compared to controls MDA, TNF-α, sFlt-1 levels were found to be significantly high and FRAP, VEGF, PlGF and NO levels were significantly low in PE, E group. E showed a significantly high level of MDA, TNF-α, sFlt-1 and low levels of FRAP, VEGF, PlGF, NO when compared to PE group.

Conclusion: Our study substantiated the fact, that, oxidative stress, imbalance between anti-angiogenic factors and pro-angiogenic factors exists in Pregnancy Induced Hypertension (PIH) condition. This imbalance is directly related to the ED, the hallmark of PE. So oxidative stress, VEGF, PlGF and sFlt-1 can be used as markers to analyze the onset and progression of the disease.

Keywords: Endothelial dysfuction, Ferric reducing ability of plasma, Malondialdehyde, Nitric oxide, Placental growth factor, Pregnancy induced hypertension, Soluble fms like tyrosine kinase, Tumour necrosis factor, Vascular endothelial growth factor

INTRODUCTION

Pregnancy Induced Hypertension (PIH) is the second most common medical disorder seen during pregnancy [1]. Expectant mothers with hypertension are pre-disposed towards the development of potentially lethal complications. The causes of hypertension during pregnancy, particularly pre-eclampsia (PE), remain unknown. PE is a complication of pregnancy with significant morbidity and mortality for both mother and the fetus [2]. It affects 5-7% of pregnancies worldwide. It is a multi-organ disorder usually recognized by new onset of hypertension and proteinuria appearing in the 2nd half of pregnancy. Eclampsia is classified as presence of seizures, non-attributable to other causes, in a women diagnosed with PE [3]. Placenta plays a central role in the pathogenesis of the disease [4]. The abnormal cytotrophoblast differentiation is an early defect that may lead to reduced placental perfusion and ischaemia [5]. Reduced placental perfusion leads to widespread dysfunction of the maternal vascular endothelium [6] which is considered as a classic hallmark of PE [7] which likely affects the cerebral endothelium as well leading to cerebral oedema and seizures seen in eclampsia [8]. PE has been associated with increased oxidative stress. Reactive Oxygen Species (ROS) results in the oxidative damage of cellular lipids, proteins, DNA and RNA irreversibly. Oxidative stress and tissue damage cause a rupture in the barrier and create a leakage of fetal and placenta-derived factors or material into the maternal circulation leading to maternal endothelial damage, elevated oxidative stress, and systemic inflammation [9]. Tumour Necrosis Factor-α (TNF-α) is a pro-inflammatory cytokine with a pleiotropic effect on the immune system, tissue homeostasis, embryonic development and placentation. When released in large amounts, TNF-α induces enhanced activation and injury to the vascular endothelium [10]. Normal endothelium is maintained by Vascular Endothelial Growth (VEGF). It is a sub-family of growth factors involved in both vasculogenesis and angiogenesis [11]. The most important member is VEGF-A. Other members are Placenta Growth Factor (PlGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. It is suspected that trophoblastic injury markedly enhances placental Soluble fms-like Tyrosine Kinase (sFlt-1) production, antagonizing the endothelial protective role of VEGF and/or PlGF eventually leading to clinical PE [12]. sFlt-1works by enhancing the Endothelial Dysfunctioning (ED) already established by oxidative stress, ROS and damage [13]. So the study had been designed to determine the factors that influence ED in PIH.

MATERIALS AND METHODS

A cross-sectional analytical study was conducted in the in-patient ward of the Department of Obstetrics and Gynaecology, Annapoorana Institute of Medical Sciences, Salem, Tamilnadu from August 2012 to April 2016. The study was approved by the Institutional Ethics Committee of AMC&H and informed consent was obtained from all participants. PIH patients were defined according to the NHBPEP (National High Blood Pressure Education Programme) guidelines [14].

Three hundred patients were recruited for the study, dividing them into 3 groups (100 in each): control (group1), pre-eclampsia (PE-group2) and Eclampsia (E-group3). The mean Systolic Blood Pressure (SBP) in 3 groups were recorded as (116 ± 5.45 vs.162.18 ± 18.26vs.170 ± 15.52) mm Hg. The mean (DBP) in 3 groups were recorded as (75 ± 5.99 vs.107.5 ± 11.35vs.112.28 ± 10.59) mmHg. The urine albumin levels in 3 groups were measured as (150.92± 33.4 vs.436 ± 96vs.432 ± 101) mg/d.

Pre-eclampsia (PE) was defined as having a SPB 140 mmHg or a DBP 90mm Hg with proteinuria 300mg/d. Sign and symptoms of PIH like swelling in the hands, face and feet, severe

headaches, abdominal pain, reduced output of urine or no urine, blood in the urine, a change in reflexes, convulsion/seizures, coma, dizziness, excessive vomiting, nausea and rapid gain in weight were recorded. Eclamptic complications like cerebro vascular, cardio vascular, visual, pulmonary, renal, hepatic, haemostatic and obstetrical were also recorded.

Individuals with past history of cardiac, renal, hepatic illness, diabetes and hypertensions were excluded.

Assay Procedures

A 10ml of antecubital venous blood samples were collected from both patients and controls. The samples were centrifuged at 3000rpm for 20 minutes and serum was separated. Aliquots were prepared and stored at –20°C till subsequent use. sFlt-1, VEGF and PlGF were measured by a sandwich-type ELISA (Quantikine® human sVEGF R 1, Quantikine® human VEGF, Quantikine® human PlGF, R&D Systems Inc., Minneapolis, MN, USA). The minimum detectable level of the assay is 13.3pg/ml for sFlt-1, 9pg/ml for VEGF and 7pg/ml for PlGF. The intra-assay and inter-assay variations were 3.8% and 7% for sFlt-1, 4.5% and 7% for VEGF and 3.6% and 11% for PlGF, respectively. TNF-alpha levels were measured using Quantikine® human TNF- alpha R&D Systems Inc., Minneapolis, MN, USA. MDA levels were measured using TBARS. Total anti-oxidant capacity was measured using FRAP. NO levels were measured using Griess method.

STATISTICAL ANALYSIS

The quantitative variables like SBP, Diastolic Blood Pressure (DBP), serum VEGF, PlGF, sFlt-1, MDA, NO, FRAP were compared between PE, E women and Normotensive controls.The same was also compared between PE and E. The data was processed on computer software package SPSS version 20. The numerical data was presented as Mean±SD. A value of $p < 0.05$ at 95% CI was considered as statistically significant.

RESULTS

[Table/Fig-1] shows the mean SBP, DBP, MDA, FRAP, TNF-α, sFlt-1, VEGF, PlGF, NO values between 3 groups. The SBP, DBP levels were significantly higher in PE and E women ($p<0.001$, $p<0.001$) than controls and the levels were significantly high in eclamptic women compared to PE patients ($p<0.001$). PE and E patients showed a significantly higher serum sFlt-1, MDA, TNF-α levels than controls ($p<0.001$, $p<0.001$) and the levels were significantly in E than PE ($p<0.001$). The FRAP, VEGF, PlGF, NO levels were significantly low in PE and E women than controls ($p<0.001$, $p<0.001$) and the same was significantly low in women with eclampsia than PE women ($p<0.001$).

Parameter	Controls	Preeclampsia	Eclampsia
Systolic BP (mm Hg)	116 ± 5.45	162.18 ± 18.26 *	170 ± 15.52 * a*
Diastolic BP (mm Hg)	75 ± 5.99	107.5 ± 11.35 *	112.28 ± 10.59 * a*
MDA (µmol/L)	1.08 ±0.86	4.49 ± 1.75*	5.50 ± 1.97* a*
FRAP(µmol/L)	2.21 ±0.89	0.67 ± 0.42*	0.407 ± 0.38* a*
TNF-α (pg/ml)	10.51 ± 3.00	22.17 ± 8.04*	27.50 ± 14.07* a*
sFLT-1 (pg/ml)	1271.22 ±365.22	3854.12 ± 741.97*	7827.57 ± 1841.29*a*
VEGF(pg/ml)	274.05 ±36.15	179.12 ± 18.87*	131.48 ± 36.93*a*
PlGF(pg/ml)	682.97 ± 212.19	225.56 ± 56.46*	141.63 ± 121.74* a*
Nitric oxide (µmol/L)	117.37 ±14.77	43.87 ± 6.13 *	38.6 ± 9.94 * a*

[Table/Fig-1]: Clinical characteristics and concentrations of parameters between three groups.
The numerical data was presented as mean + SD. A value of $p < 0.05$ at 95% CI was considered as statistically significant. Values that are marked with a star* differ significantly from the control at $p= <0.05$.Values that differ significantly at $p=<0.05$ between PE and E groups are marked with a*

DISCUSSION

PIH continues to be a main obstetric problem in present-day healthcare practice. It affects not only maternal health but also puts fetal development at risk [15]. PE is a hypertensive disorder of pregnancy in which the normal haemodynamic response to pregnancy is compromised and it is a two-stage disorder. The first stage involves, an abnormal placentation that results in hypoxic placenta. In the second stage, the maternal syndrome develops which is characterized by ED and clinical signs including hypertension and proteinuria. The link between these stages is unclear. Cohen et al., had stated that the chronic hypoxia that occurs in placenta induces oxidative stress [16]. In this regard our study displayed significantly high levels of MDA in PE & E women compared to controls. The MDA levels were found to be significantly high in eclamptic women compared to PE women. This might be due to the extent of hypoxia which causes the overload and accumulation of free radicals in the body [17]. Free radicals induce the lipid peroxidation process and generate Malondialdehyde (MDA), which is a marker for oxidative stress [18]. A biological antioxidant has been defined as “any substance that when present at low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substrate”. The FRAP value is a marker for antioxidant capacity [19]. Antioxidant status as measured by FRAP is significantly lower in PE and E women compared to the controls. The FRAP levels were significantly lower in E than in PE women. This reduction might be due to the utilization of anti-oxidants to a greater extent in order to counteract free radical mediated cellular changes [20].

A study by Tai-Ho-Hung et al., had stated that oxidative stress leads to increased production of TNF-α by placental villous explants [21]. Our study displayed significantly high levels of TNF-α in PE and E women than controls and the levels were significantly high in E when compared to the PE women. This might be due to hypoxia-induced up regulation of placental inflammatory cytokines. Furthermore, intermittent perfusion of the placenta, secondary to reduced trophoblast invasion, causes increased secretion of TNF-α [21]. A study by Sydney et al., had stated that TNF-alpha may stimulate sFlt-1 production through an indirect mechanism, possibly mediated by the Angiotensin type II receptor agonistic auto-antibodies (AT1-AA). Alternatively, under chronic conditions TNF-α can directly stimulate the sFlt-1 production [22]. sFlt-1, a soluble form of VEGFR-1 canbind and reduce the free circulating levels of proangiogenic factors (VEGF and PlGF). Thus, it blunts the beneficial effects of these factors on maternal endothelium, with consequent maternal hypertension and proteinuria. In normal pregnancies Flt-1 prevents damage to the placenta and fetus by inhibiting excess VEGF signaling, which would otherwise leads to excessive placental invasion. This may lead to catastrophic haemorrhage at delivery. Thus, in normal pregnancies VEGF level are tightly controlled at the maternal-fetal interface in order to regulate placental invasion and facilitate detachment of placenta after delivery of the fetus, through the modulation by sFlt-1 [23].

Endothelium is a single-cell lining covering the luminal side of blood vessels. A critical balance exists between endothelium-derived relaxing and contracting factors to maintain vascular homeostasis. In case of any disbalance, the vasculature is predisposed to vasoconstriction, leukocyte adhesion, mitogenesis, pro-oxidation and vascular inflammation [24].

VEGF is a highly specific mitogen for micro- and macro vascular endothelial cells derived from arteries, veins, and lymphatics. It has five members in its family, VEGF-A, -B, -C, and -D and PlGF. The most widely studied form, VEGF-A (or simply VEGF) is the dominant angiogenic molecule in physiological and pathological angiogenesis and its production is induced by hypoxia/ischemia [25]. It was stated that during pregnancy the maternal plasma concentration of VEGF is increased because, the provision of nutrients

to the fetoplacental unit depends partly on uterine blood flow. So, the increased concentrations of VEGF induces vasodilation through the production of NO and PGI2 in order to increase the uterine blood flow to the feto placental unit [26].

PlGF, a member of VEGF family has 42% homology with VEGF. The name refers to placenta since it was cloned from a human placental cDNA library. PlGF is highly expressed in placenta throughout all stages of gestation. It controls trophoblast growth and differentiation dilates uterine vessels, promotes EC growth, vasculogenesis and placental development. It has weak mitogenic activity and potentiates the actions of VEGF. PlGF stimulates angiogenesis in different physiological and pathological conditions [27].

Preeclampsia is associated with placental hypoxia, which is said to be the putative culprit in initiating the cascade of events that ultimately results in the maternal manifestations of the disease [16]. Usually hypoxic conditions stimulates the production of VEGF and PlGF levels, but our study showed a significant decrease in free VEGF and PlGF levels in PE and E women as compared with controls. However, studies on circulating levels of VEGF in PE have been inconsistent, with reports of both increased and decreased levels. This discrepancy could be explained by the fact that VEGF-protein complexes are undetectable by the sandwich-type ELISA because there is a substantial increase in circulating VEGF binding proteins during pregnancy. All prior studies reporting on decreased VEGF have used an ELISA kit, which measures free (unbound) VEGF whereas all studies reporting on an increased VEGF in PE used either a radioimmunoassay or an ELISA system measuring total (bound and unbound) VEGF [28]. In our study, we estimated free VEGF and PlGF levels. The reduction in VEGF levels might be a reason for observed hypertension and proteinuria among PE and E women because VEGF is important in regulation of blood pressure and maintaining the integrity of glomerular filtration barrier. It also has a role in glomerular healing. Alterations in the VEGF bioavailability might have resulted in endothelial as well as podocyte damage [11]. Our study also displayed a significantly low free VEGF and PlGF levels in E women than PE women. This might be the reason for the disruption of endothelial cells by disrupting the endothelial cells that maintains blood-brain barrier and/or endothelial cells lining the choroid plexus of the brain thus leading to cerebral oedema and seizures seen in eclampsia [29].

The signal transduction of VEGFs involves binding to VEGFR1, VEGFR2 and VEGFR3, tyrosine kinase receptors. VEGF-A binds to both VEGFR1 and VEGFR2, whereas PlGF binds only to VEGFR1 [30]. Soluble Flt-1 (sFlt-1) is a splice variant of VEGFR1 (Flt-1) which is produced by a variety of tissues. It contains the extracellular ligand-binding domain but lacks the trans-membrane and cytoplasmic portions of VEGFR1 [23]. Our study showed significantly high levels of sFlt-1 in PE and Ewomen than controls. The sFlt-1 levels were significantly high in E women than PE women. This may be due to the TNF-α mediated up regulatory mechanism. This high level of sFlt-1 might be a reason for reduced level of free VEGF and PlGF in our study. This phenomenon of sFlt-1-VEGF complex formation substantiates the ironical observation in PE "Low circulatory VEGF in conditions of high VEGF mRNA expression" [31].

Our study displayed significantly, low levels of NO, which is a marker for ED in PE and E women than in controls. The levels of NO were significantly low in eclamptic women than in PE women. This observed low level of NO can be explained as a consequence of reduced level of free VEGF as it plays a major role in the expression of Enos [32]. This might have led to decreased vascular tone and hypertension [33].

CONCLUSION

Our study substantiated the fact that oxidative stress, imbalance between anti angiogenic factors (increased sFlt-1 levels) and pro angiogenic factors (suppressed PlGF and VEGF levels) exists in PIH condition. These factors have enormous effects on endothelium. This imbalance is directly related to the ED which is said to be the hallmark of PE. So oxidative stress, VEGF, PlGF and sFlt-1 can be used as markers to analyze the onset and progression of the disease.

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