Original Article

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Relation of Circulatory Levels of Endothelin-1, Antioxidants, and Inflammatory Markers with Varying Blood Pressure Levels in Preeclampsia: A Case-control Study

Biochemistry Section

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ABSTRACT

Introduction: Preeclampsia is a pregnancy disorder with gestational hypertension and proteinuria, it affects maternal-foetal vasculature. The risk of preeclampsia increases upon exposure to different genetic mutations, environmental changes, obesity, gestational diabetes mellitus, multifoetal gestations, and hydatidiform mole. Preeclampsia affects the mother and foetus; its pathogenesis is complex involving inflammation, stress, and molecular factors.

Aim: To measure the circulatory levels of Endothelin-1 (ET-1), Glutathione (GSH), Interleukin-6 (IL-6), and Tumor Necrosis Factor-α (TNF-α) by Enzyme Linked Immunosorbent Assay (ELISA) kit (sandwich) in preeclamptic women and healthy controls.

Materials and Methods: The present study was a case-control study carried out in the Department of Biochemistry at Government Institute of Medical Science (GIMS), Greater Noida, and Santosh Medical College, Ghaziabad, Uttar Pradesh, India, from November 2018 to November 2020. The demographic data was recorded, and blood samples were studied. Preeclamptic women (cases, n=90) were grouped as group A: with BP 140/90-149/99 mmHg, group B: with BP 150/100-159/109 mmHg, and group C: with BP 160/110 mmHg along with controls (n=70). The normotensive pregnant women without family history of preeclampsia were included as controls. The competitive ELISA principles were used to quantify ET-1, GSH, IL-6, and TNF-α levels. One-way Analysis of Variance tests (ANOVA) and Tukey Posthoc Test was carried out to compare the clinical characteristics, ET-1, GSH, IL-6, and TNF-α between the cases and controls.

Results: The demographic data of age, gestational age, and Body Mass Index (BMI) were not significantly different between cases and control. The ET-1 levels were significantly (p-value=0.042) higher in groups (A: 116±79.4, B: 99.2±72, C: 159±96) than controls (54±49). In contrast, GSH levels were low in preeclampsia. In addition, cases had elevated IL-6 and TNF-α levels (IL-6; groups A: 112.3±49.3, B: 127±17.5, C: 324±52.9, and control 40.3±13, TNF-α; groups A: 241±179, B: 200.9±196, C: 391±299 and controls: 167±104). GSH levels decreased with the rise in Blood Pressure (BP), while IL-6 increased significantly with an increase in BP.

Conclusion: Every 10 mmHg increase in BP led to increasing in IL-6 levels. The ET-1 and TNF-alpha levels were increased in all three groups when compared to controls. Similarly, the GSH levels were decreased. Thus, the present study provided evidence that links oxidative stress, inflammation, and a potent vasoconstrictor ET-1 with altered antioxidants (GSH) that led to the pathophysiology of preeclampsia.

Keywords: Glutathione, Interleukin-6, Tumor necrosis factor-α

INTRODUCTION

Preeclampsia is a pregnancy disorder with gestational hypertension and proteinuria, although it affects maternal-foetal vasculature [1]. The risk of preeclampsia increases upon exposure to different genetic mutations, environmental changes, obesity, gestational diabetes mellitus, multifoetal gestations, and hydatidiform mole [2,3]. Pathogenesis of preeclampsia can be aggravated by different mechanisms such as improper placentation, syncytiotrophoblast stress, immunogenic maladaptation at maternal-foetal interphase, genetic predispositions, improper balance of angiogenic and antiangiogenic markers [4]. The early stages of healthy pregnancy begin with the maternal spiral artery reshaping deep into the uterine myometrium. As a result, the spiral artery can transform into wide vessels to accommodate increased blood flow to intervillous space and perfusion to the foetal during embryonic development.

However, in preeclampsia, inadequate maternal spiral artery transformation leads to decreased/turbulent perfusion to the foetus. In addition, the release of microvesicles (apoptotic cell/atheromatous plaques), Hypoxia-Inducible Factor 1-alpha (HIF-1α), reduced antioxidants, and Oxidative Stress (OS) altogether, which further propagates the inflammation and release of cytokines into circulation [5]. Vasoconstrictors, antioxidants and inflammatory markers have their significant roles in preeclampsia. Endothelin-1 (ET-1) is synthesised from proendothelin in the presence of endopeptidases [6]. The endothelial cells are ruptured, which releases ET-1 into circulation. Decreased urinary clearance as well as decreased Nitric Oxide (NO) and prostacyclin (PGI2), leads to vasoconstriction and clinical symptoms of preeclampsia [7].

Glutathione (GSH) is the central cellular thiol redox buffer in cells, which is synthesised by L-glutamate, L-cysteine and glycine in the cytosol. GSH detoxifies many reactions forming glutathione disulphide, which is converted back to GSH by the action of glutathione reductase at the expense of Nicotinamide Adenine Dinucleotide Phosphate (NADPH). It has a role in regenerating vitamins C and E in their active forms [8]. During pregnancy, GSH, vitamin E and C, enzymes such as superoxide dismutase, and GSH peroxidase are responsible for Reactive Oxygen Species (ROS) elimination and its alteration leading to oxidative stress in preeclampsia [9]. During implantation, TNF-α promotes the expression of inflammatory factors by differentiation of endometrial stromal cells to trophoblast acquiring the capacity to

migrate and invade the uterine compartment. Thus, enhances human Embryonic Stem Cell (hESC) secretion of inflammatory cytokines and chemokines that promotes trophoblast migration and invasion [10]. There is impaired remodelling in spiral artery leading to hypoxia. This hypoxic tissue releases proinflammatory cytokines such as TNF-α, IL-6, C-Reactive Protein (CRP), which will further induce OS [11,12].

Literature on the association between GSH, IL-6, TNF-α and ET-1 with an increase in BP among preeclampsia patients is sparse. Hence, this study was planned to measure the circulatory levels of ET-1 (a potent vasoconstrictor), inflammatory markers (IL-6, TNF-α) and antioxidant (GSH) by ELISA kit (sandwich) among preeclampsia patients and find an association between ET-1, IL-6, TNF-α, and GSH with BP change (increase every 10 mmHg) in preeclamptic women.

MATERIALS AND METHODS

The present study was a case-control observational study, which was carried out for two years (November 2018 to November 2020) in the Department of Biochemistry, Government Institute of Medical Science (GIMS), Greater Noida, and Santosh Medical College, Ghaziabad, Uttar Pradesh, India. Institutional Ethics Committee (IEC) clearance from both the teaching institutions {GIMS/IEC/2019/11 and SU/2018/1456(8)} were obtained and signed informed consent was taken prior to the study.

Inclusion criteria: The American College of Obstetricians and Gynaecologists (ACOG) recommendations for the diagnosis of preeclampsia was ≥20 weeks of gestational age, new-onset hypertension (BP ≥140/90 mmHg) and proteinuria [13] were included.

Exclusion criteria: Family history of preeclampsia, history of preeclampsia, tobacco consumption, alcohol consumption, irrespective of parity were excluded from the study.

Control selection criteria: Gestational age ≥20 weeks, normotensive pregnant women without family history of preeclampsia, previous history of preeclampsia, tobacco consumption, alcohol consumption, irrespective of parity, any other chronic disease, and proteinuria were selected as controls.

Sample size calculation: The sample size was obtained by Z power software using the significance level of 0.05, power of study at 80%, Standard deviation for two independent variables, and mean. The sample size derived by the Z power software was 90 for each group.

Procedure

Preeclamptic women (cases, n=90) were categorised into group A: 140/90-149/99 mmHg, group B: 150/100-159/109 mmHg, and group C: ≥160/110 mmHg. The controls (n=70) were included as per control selection criteria. Three mL of blood samples were drawn from all the subjects in a clot activator vacutainer and were centrifuged at 3000 rpm for 10 minutes. Samples were stored in the deep refrigerator (-80°C) until further analysis.

Quantification of serum ET-1 levels, serum GSH levels, serum IL-6 levels, and serum TNF-α levels were measured by Competitive ELISA kit (Puregene, Genetix Biotech Asia Pvt. Ltd., India) as per kit insert and manufacturer's protocol. All samples and standards were processed on ImmunoWash™ 1575 Microplate Washer, iMark Microplate Reader (BIO-RAD, USA).

ET-1 quantification protocol: 50 µL standard was added to the standard labelled wells, and no Biotinylated Detection Ab were added to the standard well. 40 µL of a serum sample was added to a sample labelled well, 10 µL of anti ET1 antibody plus 50 µL of a streptavidin-Horseradish Peroxidase (HRP) sample as well as standard well. The plate was sealed with a sealer and incubated for 60 minutes at 37°C. After incubation, the solution was aspirated by an ELISA washer with 350 µL of wash buffer (five cycles) with a soak cycle for 45 seconds to each well by Immuno Wash™ 1575 Microplate Washer. 50 µL of substrate solution A and B was pipetted to each well. The plate was incubated at 37°C for 10 minutes in the dark. Stop solution (50 µL) was pipetted to each well and determined, and the blue colour turned to yellow immediately. The intensity of yellow colour developed was measured in each well by the iMark Microplate Reader (BIO-RAD, USA) at 450 nm.

Glutathione quantification protocol: Samples (50 µL) and standards (50 µL) were pipetted into respective wells. Immediately Biotinylated Detection Ab working solution (50 µL) was added to each well. The ELISA plate was sealed with a sealer provided in the kit and then the plate was incubated at 37°C for 45 min. After incubation, the solution was aspirated by an ELISA washer with 350 µL of wash buffer with a soak cycle for 2 minutes to each well by ImmunoWash™ 1575 Microplate Washer. HRP conjugate working solution (100 µL) was added to each well. ELISA plate was sealed with a sealer provided in the kit, and then incubated at 37°C for 30 minutes. After incubation, the solution was aspirated by an ELISA washer with 350 µL of wash buffer with a soak cycle for 2 minutes to each well by ImmunoWash™ 1575 Microplate Washer. Substrate (90 µL) was added to each well. Stop solution (50 µL) was pipetted to each well and the OD of each well was determined by iMark Microplate Reader (BIO-RAD, USA) at 450 nm.

IL-6 quantification protocol: Incubation buffer (50 µL) was pipetted into all the wells. 100 µL set pipette was used to dispense of calibrator, control, and sample into the appropriate wells. ELISA plate was incubated for one hour between 18-25°C on a horizontal shaker set at 800 rpm. After incubation, the solution was aspirated by an ELISA washer with 400 µL of wash buffer into each well by ImmunoWash™ 1575 Microplate Washer. ELISA plate was pipetted with 100 µL of anti-IL-6-HRP conjugate and 50 µL specimen was used as diluent into all the wells. ELISA plate was incubated at 22°C for one hour on a horizontal shaker set with 800 rpm. After incubation, the solution was aspirated by an ELISA washer with 400 µL of wash buffer into each well by ImmunoWash™ 1575 Microplate Washer. After washing, the ELISA plate well was pipetted 200 µL of the chromogenic solution into each well within 15 minutes. The whole ELISA plate was incubated at 22°C for 15 minutes on a horizontal shaker set with 800 rpm. Stop solution (100 µL) was pipetted to each well and the OD of each well was determined by iMark Microplate Reader (BIO-RAD, USA) at 450 nm within three hours and the results were calculated.

TNF-α quantification protocol: 50 µL standard was added to the standard labelled wells, and no Biotinylated detection Ab were added to the standard well. 40 µL of a serum sample was added to a sample labelled well, 10 µL of anti TNF-α antibody plus 50 µL of a streptavidin-HRP sample was added in all the wells as well as in standard well. The plate was sealed with a sealer and incubated for 60 minutes at 37°C. After incubation, the solution was aspirated by an ELISA washer with 350 µL of wash buffer (five cycles) with a soak cycle for 45 seconds to each well by ImmunoWash™ 1575 Microplate Washer. 50 µL of substrate solution A and B to each well was added. The plate was incubated at 37°C for 10 minutes in the dark. Stop solution (50 µL) was pipetted to each well, and the blue colour turned to yellow immediately. The yellow colour's intensity was measured in each well by the iMark Microplate Reader (BIO-RAD, USA) at 450 nm.

STATISTICAL ANALYSIS

Statistical analysis was done using International Business Machines (IBM) Statistical Package for the Social Sciences (SPSS) statistics software version 26.0. One-way ANOVA was carried out to compare the clinical characteristic, ET-1, GSH, IL-6, and TNF-α between the cases and control. Tukey Posthoc Test was performed on the preeclampsia (case subgroups) such as A, B, and C and the control. The level of significance considered was p-value<0.05. Clinical characteristics, were shown as Mean±Standard Deviation

(SD). In addition, the Odds Ratio (OR) and 95% confidence intervals were calculated by Multinomial Logistic regression analysis.

RESULTS

The demographic data was similar for case and control group [Table/Fig-1]. Mean ET-1 levels were significantly different in the cases than in control [Table/Fig-1]. However, a Tukey Posthoc Test displayed an insignificant difference in ET-1 levels between subgroups of A, B and C and normotensive control group. Mean GSH levels showed a significant low serum concentration in cases than control. A Tukey Posthoc Test displayed a little distinction in GSH levels between subgroups. Groups A, B, and C showed significant decrease in GSH levels when compared to the controls [Table/Fig-1].

Mean IL-6 levels were significantly elevated in cases than controls. Tukey Posthoc Test displayed a significant difference in IL-6 levels in group A, group B and group C when compared with the normotensive controls. Increased levels of IL-6 were seen in group C than in group B and group A. Groups A, B, and C showed significantly increased IL-6 levels compared to the controls [Table/Fig-1]. Mean TNF-α levels were significantly different in cases and controls. A Tukey Posthoc Test displayed an insignificant difference in TNF-α levels in group A and group B. Similarly, increased levels of TNF-α were seen in group C than in group B. Groups C showed significantly increased TNF-α levels than the controls [Table/Fig-1].

Characteristics	Normal pregnant women (control n=70)	Preeclampsia (A, B, and C, n=30 each)	One way ANOVA p-value	Tukey Posthoc Test (control vs. A, control vs. B, and control vs. C n=30 each)
Age (years)	24.9±3.9	24.2±3.3	0.065	--
		27.2±5.7		
		24.3±3.4		
Gestational Age (weeks)	33±5.6	35.4±4.2	0.156	--
		34.5±4.7		
		35±4.5		
BMI (kg/m^2)	24±3.5	26±3.2	0.071	--
		26±3.4		
		24±2.3		
Proteinuria (Dipstick)	Negative	+3	---	--
		+3		
		+3		
ET-1 (ng/L)	54±49	116±79.4	0.042	0.667
		99.2±72		0.856
		159±96		0.469
GSH (µg/mL)	71±37	22±20	<0.001	<0.001
		19±15		<0.001
		23±10		<0.001
IL-6 (pg/mL)	40.3±13	112.3±49.3	<0.001	<0.001
		127±17.5		<0.001
		324±52.9		<0.001
TNF-α (ng/L)	167±104	241±179	0.002	0.153
		200.9±196		0.907
		391±299		0.002

[Table/Fig-1]: Demographic and biochemical data of study subjects.
Data are depicted as mean±SD for ET-1: Endothelin-1; GSH: Glutathione; IL6: Interleukin-6; TNF-alpha: Tumor necrosis factor-alpha; *p-value <0.05 considered significant; study subjects: normal pregnant women (control) versus (vs.) pre-eclamptic women (three subgroups A: BP 140/90 – 149/99, B: BP 150/100 – 159/109, C: BP ≥160/110 mmHg)

Unadjusted OR were derived for ET-1, GSH, IL-6, and TNF-α [Table/Fig-2]. Mean GSH levels were significantly less than 1, indicating a lower risk factor. In addition, OR for ET-1, IL-6, and TNF-α were more than 1, indicating a higher risk factor [Table/Fig-2].

Parameters	df	Unadjusted OR	95% CI	p-value
ET-1 (ng/L)	1A	1.1	1-1.1	0.185
	1B	1.1	1-1.2	0.029
	1C	1.2	1-1.2	0.127
GSH (µg/mL)	1A	0.915	0.8-0.9	<0.001
	1B	0.902	0.8-0.9	<0.001
	1C	0.909	0.8-0.95	<0.001
IL-6 (pg/mL)	1A	1.2	1-1.2	<0.001
	1B	1.2	1-1.2	<0.001
	1C	1.2	1.1-1.3	<0.001
TNF-α (ng/L)	1A	1.1	1-1.1	0.032
	1B	.9	0.9-1.1	0.400
	1C	1.2	1-1.1	0.001

[Table/Fig-2]: Results of multinomial logistic regression analysed with reference to control subjects.
Data represented as Df: Degrees of freedom, Unadjusted Odds ratio (OR), 95% confidence interval (CI),1 was considered as normal pregnant women, ET-1: Endothelin-1, GSH: Glutathione, IL6: Interleukin 6, TNF alpha: Tumor necrosis factor alpha; p-value<0.05 was considered significant; study groups: normal pregnant women and preeclamptic women (three subgroups are marked as superscript A: BP 140/90-149/99, B: BP 150/100 – 159/109, C; BP ≥160/110 mmHg)

DISCUSSION

Pathophysiology of preeclampsia arises from implantation of the placenta, exposure to risk factors, and syncytiotrophoblast stress, thus leading to improper spiral artery remodelling [4]. Altogether, hypoxic placental tissue may enhance the excessive production of oxidative markers [14]. In addition, hypoxic placental tissue can induce inflammation by different cytokines such as IL-6, TNF-α, IL-1, IL-10, and OS [15,16]. Furthermore, lower Flow-Mediated Dilatation (FMD) was reported by Mannaerts D et al., in preeclamptic women than in non preeclamptic women, which lead to enhanced endothelial dysfunction, vasoconstriction, and hypertension [17].

Further, the protective antioxidant GSH plays a crucial role against ROS. Low GSH and increased Reactive Oxygen Species (ROS) were linked to aetiology of preeclampsia [18,19]. It also protects the vessel wall, thereby preventing high BP, and antioxidant function of glutamate-cysteine ligase [20]. Angiogenic antiangiogenic misbalance elevates BP, thereby excessive release of a potent vasoconstrictor such as ET-1 in maternal circulation study was reported by Amraoui F et al., in mice [21]. Suggestive of ET-1 play a role in BP elevation. Aggarwal PK et al., Lu YP et al., reported plasma ET-1 concentration is elevated in obstructive complications [22,23]. In particular, pregnant women with preeclampsia (hypertensive, proteinuria) have increased plasma ET-1 than normotensive pregnant women [23]. Hypoxia-intermittent perfusion injury, OS, lipotoxicity, and excessive ROS generation are linked to the pathogenesis of preeclampsia. Ferroptosis is iron-dependent programmed cell death with failure of antioxidants (glutathione) defence. Ferroptosis and placenta dysfunction may provide a mechanistic, molecular, and biochemical overview for better insight into trophoblast oxidative stress, lipotoxicity, and placental health [24].

Thus, this study shows an association between ET-1, antioxidants, and inflammation in preeclampsia with a significant increase in ET-1, IL-6, and TNF-α and altered GSH in all three groups. The IL-6, TNF-α and ET-1 was higher than control with decreased GSH levels in preeclampsia than control, which might be a risk factor for PE.

Limitation(s)

The parity of pregnant women was not studied. No data was collected from newborn babies like weight, height, etc. The preeclamptic pregnant women may affect newborn health, therefore demographic and inflammatory markers may help to reduce newborn complications.

Furthermore, studies are warranted to see the beneficial effect of antioxidants and anti-inflammatory drugs.

CONCLUSION(S)

The ET-1, GSH, IL-6, and TNF-α levels are associated in preeclampsia patients. Every 10 mmHg increase in BP led to increasing in IL-6 levels. The ET-1 and TNF-α levels were increased in all three groups when compared to controls. Similarly, the GSH levels were decreased. The present study findings suggest that Oxidative Stress (OS), inflammation, and a potent vasoconstrictor ET-1 with altered antioxidants (GSH) led to the pathophysiology of preeclampsia. The GSH is involved in protection from free radicals' generation in preeclampsia. Hypoxic placental tissue enhances inflammation and decreases GSH. Preeclamptic women's inflammatory status and stress may reduce if BP is maintained within the normal range.

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