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A comparative study of novel biomarkers on preeclampsia in relation to body mass index

Sabir Ali Shaikh*[1], Rajagopalan Vijayaraghavan[2], Das Subir Kumar[1], Pal Manidip[3]

[1]Department of Biochemistry, College of Medicine and JNM Hospital, West Bengal University of Health Sciences, Kalyani, Nadia-741235, India
[2]Saveetha Institute of Medical and Technical Sciences, Saveetha University, Thandalam, Chennai-602105, Tamil Nadu, India
[3]Department of Obstetrics and Gynaecology, College of Medicine and JNM Hospital, West Bengal University of Health Sciences, Kalyani, Nadia-741235, India

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ABSTRACT

Objective of the study was to compare serum level of nitric oxide (NO), endothelial nitric oxide synthase (eNOS), asymmetric dimethylarginine (ADMA), arginine, placental growth factor (PIGF), soluble Fms like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng), malondialdehyde (MDA), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase, in normotensive and preeclamptic pregnancies in relation to body mass index (BMI). The study was done on 100 healthy normotensive and 100 preeclamptic pregnant women. Based on BMI, they were categorized into three groups. Group 1 (<25 kg/m^2), group 2 (25 – 30 kg/m^2) and group 3 (>30 kg/m^2). ADMA, eNOS, arginine, PlGF, sFlt-1, sEng were estimated by ELISA method, and MDA, NO, GPx, SOD, and catalase by spectrophotometric method. Statistically, a significant difference was not observed within normotensive and preeclampsia in relation to BMI. But when respective normotensive was compared with preeclampsia, there was a high increase of ADMA, sFlt-1, sEng, and MDA. A significant decrease was observed in arginine, eNOS, NO, GPx, SOD, and catalase in preeclampsia. This study shows that though preeclampsia is associated with overweight and obesity, but during pregnancy, higher BMI has no additional deleterious effect in preeclampsia.

*Corresponding Author

Name: Sabir Ali Shaikh
Phone: +91- 8017597407
Email: sabirali23@gmail.com

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INTRODUCTION

Preeclampsia (PE) is a pregnancy-related disorder, characterized by sudden onset of hypertension, proteinuria, and vascular dysfunction. PE develops after 20 weeks of pregnancy in women without any systemic illness. It still remains a major cause of maternal and fetal morbidity and mortality. In the affected offspring, it may cause cardiovascular and metabolic dysfunction (Roberts and Cooper, 2001). If PE remains untreated, it may progress to a more complicated condition known as eclampsia. Diabetes mellitus, hypertension, proteinuria, family history, obesity, nulliparity, multiple pregnancies, and thrombotic vascular disease are the risk factors for PE. Preeclamptic women are prone to develop chronic kidney disease and cardiovascular disease in the future (Bellamy *et al.*, 2007).

PE is one of the common pregnancy complications, and it is a multiorgan disease. Organs affected

are endothelium, brain, liver, and kidneys. Delivery is the only curative measure in the case of severe PE, but the foetus may be affected. Numerous factors, including immunological, serum magnesium, early antenatal serum lipid levels, oxidative stress, and genetic factors, play a role for the development of PE (Reddy *et al.*, 2017; Martínez-Varea *et al.*, 2014). Thrombocytopenia one of the clinical features of preeclampsia, drugs like Nifedipine and verapamil enhances the platelets aggregation (Mohammed *et al.*, 2019).

According to a hypothesis, in the early age of the pregnancy, impaired placentation leads to placental dysfunction and hypoxia, which is the basis of the pathogenesis of PE. Improper placentation leads to an imbalance between placentally derived angiogenic and antiangiogenic growth factors, which may be responsible for all the clinical manifestations. Inadequate uterine and placental perfusion responsible for oxidative stress, hypoxia, and subsequent release of antiangiogenic factors in maternal circulation (Huppertz, 2008). It has been suggested that diseased placenta releases anti-angiogenic factors like soluble endoglin (sEng) and soluble Fms like tyrosine kinase-1 (sFlt-1), they inhibits the synthesis of pro-angiogenic factors like placental growth factor (PIGF) and vascular endothelial growth factor (VEGF), results in endothelial dysfunction, hypertensive syndrome and microangiopathy. Early-onset and severity of PE may be assessed by the alteration of sFlt-1 and PIGF in maternal circulation (Romero *et al.*, 2008).

Nitric oxide (NO) modulates placental blood flow, thereby activation of trophoblast and placental development. Clinical manifestations of PE may be due to insufficient NO synthesis or bioavailability (Demir *et al.*, 2012). NO is produced by the endothelial and inducible NO synthases (eNOS and iNOS, respectively). Arginine is converted to NO and citrulline, which regulate the development of embryo, implantation and trophoblast invasion. Despite evidence of NO in the implantation and angiogenesis, its role on vascular development in the placenta is not completely understood and remains to be elusive (Kaufmann *et al.*, 2003). According to a hypothesis, Asymmetric dimethylarginine (ADMA) reversibly inhibits NO production from L-arginine by endogenously suppressing the NOS enzyme, results in reduced vasodilation and endothelial dysfunction. Low levels of ADMA in normotensive pregnant women suggest that it has a role in vascular dilatation. During pregnancy, ratio of L-arginine and ADMA is an important factor for proper endothelial function (Savvidou *et al.*, 2003).

At the end of the first trimester of pregnancy due to increase maternal intraplacental circulation, there is an increased oxygen supply resulting in increased reactive oxygen species (ROS) formation, and normal placenta must adapt to it. In PE, due to poorly perfuse placenta, there is an imbalance between the generation of ROS and intrinsic antioxidant defense mechanisms, which leads to oxidative stress and results in inflammation, vasoconstriction, and reduction of uteroplacental blood flow (Myatt, 2010; Chaiworapongsa *et al.*, 2014). Oxidative stress generates free radicals which attack the polyunsaturated membrane lipids producing malondialdehyde (MDA) (Niedernhofer *et al.*, 2003). How oxidative stress and generation of ROS leads to placental dysfunction remains to be analyzed.

One of the predisposing factors of PE is high body mass index (BMI). Women with an increasing BMI before pregnancy more prone to develop PE compared to normal BMI (Bodnar *et al.*, 2007). In developing and developed countries, obesity is increasing at a faster rate. A British study showed that highly obese women are at a four-fold risk of developing PE compared to normal-weight women (Knight *et al.*, 2010). The incidence of PE is increasing in the United States (Berg *et al.*, 2009). However, studies showing diverging results on obesity and overweight as risk factors.

Hence, there is a need to study in detail and to compare the level of selected biomarkers viz., NO, eNOS, ADMA, arginine, PlGF, sFlt-1, sEng, MDA, GPx, SOD and catalase in normotensive and preeclamptic pregnancies in relation to body mass index. The null hypothesis states that there is no difference in the biomarkers with respect to BMI within normotensive and preeclampsia, though compared to normotensive, the preeclamptic pregnant women will have altered levels.

MATERIALS AND METHODS

Participants of the study

Prior approval was obtained for this investigation from the Institutional Ethics Committee of College of Medicine and Jawaharlal Nehru Memorial Hospital (No.F24/Pr/CMJNMH/IEC/14/93/(5) dated 23 April 2015). The inclusion criteria were women with singleton and uncomplicated pregnancy. For the control group, arterial blood pressure should not exceed 135/85 mmHg and no proteinuria. Exclusion criterion were pregnant women with multiple pregnancies, smoking, alcohol consumption, history of renal, cardiovascular, endocrinological and neurological disorders, liver disease, psychiatric illness, hypertension, and diabetes mellitus. For PE,

no women had any systemic illness and hypertension, 20 weeks before pregnancy. A participant information sheet was prepared in two languages (English and Bengali) about the investigation, and signed consent was obtained from each individual participant. One hundred healthy normotensive and 100 preeclamptic pregnant women were recruited for the study. At the end of the investigation, the 100 normotensives and 100 preeclamptic women were categorized into three groups as per WHO guidelines, based on BMI. Group 1 (BMI less than 25 Kg/m^2), group 2 (BMI 25 to 30 Kg/m^2), and group 3 (BMI more than 30 Kg/m^2). It was a prospective, case-control study. The duration of the study was from May 2015 to April 2018. Maternal age for normotensive and preeclamptic women was 25 to 30 years. Both for normotensive and preeclampsia, first-time pregnant women, were included in this study.

According to American College of Obstetricians and Gynaecologists (ACOG, 2002), PE was diagnosed by an increased of systolic blood pressure more than 140 mmHg and diastolic more than 90 mmHg, measured on two separate times, two hours apart, with proteinuria, 0.3 g in 24 hours urine sample or 1+ proteinuria by dipstick analysis. Pregnant women with PE admitted to the Department of Obstetrics and Gynaecology, College of Medicine and JNM Hospital (Kalyani, Nadia) as per the inclusion and exclusion criteria were the participants.

Biochemical analysis

Immediately after the diagnosis of PE, blood samples were collected from the preeclamptic pregnant women and from normotensive pregnant women during their routine check-up. The gestational age of normotensive and preeclamptic women was 30 to 31 weeks. Blood sample were collected in EDTA-K2 and serum separator vacutainers. EDTA sample was used to prepare hemolysate.

EDTA sample was used for the preparation of erythrocyte suspension according to the method of Beutler *et al.* (1977). The haemolysate was used for assay for glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase. The clot sample divided into two parts; one part was kept for 1 hour at room temperature and then centrifuged at 1500xg for 15 minutes. Serum was separated and stored in -80^0C until analysis, batch-wise for the estimation of MDA, eNOS, ADMA, L-arginine, PlGF, sFlt-1, and sEng. The other part of the clot sample was kept on ice and done centrifugation at 1000xg for 10 minutes. Separated serum was used for the assay of nitric oxide (NO) immediately. Nitric oxide (NO) was assayed using the method described by Griess (1879).

For the estimation of maternal serum eNOS, ADMA, arginine, PlGF, sFlt-1, and sEng, enzyme-linked immunosorbent assay (ELISA) method was used. The respective ELISA system packs were used as per the manufacture's guideline. An instrument used was ELISA washer and reader (Tecan Life Sciences, Sunrise microplate reader, washer, Switzerland).

MDA was estimated by the method of Kei (1978). GPx was measured by the method of Paglia and Valentine (1967). SOD was estimated by the method of Marklund and Marklund (1974). Catalase activity was determined by the method of Góth (1991).

Statistical analysis

Data were expressed as mean ± SEM. One way analysis of variance (ANOVA) was done to test the differences between 3 independent BMI groups of normotensive (control) and preeclampsia, with Student Newman Keul's multiple comparison tests. Unpaired 't' test used to compare the respective BMI groups of control and preeclampsia. Probability of 0.05 or less taken as statistically significant. SigmaPlot 13 (Systat Software, USA) was used for statistical analysis and for graph plotting.

RESULTS AND DISCUSSION

The number of participants in normotensive (control) pregnancy of groups 1, 2, and 3 (BMI <25, 25-30, >30, respectively) were 21, 38, and 41, respectively. The number of participants in preeclamptic pregnancy in group 1, 2, and 3 were 6, 37, and 57, respectively. Statistically, the significant association observed by Chi-square analysis (P <0.001), showing that PE is associated with overweight and obese women.

The mean value of PIGF of control group 1, group 2, and group 3 were 220.1, 234.0, 239.9 pg/mL, respectively. It was statistically significant (P <0.001), showing an increase in PlGF with an increase of BMI. When compared to group 1, there was a statistically significant increase of PlGF in group 2 and group 3. The mean value of PlGF of PE group 1, group 2, and group 3 were 88.3, 84.4, and 88.2 pg/mL, respectively, and was not statistically significant (P = 0.376). But there was a significant decrease of PlGF in PE in all the 3 groups compared to the control. There was 59.9%, 63.9%, and 63.2% decrease of PlGF in PE when compared to the respective control groups (P <0.001) (Figure 1).

The mean value of sFlt-1 of control group 1, 2 and 3 were 1.72, 1.70, 1.71 ng/mL respectively. It was not found to be statistically significant (P = 0.681). Mean value of sFlt-1 of PE group 1, group 2, and group 3

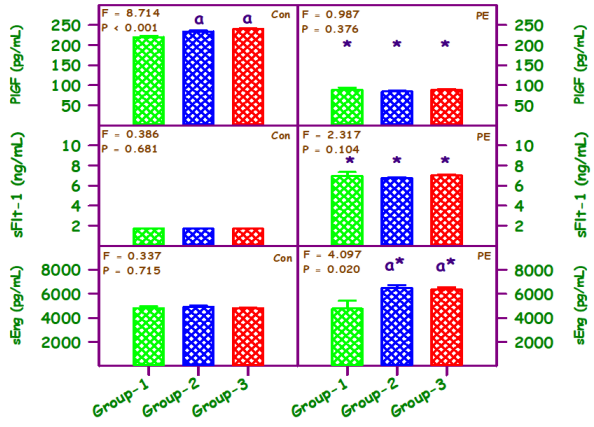

Figure 1: Comparison of placental growth factor (PlGF), soluble FMS like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) in normal pregnant (Con) and preeclampsia (PE) in Group-1, Group-2, and Group-3.^{a}significantly different from Group-1

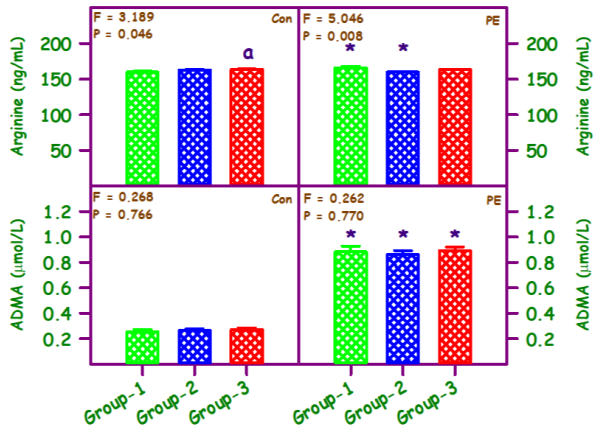

Figure 2: Comparison of arginine and asymmetric dimethylarginine (ADMA) in normal pregnant (Con) and preeclampsia (PE) in Group-1, Group-2, and Group-3.^{a}Significantly different from Group1

were 6.97, 6.73, and 7.02 ng/mL, respectively, and it was also not found to be statistically significant (P = 0.104). But there was an enormous increase of sFlt-1 in PE. There was 4.1, 3.9, and 4.1 fold increase of sFlt-1 in PE when compared to respective control groups (P <0.001) (Figure 1).

The mean value of sEng of control group 1, group 2, and group 3 were 4804, 4907, 4793 pg/mL, respectively. It was not statistically significant (P = 0.715). The mean value of sEng of PE group 1, group 2, and group 3 were 4769, 6500, 6354 pg/mL, respectively. It was found to be statistically significant (P = 0.020). When compared to group1, there was a statistically significant increase of sEng in group 2 and group 3. When compared to control group 1 with PE group 1, it was not significant (P = 0.941). In group 2 and group 3, there was 32.4% and 32.6% increase of sEng in PE when compared to respective control groups (P <0.001) (Figure 1).

The mean value of arginine of control groups 1, 2, and 3 were 159.9, 162.9, 163.7 ng/mL, respectively, and was statistically significant (p=0.04). Group 1 and group 2 were statistically not significant. When compared to group 1, there was a statistically significant increase of arginine in group 3 (P = 0.038). The mean value of arginine of PE group 1, group 2, and group 3 were 165.8, 159.9, 163.3 ng/mL, respectively, and the comparison was statistically significant (P=0.008). However, group 1 and group 2 of PE showed a statistically significant difference when compared with respective control group 1 and group 2 (P = 0.046, P = 0.031 respectively), but group 3 of PE was not statistically significant (P = 702) (Figure 2).

The mean value of ADMA of control group 1, group 2, and group 3 were 0.256, 0.266, 0.277 μmol/L, respectively, and it was not statistically significant (P = 0.766). The mean value of ADMA of PE group 1, group 2, and group 3 were 0.883, 0.862, and 0.892 μmol/L, respectively, and was also not found to be statistically significant (P = 0.770). There was 3.4, 3.2, and 3.2 fold increase of ADMA in PE when compared to respective control groups (P <0.001) (Figure 2).

The mean value of eNOS of control groups 1, 2, and 3 were 183.9, 184.6, 183.8 U/mL, respectively. The comparison was not statistically significant (P = 0.900). The mean value of eNOS of PE of group 1, group 2, and group 3 were 158.7, 157.9, and 157.5 U/mL, respectively, and it was not statistically significant (P = 0.541). There was 13.7%, 14.5% and 14.3 % decrease of eNOS in PE when compared to respective control groups (P <0.001) (Figure 3).

The mean value of control group 1, group 2, and group 3 were 33.5, 34.0, 34.2 μmol/L, respectively. No statistical significant difference was observed (P = 0.623). The mean value of NO of PE group 1, group 2, and group 3 were 21.3, 21.2, and 21.0 μmol/L, respectively, and the difference was not statistically significant (P = 0.567). There was 36.4%, 37.6%, and 38.7% decrease of NO in PE when compared to respective control groups (P <0.001) (Figure 3).

The mean value of MDA of control group 1, group 2, and group 3 were 1.68, 1.68, 1.71 nmol/mL, respectively. No statistically significant difference was observed (P = 0.121). The mean value of MDA of PE group 1, group 2, and group 3 were 3.18, 3.58, and

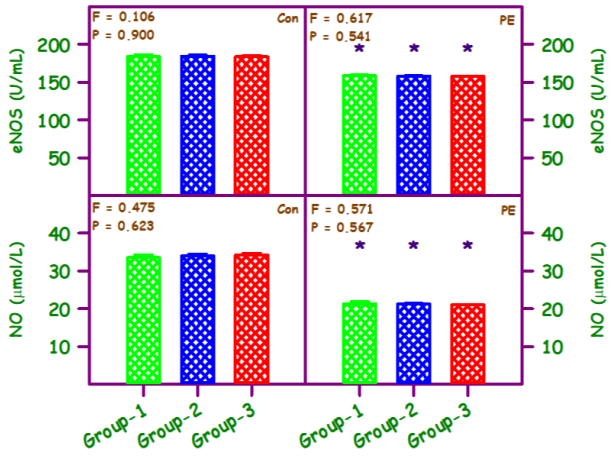

Figure 3: Comparison of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) in normal pregnant (Con) and preeclampsia (PE) in Group-1, Group-2 and Group-3

3.85 nmol/mL, respectively, and was also not found to be statistically significant (P = 0.176). There was 1.9, 2.1, and 2.3 fold increase of MDA in PE when compared to respective controls (P <0.001) (Figure 4).

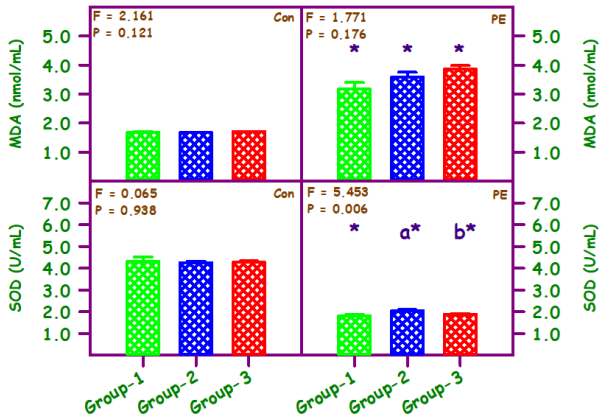

Figure 4: Comparison of malondialdehyde (MDA), superoxide dismutase (SOD) in normal pregnant (Con) and preeclampsia (PE) in Group-1, Group-2, and Group-3. [a]Significantly different from Group-1, [b]Significantly different from Group-2

The mean value of SOD of control groups 1, 2, and 3 were 4.33, 4.26, 4.27 U/mL, respectively. It was not found to be statistically significant (P = 0.938). The mean value of SOD of PE of group 1, group 2, and group 3 were 1.82, 2.05, and 1.89 U/mL, respectively, and was found to be statistically significant (P = 0.006). When compared to group 1 statistically significant increase of SOD observed in group 2. Group 3 showed a significantly low level compared to group 2. There was 58%, 51.9%, and 55.7% decrease of SOD in PE when compared to respective controls (P <0.001) (Figure 4).

The mean value of GPx of control groups 1, 2, and 3 were 28.5, 26.5, 26.7 U/gHb, respectively. It was not found to be statistically significant (P = 0.759). The mean value of GPx of PE group 1, group 2, and group 3 were 14.1, 15.3, and 14.8 U/gHb, respectively, and was also not found to be statistically significant (P = 0.157). There was 50.1%, 42.2% and 44.7% decrease of GPx in PE when compared to respective controls (P <0.001) (Figure 5).

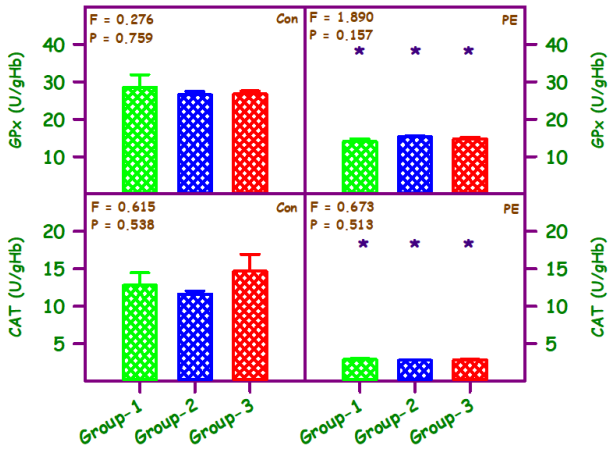

Figure 5: Comparison of glutathione Peroxidase (Gpx), catalase (CAT) in normal pregnant (Con), and preeclampsia (PE) in Group-1, Group-2, and Group-3

The mean value of catalase of control groups 1, 2, and 3 were 12.8, 11.6, 14.7 U/gHb, respectively. It was not statistically significant (P = 0.538). The mean value of catalase of PE group 1, group 2, and group 3 were 2.9, 2.8, and 2.8 U/gHb, and the comparison was not statistically significant (P = 0.513). There was 77.5%, 76.3%, and 80.6 % decrease of catalase in PE when compared to respective controls (P <0.001) (Figure 5).

Maternal weight plays a key role for better pregnancy outcomes. The prevalence of obesity in pregnant women has been on the rise. There are reports that more than 20% of pregnant women fall into the obesity criteria. Studies are suggesting that obesity may be responsible for the progression of PE (Kim *et al.*, 2007). BMI has proposed to be one of the clinical predictors for the development of preeclampsia, but the data is limited.

In the present study, the pro and antiangiogenic growth factors like PlGF, sFlt-1, and sEng were compared with BMI groups 1, 2, and 3 of control and PE. No significant difference was there with respect to PlGF and sFlt-1, but a significant difference was observed in sEng in PE. When compared to group

1, there was a statistically significant increase of sEng in group 2 and group 3. In PE, there is maternal endothelial dysfunction, and obesity increases the risk of venous thromboembolism (VTE) in pregnancy, which may be responsible for the increase of sEng in group 2 and group 3 of PE (Morgan *et al.*, 2017).

The findings regarding the decrease of PlGF, increase of sFlt-1, and increase of sEng in preeclampsia when compared with normotensive pregnancies are in agreement with other studies. One study observed that there was a significant elevation of sFlt-1, sEng, and a significant decrease of PlGF in preeclampsia (Lecarpentier *et al.*, 2016). Another study found low circulating PlGF, and high circulating sFlt-1and sEng in the maternal serum of PE (Chau *et al.*, 2017). The imbalance of these pro and antiangiogenic factors took place at an early stage of pregnancy, which is the basis of the development of PE at a later stage of pregnancy. sFlt1 endogenously inhibits the production of PlGF in the placenta. sFlt1 level increased in maternal circulation five weeks before the onset of PE. High-circulating levels of sFlt1 and sEng have been documented in women with preeclampsia.

BMI groups 1, 2, 3 showed no statistically significant difference in NO, eNOS, ADMA, and L-arginine in normal healthy pregnancy and in preeclampsia. Statistically significant increase of ADMA was observed when respective control groups 1, 2, 3 were compared with PE groups. The level of eNOS and NO was significantly reduced in preeclampsia when compared with respective control groups. Arginine level significantly decreased in groups 1 and 2 in preeclampsia, but in group 3, it was statistically not significant. Decreased level of NO and eNOS in PE is in accordance with other studies (Hodžić *et al.*, 2017). Studies also have shown that there was a decrease of NO level in PE compared with normal healthy pregnant women (Schiessl *et al.*, 2006). In a normal pregnancy, NO maintains the vascular tone and endothelial function. ADMA inhibits NO production leads to endothelial dysfunction and results in PE. The balance between the formation and NO and ADMA is crucial for maintaining a healthy pregnancy (Kielstein and Zoccali, 2005).

In the present study, MDA, GPx, SOD, and catalase were estimated to evaluate oxidative stress in relation to BMI groups of 1, 2, and 3 of control and PE. The results did not show any BMI related statistical significance in MDA, GPx, and catalase, but SOD showed significant change with BMI, in group 2 and group 3 of PE. Decreased SOD level indicates excessive oxidative stress in PE. When respective control groups were compared with PE groups, there was a significant elevation of MDA and a significant decrease of cellular antioxidants GPx, SOD, and catalase in PE. Earlier studies also showed similar findings suggesting that a low level of GPx was associated with PE (Malinova and Paskaleva, 2013). SOD level was also significantly decreased in PE, suggested by earlier studies (Bakacak *et al.*, 2015; Keshavarz *et al.*, 2017). A decrease of catalase in PE compared to control was also in agreement with the other studies (Lucca *et al.*, 2016). An increased level of MDA was observed in PE, established by other studies (Rudra *et al.*, 2006). Most of the above studies were generalized without reference to overweight or obesity.

Less bioavailability of NO and increased production of ROS responsible for platelets adhesion to endothelium and subsequent release of more antiangiogenic factors, the release of cytokines, lipid peroxidation, DNA oxidation which leads to endothelial dysfunction in PE. Imbalance in oxidant and antioxidant, responsible for oxidative stress and results in the generation of free radicals. Free radicals, because of their highly reactive nature on cellular organelles, behave like oxidants. Free radicals attack on polyunsaturated lipids membrane and produce hydroperoxide with subsequent production of MDA. This effect on membrane lipid is termed as lipid peroxidation, and malondiadehyde is measured as the product of free radical injury on membrane lipid. Antioxidants like catalase, SOD, and GPx protect the placental vasculature from ROS and maintain the vascular tone of the placenta. To minimize the cellular oxidative stress in PE, cellular antioxidant enzymes SOD, GPx, and catalase are depleted (Matsubara *et al.*, 2015).

CONCLUSIONS

The present study showed that there was a very high increase in sFlt-1, ADMA, and MDA and a significant increase in sEng. The level of eNOS, NO, arginine, GPx, SOD, and catalase are significantly decreased. This study shows that though preeclampsia is associated with overweight and obesity, but higher BMI has no additional deleterious effect in PE during pregnancy. There is no requirement for weight reduction in overweight and obese women, as there is no aggravation of any of the biochemical parameters in PE, which may affect the pregnancy outcome.

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