Decreased Level of Nitric Oxide in Preeclamptic Pregnancy: A Relationship with PON1 Arylesterase Activity

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Authors' contributions

This work was carried out in collaboration between both authors. Author SDS performed analysis of samples, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author MRM designed the study, read. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of the study was to evaluate the status and diagnostic utility of PON1. (Paraoxonase-1) Arylesterase and nitric oxide as indicator of antioxidant status in preeclampsia.

Study Design: Analytical case control study.

Place and Duration of Study: Sample: Department of obstetrics and gynecology Department, G. M. C. Ambajogai, between July 2010 and July 2012.

Methodology: We conducted a case-control study of 57 women with preeclampsia and 57 women with uncomplicated deliveries. We measured PON1 Arylesterase activity, Nitric oxide and lipid profile.

Results: Serum levels of LDLc (low density lipoprotein cholesterol) are higher in cases than in controls and are statistically significant (p=0.023). However serum HDLc (high density lipoprotein cholesterol) levels are decreased significantly (p = 0.017). Serum PON1 Arylesterase showed significant decrease in cases152.68 KU/L versus controls 180.89 KU/L, p value=0.002. Serum
1. INTRODUCTION

Pre-eclampsia is a multisystem disorder of unknown etiology exclusive to pregnancy. It is a leading cause of adverse pregnancy outcomes worldwide, remains a major cause of maternal and perinatal mortality [1]. It is defined as denovo hypertension (140/90 mmHg) appearing after 20 weeks of gestation accompanied by proteinuria (0.3 g/24 hr) [2]. Today, it is unanimously viewed as a multisystem disorder with vascular dysfunction at its centre in which systemic endothelial dysfunction, platelet aggregation and reduced placental perfusion is the underlying pathology [3]. Human paraoxonase-1 (PON-1) is thought to play a role in preeclampsia and atherosclerosis, mainly through a reduction in low density lipoprotein oxidation [4]. Oxidized low-density lipoprotein (LDL) plays important role in endothelial dysfunction of preeclampsia [5]. A PON-1 enzyme metabolizes pro-inflammatory lipids formed during the oxidation of low density lipoproteins (LDL) and destroys LDL lipid peroxide and therefore, it is considered as antiatherogenic [6]. PON-1 functions in preventing not only lipid peroxidation but also HDL itself [7]. It is known that in a healthy endothelium nitric oxide (NO) rapidly reacts with Homocysteine to form S-nitrosohomocysteine which constitutes a protective mechanism [8]. However, high Homocysteine levels can compromise NO bioavailability inhibiting its regulatory endothelial vascular action thus leading to injury and dysfunction [9]. Increased Homocysteine levels may also decrease NO bioavailability by increasing asymmetric dimethylarginine (ADMA) an analogue of L-arginine which acts as a competitive inhibitor of high Homocysteine levels can compromise NO bioavailability inhibiting its regulatory endothelial vascular action thus leading to injury and dysfunction [10]. This evolutionary perspective raises the question of establishment of interlink between PON1 Arylesterase and NO in pathophysiology of pre-eclampsia hence we planned this study to determine the correlation between PON1 Arylesterase and NO (Nitric Oxide) parameters in preeclampsia.

2. MATERIAL AND METHODS

This is a hospital based case control study. A total of 114 pregnant females were enrolled in this study. 57 patients diagnosed as having Preeclampsia admitted to Medical college Hospital, were selected as cases for this study. Preeclampsia is defined as de novo hypertension (140/90 mmHg) measured on two occasion each 6 hours apart appearing after 20 weeks of gestation accompanied by proteinuria (0.3 g/24 hr). Sample size calculated with values of pilot study arylerase activity mean values and Standard deviation with 95% Confidence interval and 5% error of detection.

Control population consisted of 57 healthy pregnant females matched for age, gender attending the routine health check-up in our outpatient department. Controls No participants smoked, used caffeine or alcohol, and had history of thyroid disease, diabetes mellitus, and hypertension. None of the women from cases and control had a positive medical history of cardiac and metabolic disease. The sample size calculation was based on type I alpha error of 5% and a test power of 80%. Controls No participants smoked, used caffeine or alcohol, and had history of thyroid disease, diabetes mellitus, and hypertension. All women gave informed consent to participate in the study, which had been approved by the institutional Ethics Committee. Exclusion criteria included multiple pregnancies, maternal chronic disease (hypertension, endocrine diseases, connective tissue diseases, thrombophilies, hyperlipidemia, acute or chronic hepatic diseases), The results obtained in the study were evaluated using MYSTAT STATISTICAL PACKAGE at 95% confidence interval and at a significance level of p<0.05.

Blood samples, which were obtained from antecubital veins of the subjects in the patient and control groups. A fasting venous blood sample was collected in the morning from the pre-eclampsia group immediately after the diagnosis before giving any medication and from normal pregnant women at their routine prenatal
visits. The remaining blood was allowed to clot at room temperature in plain bulb for one hour and serum was collected by centrifugation at 1500 x g for 10 minutes which was then used for estimation of PON1 activity [11].

Serum arylesterase activity assay (By Eckerson et al. 1983).

\[
\text{Phenyl Acetate} \xrightarrow{\text{H}_2\text{O}} \text{Acetic Acid} + \text{Phenol}
\]

Procedure:
The assay mixture contains 4.0 mM/L phenylacetate, 1 mM/L CaCl\(_2\) dissolved in 20 mM/L Tris HCl buffer, PH 8.0 at 25\(^\circ\)C. Reaction was initiated by adding 5 µl sample in 3 ml assay mixture. The rate of phenol formation was recorded at 270 nm following 20s lag time. One U of arylesterase activity is equal to 1 mM of phenylacetate hydrolysed per min. The activity is expressed as kU/L, based on the extinction coefficient of phenol of 1310 M\(^-1\)cm\(^{-1}\) at 270 nm, pH 8.0, and 25\(^\circ\)C. Blank samples containing water are used to correct for non-enzymatic hydrolysis.

Calculations

\[
\text{Arylesterase activity (kU/L)} = \Delta A /\text{min} \times 458.778
\]

Serum nitric oxide estimation (nitrate+ nitrite) [12].

Principle:
This assay determines nitric oxide based on the chemical conversion of nitrate to nitrite by vanadium chloride. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on the two-step diazotization reaction in which acidified NO2 produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm.
Chemicals used: \textit{N}-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD), sulfanilamide (SULF), vanadium(III) chloride (VCl3), sodium nitrte, sodium nitrate, sodium iodide (NaI), reduced form glutathione (GSH), hydrochloric acid, phosphoric acid, and glacial acetic acid were all purchased from Sigma–Aldrich and were used without further purification.

2.1 Stock Solutions

Saturated solutions of VCl3 (0.8 g\%) was prepared in 1 M HCl (50 ml). The blue solution was stored in the dark at 4\(^\circ\)C for less than 2 weeks. Development of a lighter blue color indicated oxidation, after which the solution was discarded. Separate solutions for Griess reagent are prepared.

Complete dissolution of NEDD (0.1\% w/v) in H\(_2\)O and SULF (2\% w/v) in 5\% HCl (v/v) required stirring and heating, after which each solution was filtered to remove trace particulates. Both solutions were stable for several months when stored in the dark at 4\(^\circ\)C and were discarded if colored. Solutions of Na\(^+\) nitrite 100 \(\mu\)mol/L in water (50 mg/ml) were prepared fresh daily. ZnSO\(_4\) (1.5 g\%) used for deproteinisation of serum.

Procedure:

<table>
<thead>
<tr>
<th>Test standard</th>
<th>blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>deproteinised serum sample</td>
<td>500</td>
</tr>
<tr>
<td>Sodium Nitrite Standard</td>
<td>500</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>500</td>
</tr>
<tr>
<td>Vanadium Chloride</td>
<td>500</td>
</tr>
<tr>
<td>SULF</td>
<td>250</td>
</tr>
<tr>
<td>NEDD</td>
<td>250</td>
</tr>
</tbody>
</table>

Experiments were performed at room temperature. Nitrate present in serum is reduced ultimately to nitrite with vanadium chloride which is finally estimated with Griess reagent.

After loading the tubes with sample blank values were obtained by substituting diluting medium for Griess reagent. In either case the absorbance at 540 nm was measured using ERBA spectrophotometer following incubation (usually 30–45 min). Linear regression of the mean values of the absorbance at 540 nm for standard set minus the blank values was utilized to determine the nitrite or total nitric oxide concentrations in samples. Final results multiplied by dilution factor.

Lipid parameters:

Serum total cholesterol (By modified Roeschlaus’s method), serum triglycerides (TG) (Method of Wako, modified by McGowan and Fossati), serum hdl cholesterol (HDL-c) (By phosphotungstic acid method)

VLDL-c and LDL-c calculated with Friedewald formula.

VLDL (mg/dl) = TG/5

LDL-c (mg/dl) = Total cholesterol − (HDL-c + VLDL-c)

Serum analytes were estimated by ERBA Smartlab auto analyser. Analysis was performed within 24 hours of sample collection. All chemicals used were of reagent grade.

Results are presented as mean± standard deviation. The continuous variables are tested for normality with Shapiro-Wilk test. Student’s unpaired t test used for statistical analysis between cases and controls for numerical variables in Gaussian distribution. The strength of association between two parameters is expressed by the Pearson’s correlation coefficient. The logistic regression analysis is used for prediction of risk of pre-eclampsia contributed by various risk factors. The three models prepared in the logistic regression for the analysis of data are as follows.

Model 1: total cholesterol, HDL-C, LDL-C, nitric oxide (known risk factors).

Model 2: All parameters in Model 1 + PON1 Arylesterase activity.

At each step, variable in the model is assessed for its contribution to the model reflected by the Naglekerke R2 value and p value of the model. Odds ratios (ORs) and 95\% confidence intervals are calculated. \(p<0.05\) is considered as statistically significant.

3. RESULTS

There were no differences in maternal characteristics between the two groups, with regard to age, number of pregnancies and delivery type.
Mothers participating in the study were predominantly, 20–35 years old. Serum levels of LDLc are higher in cases than in controls and are statistically significant (p=0.023) in pre-eclampsia patients when compared with control group. However serum HDLc levels are decreased significantly (p=0.017). Other lipid parameters are increased in preeclamptics but not statistically significant. Serum PON1 Arylesterase showed significant decrease in cases 152.68 KU/L versus 180.89 KU/L p value=0.002. Serum nitric oxide also showed significant decrease in cases 22.77±4.792 umol/L versus 25.127 umol/L, p =0.010 (Table 2). PON1 Arylesterase activity is found to be positively correlated with serum HDL cholesterol (correlation coefficient r=0.449, p value< 0.001) (Fig. 1). In our study we found significantly positive correlation between PON1 Arylesterase and Nitric oxide level (correlation coefficient r=0.20, p value=0.04) (Fig. 2). Significant association between PON1 Arylesterase activity, and nitric oxide levels, and the risk of preeclampsia identified in univariate regression analysis remain significant after adjustment of other risk factors of preeclampsia, for PON1 (OR=1.012 , p =0.027 ) and for nitric oxide (OR=1.094 , p = 0.038). This finding suggests that, PON1 Arylesterase activities and nitric oxide level are independent predictor of preeclampsia. Multivariate logistic regression analysis demonstrates that low PON1 Arylesterase activity is associated with greatest risk for the development of preeclampsia. Model I, total cholesterol, HDL-C, LDL-C, nitric oxide ($R^2$=0.161, p= 0.006, Area under ROC=0.711) as shown in Table 3 and Fig. 3. In model II All parameters in Model I + PON1 Arylesterase activity ($R^2=0.215$, p = 0.001, Area under ROC=0.745) shown in Table 4 and Fig. 4.

Table 2. Biochemical parameters of pre-eclampsia cases and controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cases</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>23.85±3.38</td>
<td>22.75±2.89</td>
<td>0.057</td>
</tr>
<tr>
<td>T. Cholesterol (mg/dl)</td>
<td>184.78±47.59</td>
<td>169.58±39.36</td>
<td>0.066</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>176.68±47.55</td>
<td>162.17±44.44</td>
<td>0.095</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dl)</td>
<td>33.82±5.99</td>
<td>36.84±7.27</td>
<td>0.017</td>
</tr>
<tr>
<td>VLDL-Cholesterol (mg/dl)</td>
<td>35.29±9.43</td>
<td>32.56±9.09</td>
<td>0.117</td>
</tr>
<tr>
<td>LDL-Cholesterol (mg/dl)</td>
<td>111.78±32.28</td>
<td>98.05±31.20</td>
<td>0.023</td>
</tr>
<tr>
<td>Nitric oxide (umol/L)</td>
<td>22.77±4.792</td>
<td>25.127±4.838</td>
<td>0.010</td>
</tr>
<tr>
<td>PON1 Arylesterase(KU/L)</td>
<td>152.68 ±39.361</td>
<td>180.899±53.763</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Fig. 1. PON1 Arylesterase activity is positively correlated with serum HDL cholesterol ($r=0.449$, p value< 0.001)
Fig. 2. Between PON1 Arylesterase is positive correlated with Nitric oxide level ($r=0.20$, $p$ value=$0.04$)

Table 3. Model 1, Logistic regression analysis (Naglekerke $R^2=0.161$, $p=0.006$)

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Coefficient</th>
<th>Z value</th>
<th>SE</th>
<th>OR(95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-2.681</td>
<td>-1.564</td>
<td>1.714</td>
<td>-</td>
<td>0.118</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>-0.001</td>
<td>-0.070</td>
<td>0.009</td>
<td>0.999(0.982-1.017)</td>
<td>0.944</td>
</tr>
<tr>
<td>HDL-Cholesterol</td>
<td>0.047</td>
<td>1.468</td>
<td>0.032</td>
<td>1.049(0.984-1.117)</td>
<td>0.142</td>
</tr>
<tr>
<td>LDL-Cholesterol</td>
<td>-0.012</td>
<td>-0.957</td>
<td>0.012</td>
<td>0.988(0.965-1.012)</td>
<td>0.339</td>
</tr>
<tr>
<td>Nitric Oxide</td>
<td>0.098</td>
<td>2.318</td>
<td>2.318</td>
<td>1.103(1.015-1.198)</td>
<td><strong>0.020</strong></td>
</tr>
</tbody>
</table>

Fig. 3. Model 1, total cholesterol, HDL-C, LDL-C, nitric oxide (R2=0.161, $p=0.006$, Area under ROC=0.711)
paraoxonase and arylesterase activities in performed a study on the changes of serum pathogenesis of preeclampsia and arylesterase activities may have a role. Findings support those of one study which shows recent studies oxidative stress was considered in preeclampsia is not understood completely, in systematic analyses of women with pre-eclampsia. To the best of our knowledge, the present study is one of the initial studies that make use of all these markers together for the present study. In this case control study, we tested the hypothesis that the relationship between PON1 activity, lipid profile and nitric oxide with the pre-eclampsia differs from normal pregnancy. To the best of our knowledge, the present study is one of the initial studies that make use of all these markers together for the systematic analyses of women with pre-eclampsia. Although the pathogenesis of preeclampsia is not understood completely, in recent studies oxidative stress was considered in the pathogenesis of preeclampsia [13]. Our findings support those of one study which shows an abnormal lipid profile and decreased PON and arylesterase activities may have a role in pathogenesis of preeclampsia [14]. They performed a study on the changes of serum paraoxonase and arylesterase activities in severe preeclamptic women. Decreased PON1 activity would result in decreased protection against lipid peroxidation with increased accumulation of reactive oxygen species within the placenta [15]. Hyperlipidemia, increment in lipid peroxidation product levels, and oxidized lipoproteins are factors that are related with each other and endothelial dysfunction in preeclampsia [16]. Serum lipids have a direct effect on endothelial function and abnormal serum lipid profiles are associated with endothelial dysfunction [17]. Hyperlipidemia of pre-eclampsia may make the components of systemic circulation sensitive to pro-oxidative compounds [18]. In our study we found no significant difference in total cholesterol, VLDLc and TG this may be because of limited sample size. HDLc can prevent the in vitro oxidative modification of LDLc caused both by transition metal ions and by cells [19]. This effect cannot fully be explained by chain-breaking antioxidants present in HDLc. Instead, there are several other mechanisms by

Table 4. Model 2, Logistic regression analysis (Naglekerke $R^2 =0.215$, $p= 0.001$)

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Coefficient</th>
<th>Z value</th>
<th>SE</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>constant</td>
<td>-2.902</td>
<td>-1.657</td>
<td>1.755</td>
<td></td>
<td>0.098</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>-0.002</td>
<td>-0.182</td>
<td>0.009</td>
<td>0.998(0.980-1.017)</td>
<td>0.856</td>
</tr>
<tr>
<td>HDL-Cholesterol</td>
<td>0.013</td>
<td>0.358</td>
<td>0.036</td>
<td>1.013(0.944-1.087)</td>
<td>0.720</td>
</tr>
<tr>
<td>LDL-Cholesterol</td>
<td>-0.012</td>
<td>-0.983</td>
<td>0.013</td>
<td>0.988(0.963-1.012)</td>
<td>0.326</td>
</tr>
<tr>
<td>Nitric Oxide</td>
<td>0.090</td>
<td>2.079</td>
<td>0.043</td>
<td>1.094(1.005-1.191)</td>
<td>0.038</td>
</tr>
<tr>
<td>PON1 Arylesterase</td>
<td>0.005</td>
<td>2.211</td>
<td>0.005</td>
<td>1.012(1.001-1.022)</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Fig. 4. Model 2 All parameters in Model 1 + PON1 Arylesterase activity ($R^2 =0.215$, $p= 0.001$, Area under ROC=0.745)

4. DISCUSSION

In this case control study, we tested the hypothesis that the relationship between PON1 arylesterase activity, lipid profile and nitric oxide with the pre-eclampsia differs from normal pregnancy. To the best of our knowledge, the present study is one of the initial studies that make use of all these markers together for the systematic analyses of women with pre-eclampsia. Although the pathogenesis of preeclampsia is not understood completely, in recent studies oxidative stress was considered in the pathogenesis of preeclampsia [13]. Our findings support those of one study which shows an abnormal lipid profile and decreased PON and arylesterase activities may have a role in pathogenesis of preeclampsia [14]. They performed a study on the changes of serum paraoxonase and arylesterase activities in severe preeclamptic women. Decreased PON1 activity would result in decreased protection against lipid peroxidation with increased accumulation of reactive oxygen species within the placenta [15]. Hyperlipidemia, increment in lipid peroxidation product levels, and oxidized lipoproteins are factors that are related with each other and endothelial dysfunction in preeclampsia [16]. Serum lipids have a direct effect on endothelial function and abnormal serum lipid profiles are associated with endothelial dysfunction [17]. Hyperlipidemia of pre-eclampsia may make the components of systemic circulation sensitive to pro-oxidative compounds [18]. In our study we found no significant difference in total cholesterol, VLDLc and TG this may be because of limited sample size. HDLc can prevent the in vitro oxidative modification of LDLc caused both by transition metal ions and by cells [19]. This effect cannot fully be explained by chain-breaking antioxidants present in HDLc. Instead, there are several other mechanisms by
which HDLc can inhibit either the formation or the atherogenic properties of ox-LDLc first of these includes an exchange of lipid peroxidation products between HDLc and LDLc [20].

Cholesterol accumulation and foam cell formation are the hallmark of early atherogenesis, paraoxonase inhibitory effect on macrophage cholesterol biosynthesis and on macrophage oxidative stress may have important antiatherosclerotic implications [21]. Several studies demonstrated that the activity of serum paraoxonase1 is decreased in preeclamptic patients than normotensive pregnant women and the possible role is explained by various mechanisms such as increased homocysteine concentration and increased lipid peroxidation in preeclampsia [22-23]. PON1 is a multifunctional antioxidant enzyme that not only can destroy oxidized lowdensity lipoprotein (ox-LDL), but can also detoxify the homocysteine metabolite, homocysteine thiolactone [24]. Homocysteine (Hcy) or its metabolites are associated with endothelial dysfunction and cardiovascular disease, hyperhomocysteinaemia was suggested to have a role in promoting endothelial dysfunction in pre-eclampsia [25]. Homocysteine thiolactone is considered as a natural substrate for PON1 enzyme. [26] It is known that PON is bound to HDLc [27] and acts as an antioxidant that protects LDLc from oxidative modifications and can reduce oxidized lipids in oxidized lipoproteins [28].

Controversial finding is observed in a study which showed that midgestational paraoxonase 1 activity is higher in women with preeclampsia before clinical signs of the disease are present [29]. The reductions in the activities of PON found in the present study and their relationship to HDL-cholesterol suggest that the decrease of these serum enzymes may participate in the pathogenesis of preeclampsia through predisposition to lipid peroxidation and increased susceptibility to oxidation of LDL-cholesterol. One possible explanation is that serum PON and arylesterase activities may be lowered as a result of an altered synthesis or secretion of HDL-cholesterol. These alterations may be due to the result of damaged liver cells which are unable to express PON, and have already been linked to decreases of activity as suggested by inhibition of microsomal PON activity in rats with experimental cirrhosis [30].

One study stated that PON1 variants seem to play an important role in vascular response and serum nitrate homeostasis in AMI patients and in serum lipids variations in young adults [31]. In our study we found significantly positive correlation between PON1 Arylesterase and Nitric oxide level, and nitric oxide values are decreased significantly in preeclamptics than control. The conclusions regarding the association between nitric oxide and preeclampsia are conflicting decreased levels found in various studies are in aggrement with our result i.e. decreased serum nitric oxide in preeclampsics compared to controls [32]. Nitric oxide (NO) mediates many functions of the endothelium, including vasodilatation and inhibition of platelet aggregation [33].

5. CONCLUSION

Our observed results showing decrease in the antioxidant PON1 arylesterase activity point towards their role in the pathogenesis of preeclampsia. Antioxidant support might be helpful to eliminate oxidative stress in preeclampsia. Further investigations are necessary to define the association between PON1 activities and preeclampsia.

CONSENT

All authors declare that ‘written informed consent was obtained from the Patients’.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the institutional ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


