HIF-1α, TNF-α and HO-1 Modulation in Placental Explants during Preeclampsia

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Abstract: Preeclampsia is a hypertension disorder associated with shallow trophoblast invasion which results in placental hypoxia. This initiates the cascade of events which ultimately results in exacerbated inflammation with lacking compensatory mechanism. This is understood to play a vital role in fetus development. The study aims to analysis the expression of Hypoxia inducible factor-1α (HIF-1α), Tumor necrosis factor-α (TNF-α) and Heme oxygenase-1 (HO-1) in both placental tissue and explant during preeclampsia. In this study, 10 placental samples were collected from normotensive and preeclamptic pregnant women. The viability of placental tissue and explant were assessed by LDH release and the antioxidant status was measured by superoxide dismutase (SOD). The structural morphology observed by transmission electron microscopy (TEM), tissue damage was monitored by the expression of HIF-1α, TNF-α through immunohistochemistry and expression of HO-1 by ELISA. The level of lactate dehydrogenase (LDH) release (p<0.05) was significantly increased during preeclampsia. The level of SOD (p<0.001) was significantly reduced in both preeclamptic placental tissues and explants. Alteration in the structural integrity was observed in preeclamptic placental samples using TEM. HIF-1α, TNF-α expression (p<0.05) were increased, with significant reduction in the expression of HO-1 (p<0.001) in both placental tissue and explant from pregnancies complicated with preeclampsia, compared with normotensive groups. Our results indicate that differential expression of HIF-1α, TNF-α and HO-1 may play a vital role in the progression of preeclampsia. These alterations are known for its significance during trophoblast invasion which is crucial for early human development.

Keywords: Heme oxygenase-1 (HO-1), Hypoxia inducible factor-1α (HIF-1α), Placenta, Preeclampsia, Tumor necrosis factor-α (TNF-α).

Introduction
Preeclampsia is a clinical syndrome defined as the new onset of hypertension and proteinuria during the second half of pregnancy [1]. It afflicts 3% to 5% of pregnancies and is a leading cause of materno-fetal morbidity and mortality [2, 3]. Placenta is the central organ characterized by tightly regulated trophoblast differentiation events during pregnancy. Stress arises from placenta through fluctuations in oxygenation [4]. It may lead to predisposing feature of reduced trophoblast invasion and incomplete conversion of the uterine spiral arteries [5, 6]. It results in poor placental perfusion and lead to placental and fetal hypoxia [7]. Hypoxia is a potent stimulus for endothelial dysfunction and during pregnancy it promotes pregnancy related disorders like preeclampsia, intrauterine growth retardation, fetal growth restriction etc. Placento-fetal hypoxia may therefore provide a suitable stimulus linking the adverse effects of preeclampsia on the vascular physiology of the mother, placenta and unborn child [8]. Failure of the oxygen-associated developmental events may contribute the expression of HIF-1α in placenta during pregnancy related disease like preeclampsia [9, 10].

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that plays a key role in mediating cellular and systemic responses to hypoxia [11]. It is a heterodimeric transcription factor consisting of 2 subunits α, an oxygen-sensitive and β, constitutively active [12]. HIF-1α is highly expressed in the low oxygen environment of the placenta in early gestation, playing an important role in placental development and function [13]. Alteration in the placental expression
of HIF-1α is believed to play a key role in preeclampsia\cite{14}.

The major research efforts have concentrated on identifying factors for the initiation and maintenance of the pathological processes in preeclampsia. Recently, it was suggested that preeclampsia is a disease of exacerbation of an inflammatory process with diminished defense mechanism\cite{15,16}. Inflammation is part of the complex biological response of vascular tissues to harmful stimuli \cite{17}. Progressive destruction of the tissue would compromise the survival of the organism. An inflammatory response is usually accompanied by increasing concentrations of proinflammatory cytokines, acute-phase proteins and may involve endothelial activation.

Hypoxia promotes excess production of placental tumor necrosis factor (TNF-α), a proinflammatory cytokine factor \cite{18}, and has a potential cytotoxic effect to vascular endothelial cells, trophoblastic cells of placenta \cite{19}. It induces apoptosis, inhibits proliferation of trophoblast cell \cite{20}, upregulates endothelial expression of platelet derived growth factor, endothelin-1 and plasminogen activator inhibitor-1, all of which are associated with vasoconstriction \cite{21}. The level of TNF-α in the maternal circulation is increased prior to the clinical manifestation of preeclampsia \cite{22}.

The cells apart from enhancing the high molecular weight HSPs, the low molecular weight HSPs like HSP32 (HO-1) also induced during hypoxia. However, the role of such low molecular weight HSPs in survival mechanism during hypoxia is not established. Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in heme catabolism with antioxidant, anti-inflammatory and anti-apoptotic function \cite{23}. It is a stress protein induced by cytokines, intake of heavy metals, hypoxia, metals, heme, hormones and oxygen-free radicals \cite{24}. It converts the pro-oxidant heme to biliverdin, which is then rapidly converted by biliverdin reductase to bilirubin, a known antioxidant \cite{25}. As a side product, HO-1 releases carbon monoxide, a vasodilator \cite{26} that has been implicated in defense mechanisms against agents that may induce oxidative injury \cite{27}. The expression of HO-1 in relation to oxidative stress generated in preeclamptic condition suggests their protective role in stress management prolonging the cell survival during early fetal development.

In this context, the current study was aimed to analyze the viability, antioxidant status, expression of HIF1-α, HO-1 and TNF-α in placental tissue and explant during preeclampsia. This may provide a new insight for pharmacological manipulation that may have therapeutic relevance.

**Material and Methods**

**Selection of subjects:** Patient registered in a public sector hospital in Chennai were enrolled in this study. Clearance was obtained from Institute Ethical Committee (IEC/A/BWC/001102/2010) prior to the commencement of study and the informed consent was received from all the subjects. Placenta was collected from both normal (n=10) and preeclamptic (n=10) pregnant women in the age group of 20-40 years, post delivery. Patients with preeclampsia were defined on the basis of the following laboratory criteria: blood pressure >140/90 mmHg but <160/110 mmHg, proteinuria >300 mg/L and xanthine oxidase activity of approximately 2.6 units/ mg protein \cite{28}. Patients with severe preeclampsia and other severe maternal complications were excluded from the study.

**Preparation of explants:** The collected placenta was washed with ice cold PBS buffer and was stored at 4°C in HEPES buffer physiological salt solution (pH 7.4) having the following composition (in mmol/L): HEPES 10, NaCl 139, KCl 5, CaCl\(_2\) 1, MgCl\(_2\) 1, glucose 4.2 and 0.5% (w/v) dialyzed albumin until use. The explants were cultured for 3 days as described by Yacobi et al. 2002 \cite{29} with slight modifications. The placental tissue (villi) was dissected from the fetal membranes from both normotensive and preeclamptic subjects and about 10 mg of placenta were cut into 4 pieces, transferred to the Millicell–CM separate culture dish inserts which was layered with polymerized Matrigel. Medium (Dulbecco’s Modified Eagle’s Medium) supplemented with L-glutamine (2 mM), sodium pyruvate (1mM), antibiotics/antimycotic (10000 U penicillin, 10mg streptomycin, 25µg amphotericin B/ mL in 0.9% saline) supplied by Himedia (Mumbai, India), fetal bovine serum (10%) were added to all the culture dish. Culture plates were incubated overnight in 5% CO\(_2\). The medium from all the culture dishes were changed after every 24 hrs following the beginning of the experiment, and the collected media were stored at -20°C until processing.

**Estimation of protein:** The placental tissue and cultured explants from normotensive and preeclampsia were pooled and recovered by lysing with lysis buffer (0.1 M Tris, 38 mM glycine, 2 mM EDTA, 2 mM N-ethylmaleimide, 2 mM iodoacetic acid, and 0.4 mM phenylmethysulfonylfluoride pH 8.7). The cell suspension was incubated for 30 minutes at 4°C, with occasional shaking and centrifuged at 15,000Xg for 15 minutes to remove cellular debris. The supernatant was washed with ice cold PBS buffer and was stored at 4°C. The level of TNF-α in the maternal circulation is increased prior to the clinical manifestation of preeclampsia \cite{22}.
Lactate dehydrogenase (LDH) cytotoxicity assay: Assessment of explants viability was routinely monitored by measuring the release of LDH into medium relative to a 1% Triton X-100 (Sigma)-lysed positive control. The TOX-7 viability kit supplied by Sigma- Aldrich (Mumbai, India) was used. The lactate dehydrogenase assay mixture was freshly prepared by mixing equal volume of LDH assay substrate solution, LDH assay dye solution and 1x LDH cofactor preparation. An aliquot of the medium was removed (half of the volume of the culture medium) for testing and twice the volume of lactate dehydrogenase assay mixture was added to it. The plate was covered with an opaque material to protect from light and incubated at room temperature for 20-30 minutes. The reaction was terminated by adding 1/10 volume of 1N HCl and the absorbance was read at 490nm.

Assay of superoxide dismutase: The activity of SOD was estimated by monitoring the oxidation of epinephrine according to the procedure of Misra and Fridovich, 1972. The reaction mixture contained 2.5mL carbonate buffer and 0.5mL of EDTA solution. Suitably diluted sample lysates were added and the change in absorbance was monitored after adding 0.5mL of epinephrine at 420 nm for 2 minutes at 15 seconds intervals. Auto oxidation of epinephrine was also monitored in the reaction mixture without adding the enzyme. The activity was expressed as Units/minute/mg protein.

TEM analysis of placental explants: Placental tissue and explant sections were fixed for transmission electron microscopy (TEM) with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C. The tissue remained in this primary fixative for 2 days. After primary fixation, the samples were washed in sodium cacodylate buffer and post fixed in 1 % osmium tetroxide for 1 h at room temperature in sodium cacodylate buffer and post fixed in 1 % osmium tetroxide for 1 h at room temperature in sodium cacodylate buffer. Fixation was followed by dehydration of samples by ascending series of graded alcohol (10-100%) and propylene oxide. The samples were infiltrated, embedded in siliconized rubber mold with epoxy resin, and incubated at 60°C for 48 hrs, for the preparation of blocks for sectioning. Thick sections (1 µm) were cut through ultramicrotome (Leica ultracut UCT) with glass knife and stained with toluidine blue dye. The sections were then examined by light microscopy to select areas for fine structural study and photomicrography. Ultrathin sections (below 100 nm) were cut through ultramicrotome (Leica) with diamond knife (Diatome). The ultrathin sections were taken on copper grid and stained with 2% alcoholic uranyl acetate and Reynold’s lead citrate solution. The samples were viewed at 80 kV with an electron microscope (201C; Philips Electronic Instruments, Inc., Mahwah, NJ).

Immuno histochemical analysis for localization of HIF-1α and TNF-α: Immunohistochemical analysis of placental tissue and explant were performed by the method of Sternberg et al. 1970. Formalin-fixed, paraffin embedded placental samples were processed using an immunohistochemical technique with mouse monoclonal anti-HIF-1α (ADL-OSA-602-E, Biogenuix) and mouse monoclonal anti- TNF-α antibodies (BPD-HYB-141-08, Biogenuix). Deparaffinized and rehydrated sections were incubated in 3% hydrogen peroxide (H₂O₂) in absolute methanol for five minutes in order to inhibit endogenous peroxidase activity, then rinsed in 0.05 M tris-buffered saline (TBS), pH 7.6, for 5 min. Antigen retrieval was performed by heat treating sections in citrate buffer at pH 6 in a microwave oven for 5 min (3 cycles). To reduce non-specific binding, slides were incubated in 10% normal goat serum for 10 min at room temperature before 1 h incubation with (2:250) antibodies in a humidified chamber at 4°C. After rinsing with TBS, biotinylated secondary link antibodies and streptavidin-peroxidase conjugate were added sequentially and the specimen was incubated at room temperature for 30 min in each of them. Peroxidase activity was detected using 0.1% H₂O₂ in 3, 3′-diaminobenzidine (DAB) solution applied to the tissue sections for 5 min, which were then counterstained with hematoxylin for 5 seconds before rinsing, dehydrating and mounting with coverslips using xylene and DPX mountant. The immunohistochemical images were acquired with Olympus (MLXitR, Olympus). The intensity of the expression was assessed using the Magnus Pro Software (CH 20i).

Quantification of HO-1 by ELISA technique: The inducible form of HO-1 in the placental tissue and explant was quantified using HO-1 ELISA kit (ADL-EKS-800, Biogenuix) according to the manufacturer’s instruction.

Statistical Analysis: All results were expressed as mean value ± standard deviation. Each experiment was performed thrice and statistical analysis of the data was carried out using SPSS 7.5 version package. Statistical significance was arrived by comparing the results of preeclamptic placental tissue and explant with the normotensive tissue and explant using Student ‘t’ test. Differences were taken to be statistically significant for values of p<0.05 and p<0.001.

Results and Discussion

One of the most prevalent complications of pregnancy is preeclampsia, a hypertensive disorder which is a leading cause for maternal and prenatal morbidity and premature birth with no effective pharmacological intervention. While the underlying cause is unclear, it is believed that placental ischemia/hypoxia induce the cytotoxic factors into the...
maternal vasculature leading to widespread maternal endothelial dysfunction \cite{35}.

Earlier studies have demonstrated that endothelial dysfunction is one of the key factors in complicating preeclampsia which results in preterm birth \cite{36}. In the present study the influence of hypoxia on stress proteins like HIF-1α, HO-1 and inflammatory protein like TNF-α in both normotensive, preeclamptic placental tissue and explant were elucidated. The explant study is used to maintain the placental tissue in an artificial condition. The changes in explants will reflect the status of stress in entire placenta \textit{in utero}. The placental explants were grown for a period of 3 days in DMEM. The LDH release in culture medium was used to measure the viability in both groups of placental explant (Figure 1). In preeclampsia, considerable cell damage resulted in significant increase in the release of LDH (*p<0.05) than normotensive placental tissue (0hr). There was a negligible difference in the level of LDH between the tissue (0hr) and the explant (24hrs). The viability of placental explants assessed immediately after culture showed a significant increase (p<0.05) in the release of LDH during preeclampsia than the normotensive subjects (Figure 1).

![Figure 1: Viability of placental tissue and explant by TOX-7 kit method](image1.png)

Each bar represents mean ±SD (for 10 samples in each group)

* p<0.05 when compared with normotensive placental tissues (0hr); # Not significant when compared with normotensive placental tissues (0hr); $\ddagger$ p<0.05 when compared with normotensive placental explant; $\ddagger$ Not significant when compared with preeclamptic placental tissues (0hr)

The contribution of stress to preeclampsia has been accepted, although the exact changes in antioxidants/oxidants are still controversial \cite{37}. In our study, we found significantly lower level for SOD (p<0.001) in preeclamptic placental tissue by 54% and explant by 55%, when compared with normotensive placental tissue and explant (Figure 2). This result may contribute to alterations in the oxidant and antioxidant status.

![Figure 2: Activity of superoxide dismutase in the placental tissue and explant of normotensive and preeclamptic women](image2.png)

Figure 2: Activity of superoxide dismutase in the placental tissue and explant of normotensive and preeclamptic women

Values are expressed as mean ± SD (for 10 samples in each group)

* Not significant when compared with normotensive placental tissue; **p<0.001 when compared with normotensive placental tissue and explant

The failure of the uterine vasculature to undergo adequate physiological remodeling in women with preeclampsia is due to imbalance in the oxidant-antioxidant \cite{38}. This ultimately may lead to placental ischemia/hypoxia. In this study, we observed a significant increase in the expression of HIF-1α in preeclampsia due to the alteration in oxygen tension (Figure 4, Panel-B, D) than the normotensive (Figure 4, Panel-A, C). This altered expression of HIF-1α acts as a potent inducer of inflammatory cytokine production particularly TNF-α. This may indicate that preeclamptic placenta respond to hypoxia with inadequate defense mechanisms, resulting in the over production of TNF-α thereby contributing the maternal intravascular dysfunction.
Figure 3: Transmission electron microscopic images of both placental tissue and explant from normotensive tissue (Panel A); preeclamptic tissue (Panel B); normotensive explant (Panel C) and preeclamptic explant (Panel D). 10X

Figure 4: Immunohistochemical analysis of HIF-1α in placental tissue and explant from both normotensive tissue (Panel A); preeclamptic tissue (Panel B); normotensive explant (Panel C) and preeclamptic explant (Panel D). Scale 5 µm
Inflammatory cytokines are notorious for affecting the placental endothelium during preeclampsia. We observed significantly higher levels of inflammatory cytokine TNF-α in preeclamptic women (Figure 5, Panel-B, D), when compared with normal pregnant women (Figure 5, Panel-A, C). These results are consistent with our hypothesis that placental response to hypoxia may be secondary to improper trophoblast invasion. This may trigger increased secretion of TNF-α with diminished HO-1 production and lead to the activation of maternal endothelial cells during preeclampsia.

A number of studies suggest that HO-1 acts as a potent endogenous factor for the resolution of stress induced inflammatory injury. The present study provides sufficient evidence for the hypothesis that preeclampsia is a disease in which the vascular protective factor like HO-1 that normally counteract the inflammatory process are defective. HO-1 protein expression is reduced (p<0.001) by 47% in both placental tissue and explant obtained from pregnancies complicated by preeclampsia (Figure 6). This suggests that HO protective pathway may be defective for counteracting physiopathological insults in placenta. Reduced utero-placental circulation may lead to poor placental perfusion which results in local hypoxia and secretion of cytotoxic factors into the maternal circulation. These factors can cause widespread circulatory disturbances secondary to endothelial dysfunction. Thus hypoxia along with inflammatory changes may impair the trophoblast invasion, which might contribute to pathological conditions of preeclampsia.

Figure 5: Immunohistochemical analysis of TNF-α in placental tissue and explant from both normotensive tissue (Panel A); preeclamptic tissue (Panel B); normotensive explant (Panel C) and preeclamptic explant (Panel D). Scale 5 µm

Figure 6: Expression of HO-1 in the normotensive and preeclamptic placental tissue and explant

*Not significant when compared with normotensive placental tissue; **p<0.001 when compared with normotensive placental tissue and explants.
Conclusion

Our present study reveals that preeclampsia could be a disease of the exacerbation of hypoxia and inflammatory response with lacking compensatory system which induces the expression of HIF-1α and TNF-α with reduction in preeclamptic placental HO-1 expression. This suggests that the impairment of HO-1 activation may predispose the placenta to cellular injury and subsequent maternal endothelial cell activation which release TNF-α during hypoxic condition of preeclampsia. Our results provide molecular evidence that aberrant placental protein expression changes in preeclampsia may be due to reduced oxygenation. Thus the hypoxic implications in terms of inflammatory changes must be carefully monitored during pregnancy to prevent progressive maternal and neonatal complications.

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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