

Differential Expression of Heat Shock Proteins and Inflammatory Changes in Preeclamptic Placental Explants

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Abstract: Preeclampsia, pregnancy related disorder is associated with increased OS leading to exacerbated inflammation and lipids alteration. Preeclamptic placentas are more susceptible to ROS mediated apoptosis causing preterm delivery due to enhanced oxidative stress. Explant culture is a process of tissue which reflects the status of stress in entire placenta in-utero. Apart from anti-apoptotic function, heat shock proteins sustain fetal development during preeclampsia by acting as anti-inflammatory protein. Thus the purpose of this study is to explore oxidation of free radical (4-HNE), cytokines expression, lipid profile and expression of HSP70 and HSP90 α in both normotensive and preeclamptic placental explants for first time. Level of 4-HNE, NF κ B, TNF α , HSP70 and HSP90 α were significantly increased with significant decrease in total cholesterol, TG, VLDL, LDL in preeclamptic placental explants. There was an insignificant change in HDL level between preeclamptic and normotensive placental explants. Increase in HSP70 and HSP90 α expression indicates the existence of protective mechanisms initiated against generated ROS produced by altered lipid profile and may promote live fetal delivery during preeclampsia. Thus the study emphasizes the understanding of inflammatory and lipid profile changes during preeclampsia. This may provide substantial insight into the nature of preeclampsia and to prevent progressive maternal and neonatal complications.

Keywords: Preeclampsia, Oxidative Stress (OS), Cytokines, Lipids, Stress proteins

1. Introduction

Preeclampsia is one of the most common and potentially life-threatening complications of pregnancy. It contributes significantly to materno-fetal mortality and morbidity. Preeclampsia is associated with reduced placental perfusion with diverse maternal factors that alter endothelial function (Figure 1) which contribute to hypertension, proteinuria and edema (1). Thus placenta plays a major role in the pathogenesis of preeclampsia. Placental ischemia will be impaired to the extent, generating commensurate placental oxidative stress due to formation of oxygen radicals and other reactive oxygen species (ROS) that is a major contributory factor to preeclampsia (2). Preeclampsia is a manifestation of all changes in placental cell. Placental explants contain syncytiotrophoblast, trophoblast, cytotrophoblast and extra villous trophoblast (3). Placental explant cultures *in-vitro* are useful for studying tissue functions including cellular uptake, production and release of secretory components, cell interactions, proliferation, growth and differentiation, gene delivery, pharmacology, toxicology, and disease processes (4) (5).

Oxidative stress is associated with preeclampsia where free radical reactions compromise the defensive functioning of the vascular endothelium (6). The highly reactive primary product such as lipid peroxide and hydroperoxides are formed when free radicals attack polyunsaturated fatty acids or cholesterol in membranes or lipoproteins (7). 4-Hydroxynonenal (4-HNE), an aldehydic end product of lipid peroxidation, is cytotoxic when cells are exposed to various oxidative insults (8). Hence it can be used as a biomarker for the extent of lipid peroxidation. 4-HNE can react through

both non-enzymatic and enzyme catalyzed reactions to modify a number of cellular components, including inhibition of protein and DNA synthesis, inactivation of enzymes, stimulation of phospholipase C, reduction of gap-junction communication, and stimulation of neutrophil migration (8) (9). It may also mediate disturbance of the maternal vascular endothelium (10), which would have implications for the development of cardiovascular disease in both mother and fetus (11).

Inflammation is a host response triggered by noxious stimuli arising during tissue injury. Inflammatory cytokines are known to be potent activators of vascular endothelium. They have been proposed as mediators of trophoblast cell death and endothelial dysfunction during preeclampsia (12). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) is a transcription factor, activated in a wide range of human diseases that are associated with increased oxidative stress and inflammation (13). Incorrect regulation of NF κ B has been linked to cancer, inflammatory and autoimmune disease, septic shock and improper immune development (14) (15). In unstimulated cell, NF κ B is found to be associated with I κ B in the cytoplasm (16). In response to stress stimuli I κ B gets phosphorylated and dissociated. The free NF κ B gets dimerized (Homo and Hetero (p65/p50)) (17) translocated to the nucleus and binds to the target DNA inducing the transcription of a diverse array of target genes (18). This process is regulated by redox homeostasis. Upon activation, NF κ B can stimulate an inflammatory response by activating TNF α or immune response to infection (19). TNF α is a proinflammatory cytokine produced e.g. by NK cells, monocytes/ macrophages and trophoblasts. It promotes apoptosis and leakage of the endothelial vessels, leading to systemic endothelial activation and thereby signs associated

with preeclampsia (20). Apart from this TNF α could also perturb the normal regulation of energy metabolism, especially the lipid metabolism, which might be one of the pathophysiological basis of atherosclerosis, diabetes, coronary heart diseases, etc (21).

Lipid levels may serve as a energy store to fulfill maternal and fetal development (22). Cholesterol is used by the placenta for steroid synthesis and fatty acids are used for membrane formation. Changes in total cholesterol concentration reflect changes in the various lipoprotein fractions like LDL, VLDL & HDL (23). Lipids are stored in the form of triacylglycerol and storage gets transferred during nutrient deprivation (24). Triglycerides and cholesterol are packed into lipoproteins, then taken up and degraded by the cells. Low-density lipoprotein (LDL) is one of the five major groups of lipoproteins. It is most abundant cholesterol carries and it transport cholesterol and triglycerides towards tissues. It is susceptible to free radicals. Very-low-density lipoprotein (VLDL) is a type of lipoprotein which transports both triglycerides and cholesterol molecules by conversion to IDL and LDL. High-density lipoproteins cholesterol (HDL) called as good cholesterol scavenges and removes LDL by transporting it to the liver where it can be reprocessed. It acts as a maintenance crew for the inner walls of blood vessels (endothelium). Preeclampsia is associated with altered lipids due to the increased oxidative stress (10). Lipid profile changes may provide useful information for the cause of preterm delivery in preeclampsia.

Heat shock proteins (HSPs) are ubiquitous and phylogenetically conserved molecules, which indicate their functional importance. They are usually considered to be intracellular proteins with molecular chaperone and cytoprotective functions during stress (25). Their expression can be induced by several physiological (growth factors and hormones), pathological (infection, inflammation, ischemia, oxidant injury, and toxins), and environmental (thermal changes and heavy metals) conditions (26). The most widely studied HSPs are HSP70 and HSP90; they are named according to their molecular weight.

Heat shock protein 70 (HSP70) is one of the most conserved, and it is also best characterized by its highly inducible expression in response to stress and by its function as a chaperone, facilitating the folding, unfolding and refolding of proteins under both normal and stressful conditions (27). Induced expression of HSP70 has been shown to promote cytoprotection, antiapoptotic, and immune regulatory effects (28). Heat shock protein 90 α (HSP90 α) is an abundant and highly conserved molecular chaperone that is essential for viability in eukaryotes (29). Its contribution to various cellular processes including signal transduction, protein folding, protein degradation and morphological evolution has been extensively studied (30). The full functional activity of HSP90 α is gained in concert with other co-chaperones, playing an important role in the folding of newly synthesized proteins and stabilization and refolding of denatured proteins after stress (31). Apart from its co-chaperones, HSP90 α binds to an array of client proteins, where the co-chaperone requirement varies and depends on the actual client.

Thus the purpose of this study is to explore a precise relationship that exist between the occurrence of oxidative stress, lipid alteration along with the differential expression of HSP70 and HSP90 α in modulating inflammation in placental explants for the first time. This may provides a fundamental understanding on the inflammatory modulations and counter balancing activities that occur during preeclampsia.

2. Materials and Methods

2.1. 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=21) and preeclamptic (n=21) 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 (32). Patients with severe preeclampsia and other severe maternal complications were excluded from the study.

2.2. 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) until use. The explants were cultured for 3 days as described by Yacobi *et al.* 2002 (33) with slight modifications (3).

2.3. Estimation of protein

Protein concentration was determined by the classical Bradford method (34) with Coomassie brilliant blue G-250, using bovine serum albumin as the standard. The protein concentration was expressed as mg protein/g of placental sample. The lysate was used for the estimation of the following parameters.

2.4. Quantification of 4-Hydroxynonenal (4-HNE) by ELISA: (Oxidative stress parameter)

The inducible form of 4-HNE in the placental explants was quantified using 4-HNE (MBS161454, Biosource, USA) according to the manufacturer's instruction.

2.5. Immunoblot Analysis of Signaling Molecules

The placental explants protein aliquots containing 50 μ g proteins were ran on 10 % SDS-polyacrylamide gels simultaneously. The gels were then blotted on to PVDF membranes (BioTrace PVDF 0.4 μ m, Pall Corporation, Germany) according to the method of Towbin *et al.* 1979 (35). The antibodies used were and rabbit polyclonal anti-TNF α antibody (KAP-TF112, Biogeniux), and mouse monoclonal anti- TNF α antibodies (BPD-HYB-141-08, Biogeniux) followed by goat anti-mouse IgG secondary antibody treatment and color development was done using BCIP-NBT substrate system. The band intensities were

scanned with the Hp Scan Imager and quantified using the Total Lab software, gels, USA. The results were confirmed by individually performing the blotting studies of the signaling proteins.

2.6. Lipid Profile

2.6.1. Estimation of cholesterol and triglycerides:

Cholesterol and triglycerides were estimated by using commercially available kit based on enzymatic method and GPO method respectively.

2.6.2. Estimation of LDL

LDL was estimated by using Friedwald's equation, if the cholesterol and triglyceride values were known. The concentration expressed in $\mu\text{g}/\text{mg}$ protein

$$\text{LDL} = \text{Total cholesterol} - (\text{HDL} + \text{Triglycerides}/5)$$

2.6.3. Estimation of VLDL

The values of VLDL can be calculated by using the formula. Concentration expressed in $\mu\text{g}/\text{mg}$ Protein.

$$\text{VLDL} = \text{Triglycerides} / 5.$$

2.6.4. Estimation of HDL

HDL was estimated by PTA method (Phosphotungstic acid method) (36) using kit

2.7. Quantification of HSP70 and HSP90 α by ELISA

The cytoprotective expression of HSP70 and HSP90 α in the placental explants were quantified using HSP70 (EKS-700B, Stressgen, Canada) and HSP90 α (EKS-895, Stressgen, Canada) ELISA kit according to the manufacturer's instruction.

2.8. Statistical Analysis

All results were expressed as mean value \pm 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 explant with the normotensive explant using Student 't' test. Differences were taken to be statistically significant for values of $p < 0.05$, $p < 0.01$ and $p < 0.001$.

3. Results

3.1. 4-Hydroxynonenal

The level of 4-HNE was analyzed in normotensive and preeclamptic placental explant. There was a significant increase in the level of the 4-HNE in the preeclamptic placental explant by 73% ($p < 0.001$) compared to normotensive subjects (Figure 2).

3.2. Blotting Analysis of Signaling Molecules

Blotting analysis showed an increase in NF κ B and TNF α in preeclamptic placental explants with the results obtained by quantification (Fig. 3a, b). Assessment of other signaling proteins showed a constant increase in all the proteins at significance range; NF κ B ($p < 0.05$), TNF α ($p < 0.05$). The

representative blots for all the proteins are given in Fig. 3a, b.

3.3. Lipid Profile

Figure 4 represents the lipid level of placental explants, levels of cholesterol ($p < 0.05$) and triglycerides ($p < 0.01$) are found to be decreased by 22%, 11% in preeclamptic placental explants than normotensive placental explants and also depicts the significantly decreased ($p < 0.05$) LDL (13%), and VLDL ($p < 0.01$) by 25% in preeclamptic placental explants when compared with normotensive placental explants. An insignificant change in HDL (10%) level between preeclamptic and normotensive placental explant was also observed.

3.4. Quantification of HSP70 and HSP90 α

In preeclamptic placental explants, a highly significant increase in the expression of HSP70 by 47% ($p < 0.001$) and HSP90 α by 74% ($p < 0.001$) was observed when compared with the normotensive placental explants (Figure 5a and Figure 5b).

4. Discussion

In Preeclampsia, there is an improper trophoblast invasion leading to shallow invasion of spiral arteries during placental development (Figure 1). Further placental hypoxia induces the cytotoxic factors into the maternal vasculature leading to widespread placental dysfunction which is the major cause for its pathophysiology (37). Oxidative stress is known to affect the placenta during preeclampsia (2) (38). It is known that the imbalance of pro- and antioxidant factors during oxidative stress can result in severe endothelial dysfunction, either directly or indirectly through reduction in vasoactive mediators (39). The oxidation of polyunsaturated fatty acids results in the production of 4-HNE, which will induce intracellular peroxide production, leading to oxidative stress in the cells (8). The expression of 4-HNE is also believed to be largely responsible for the cytopathological effects observed during oxidative stress. The present study observed that the preeclamptic placental explants has significantly higher ($p < 0.001$) level of 4-hydroxynonenal, a byproduct of free-radical peroxidation (Figure 2) than normotensive placental explants. The enhancement of 4-HNE appears to correlate with alterations in lipid metabolism and co-insides with impairments in antioxidant defence, which exert a range of pathophysiological effects during preeclampsia. The manifestations include alteration in the redox homeostasis by releasing pro-inflammatory cytokines. These changes ultimately results in systemic vascular dysfunction in placenta (40).

Inflammation plays an important role in preeclampsia by pro-inflammatory cytokines. This cytokines showed altered expression in preeclamptic placental explant. This study hypothesizes that the transcription factor NF κ B would be activated in placental explants obtained from women with preeclampsia. Figure 3a demonstrates that NF κ B activation is increased ($p < 0.05$) in preeclamptic placental explants compared to normotensive placental explants. The generation of placental NF κ B elicits sequences of events altering the expression of cytokines, chemokines, cell

adhesion molecules, growth factors, anti-apoptotic proteins, and immune receptors that contribute to the pathogenesis of this disorder (41). We also observed that there was an increase ($p < 0.05$) in the expression of TNF α in preeclamptic placental explants (Figure 3b). TNF α also had an additive effect to activate NF κ B. Therefore, placental oxidative stress in preeclampsia may set in motion a positive feedback loop between NF κ B and TNF α which progressively worsens inflammation in the placenta. The increased expression of TNF α interferes with lipid homeostasis and its disruption is implicated in the pathogenesis of preeclampsia (6).

In the recent years, several lines of evidence have suggested that part of this maternal predisposition could be explained by abnormal lipid metabolism (42). In this study, we observed the level of TG ($p < 0.01$), cholesterol ($p < 0.05$), LDL ($p < 0.05$), VLDL ($p < 0.01$) are found to be significantly decreased in preeclamptic placental explants (Figure 4). The increased lipolysis may be due to the elevated expression of TNF α , an inflammatory protein (6). Decreased LDL depicts that it is more prone for oxidation during increased free radical generation like LPO, LHP (HNE) which causes preterm delivery with low birth weight babies. The insignificant change in HDL (10%) level between preeclamptic and normotensive placental explants depicts that the antioxidant is insufficient to scavenge formation of LDL mediated free radical. This study shows for the first time that placental lipid content is generally reflective of the elevated lipid profile previously found in the maternal circulation. This can be positively correlated to an increased risk of preeclampsia. Thus the estimation of lipid profile may have a predictive role in the assessment of the extent of placental damage which may help patient by preventing or foreseeing the effects of complications during preeclampsia.

Heat shock proteins (HSPs) play a crucial role in fetal development. HSP70 and HSP90 α expressions ($p < 0.001$) are found to be upregulated by 47% and 74% in preeclamptic placental explant when compared with normotensive placental explants (Figure 5a and Figure 5b). The increased expression of HSPs may repress NF κ B successively decreasing TNF- α indicating that HSPs have an immunoregulatory potential. The protective mechanisms of HSPs initiated against the generated ROS (43) produced by altered lipid profile. HSPs alter proinflammatory cytokine production increasing endotoxin tolerance and survival (44). Though there is a significant change in the stress and altered lipid profile in the placenta, the live fetal delivery is not predominantly affected during preeclampsia. This may be due to the expression of HSPs which sustains fetal development.

5. Conclusion

The present study suggests that TNF α severely disrupts lipid homeostasis by altering lipids and triggers the stress markers which may be one of the main causes to trigger the upregulation of HSPs to support cell survival (anti-apoptotic and anti-inflammation) during preeclampsia. Thus the preeclamptic implications in terms of inflammatory and lipid profile changes must be carefully monitored during pregnancy to prevent progressive maternal and neonatal complications.

6. Acknowledgements

The project funded by National Tea Research Foundation, Tea Board of India Project referral number- NTRF: 142/10 is acknowledged. Indian Council for Medical Research is acknowledged for providing senior research fellowship to Ms. D. Lavanya.

7. 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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Figures

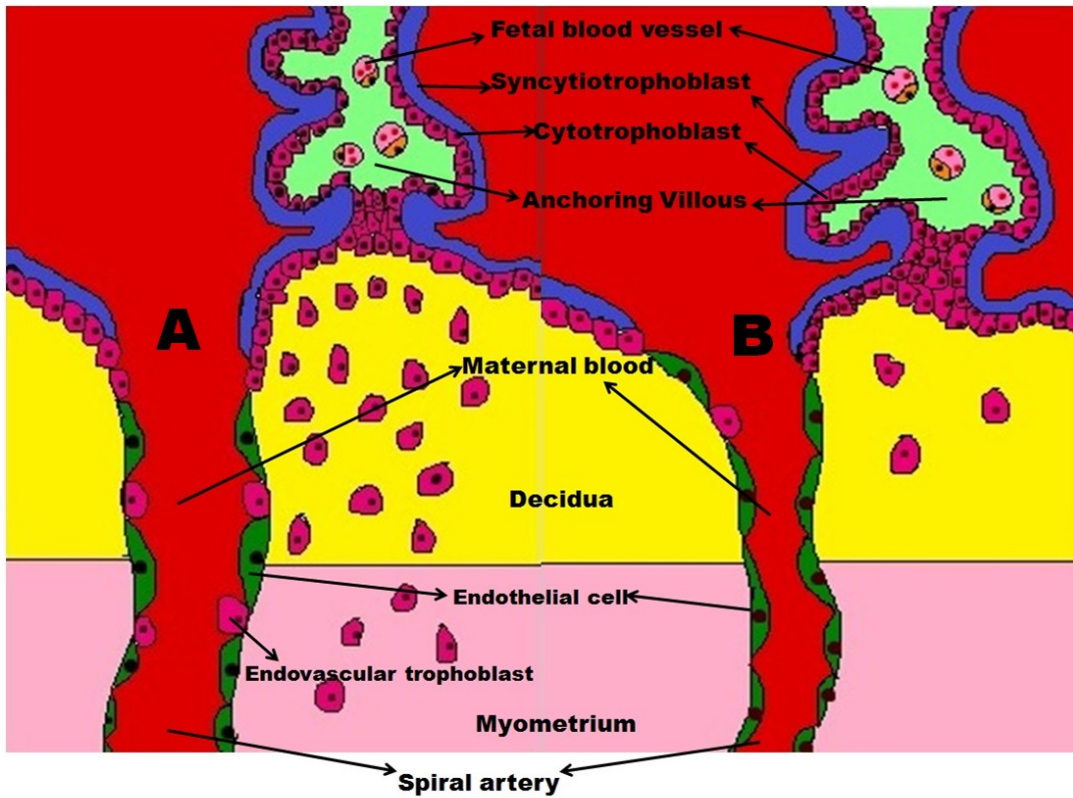
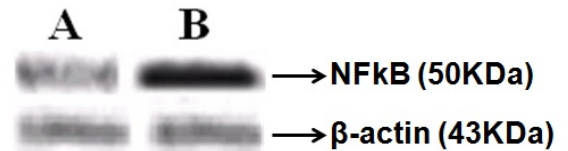


Figure 1: Pathophysiology of Preeclampsia

Where, A- Normotensive pregnancy; B- Preeclamptic pregnancy

Figure 1 shows that proper trophoblastic colonization of the uterine wall and vascular transformation occurs progressively during normal pregnancy (A). There is a small change in the kinetics of invasion that may lead to incomplete transformation of the spiral arteries during preeclampsia (B)

a) NFκB:



b) TNFα:

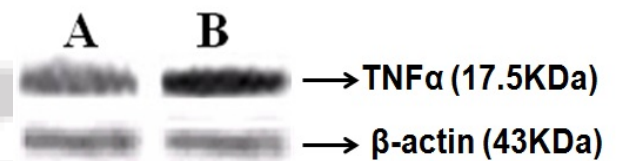


Figure 3: Western blot analysis of normotensive and preeclamptic placental explants for a) NFκB and b) TNFα expression along with β-actin as the loading control.

Where, A- Normotensive placental explants; B- Preeclamptic placental explants
A representative immunoblot is shown. β-actin has been used as the loading control.

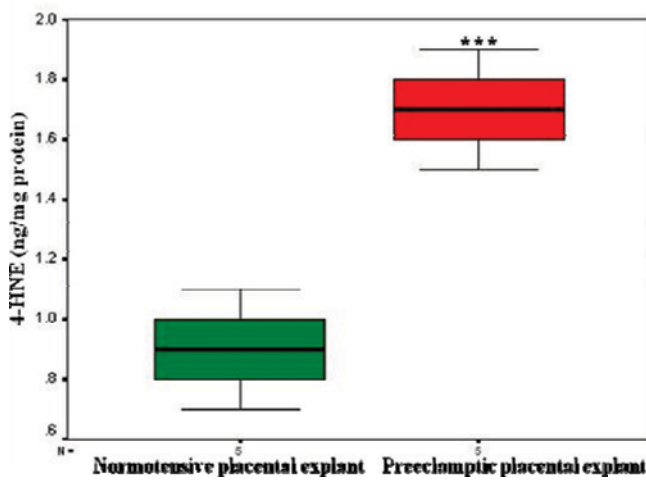


Figure 2: Level of 4-Hydroxynonenal in placental explants from normotensive women & preeclamptic women.

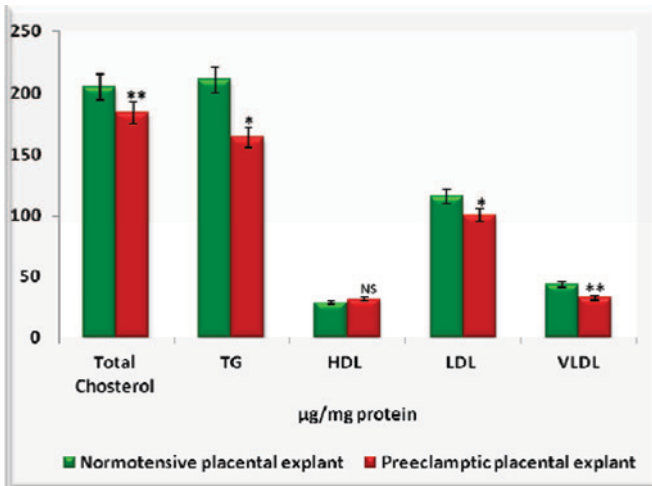
Values are expressed as Means ± SD (n=21 samples from each group)
***p<0.001 when compared with normotensive placental explant

Figure 4: Assessment of lipid profile in normotensive and preeclamptic placental explants.

Author Profile

Dr. E. Padmini received M.Sc., M. Phil., Ph.D. degrees in Biochemistry from University of Madras in 1983, 1984, 1988, respectively. I was awarded the Post Doctoral Research Associate (D.Sc FABMS) from the Department of Pharmacology, University of Tennessee, USA in the year 1993. In addition to this, I have completed PG Diploma in Bio-Informatics in the year 2004. I am working as faculty in the Department of Biochemistry, Bharathi Women's College, Chennai- 600 108, Tamilnadu, India since 1988.

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Each bar represents means ± SD (n=21 samples from each group)

**p<0.01 when compared with normotensive placental explants; *p<0.05 when compared with normotensive placental explants; ^{NS}not significant when compared with normotensive placental explants

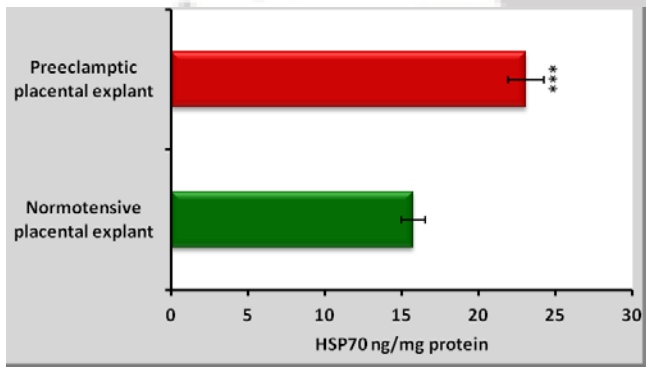


Figure 5a: Expression of HSP70 in the normotensive & preeclamptic placental explants.

Each bar represents means ± SD (n=21 samples from each group)

***p<0.001 when compared with normotensive placental explant

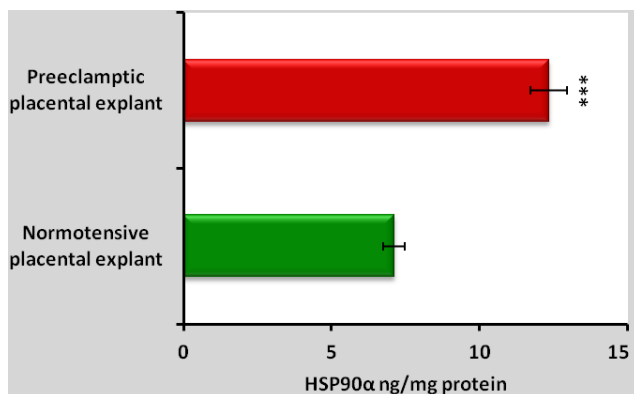


Figure 5b: Expression of HSP90α in the normotensive & preeclamptic placental explants.

Each bar represents means ± SD (n=21 samples from each group)

***p<0.001 when compared with normotensive placental explant