ABSTRACT

Preeclampsia is a common pregnancy specific syndrome that is more often associated with trophoblast dysfunction during the first trimester of pregnancy. Trophoblast plays a central role in determining fetal growth and the development of pregnancy complications as well as in programming of cardiovascular and metabolic disease in the developing fetus. Infection specific inflammation is a primary mediator of trophoblast complication. The role of U. urealyticum in altering Preeclamptic trophoblast complications are largely overlooked under such condition. Heat shock protein 70 (HSP70) is a molecular chaperone playing significant role in control of preeclamptic progression and protection of the developing fetus. c-jun N terminal Kinase (JNK) is a signaling molecule having potential role in apoptosis regulation. During preeclampsia and infection there is an altered balance between proliferation and apoptosis of villous trophoblast. There was a significant decrease in the trophoblast, endothelial cell viability in U. urealyticum infected preeclamptic (p<0.001), uninfected preeclamptic (p<0.05) cells when compared to normotensive trophoblast and endothelial cell. The changes in the expression of apoptosis regulating signal JNK in response to cytoprotective HSP70 level in the 3 study groups revealed the anti-apoptotic role of HSP70 in restoring trophoblast turnover during conditions of preeclampsia and U. urealyticum infection. The study result emphasizes the importance of HSP70 analysis under conditions of preeclamptic U. urealyticum infection.

Keywords: Trophoblast; preeclampsia; HSP70; JNK; Ureaplasma urealyticum

[1] INTRODUCTION

Preeclampsia is a major cause of materno-fetal morbidity and mortality affecting 7–10% of all pregnancies. Preeclampsia (PE) is a hypertensive pregnancy disorder marked by superficial implantation and inadequate placental perfusion that has been linked to increased oxidative stress. Pregnancy diseases, such as preeclampsia is associated with an alteration of trophoblast differentiation, and invasion [1]. Pregnancy specific complications can result in cardiovascular disease in the later life of both mother and fetus which may be genetic or programmed in-utero. The in-utero programming is restricted to the modification in trophoblast. Preeclampsia not only elevates obstetric morbidity and mortality, but also places the mother at increased risk for developing cardiovascular disease (CVD) later in life. It is mainly the trophoblast, one of the major cell types of placenta that orchestrates the complex biomolecular interactions between fetus and mother during pregnancy [2]. In preeclampsia, cytotrophoblasts fail to invade the spiral arterioles; as a result, these vessels do not enlarge, severely compromising their ability to deliver maternal blood to the intervillus space [3]. Changes in trophoblast activity play a central role in determining fetal growth and the development of pregnancy specific complication along with fetal programming of cardiovascular and metabolic disease [4]. Preeclampsia represents a major long term cardiovascular risk factor for the mother and the child. Endothelial dysfunction commonly noted during preeclampsia is a predisposing factor for abnormal placentalation which arises due to improper trophoblast invasion [5] and may represent the link between placentation defects and the development of CVD.

The presence of Ureaplasma urealyticum infection in preeclampsia and the role of U. urealyticum infection in altering placental stress have been demonstrated earlier [6]. Reports suggest that U. urealyticum infection leads to ROS mediated cell death and sperm cell inactivation [7]. U. urealyticum infection mediated apoptosis [8] and phagocytosis [9] are demonstrated. Preeclampsia is often accompanied by hypoxia of the placenta and this condition induces apoptosis in trophoblastic cells [10]. The relationship between infection and cardiovascular disease is considered to be associated with the
inflammation reaction. Earlier reports on infection and cardiovascular disease have shown a role of *C pneumoniae*, (the main cause of community acquired pneumonia) in future coronary heart disease [11, 12]. The involvement of *Mycoplasma pneumoniae* in cardiovascular diseases has been previously reported [29]. Pathological fetal growth increases the risk for perinatal complication and predisposes the baby for the development of cardiovascular disease. The contribution of both preeclampsia and *U. urealyticum* infection in oxidative stress are well demonstrated [30, 6]. Oxidative stress underlies the molecular basis via which prenatal hypoxia contributes to the developmental programming of cardiovascular disease of the mother and fetus.

Heat Shock Protein (HSP) is a major molecular chaperone that gets increased in response to a variety of stress stimuli and restores protein homeostasis. HSP70 one of the major HSP gets over expressed during preeclampsia in placental tissue [13], endothelial cell [14] and endothelial cell mitochondria [15]. An increase in HSP70 in response to *U. urealyticum* infection in preeclamptic placental tissue has also been reported [6]. HSP70 is a cytoprotective protein that has a major role in controlling programmed cell death [16]. HSF-1 (Heat shock factor-1) is the nuclear transcription factor involved in mechanistic regulation of HSP70 expression. In response to stress stimuli free HSF-1 will trimerize, translocate into the nucleus and gets phosphorylated to stimulate HSP synthesis [17]. c-Jun N-terminal protein kinase (JNK) is a subfamily of the mitogen activated protein kinase (MAPK) superfamily and plays a pivotal role in the transmission of extracellular signals through the cytosol to the nucleus that will promote either the cell growth or cell death depending on the activation stimuli [18, 19]. This study will analyze the role of *U. urealyticum* in altering preeclamptic trophoblast complication, additionally suggesting the role of *U. urealyticum* in fetal programming of cardiovascular disease. Further the study will also analyze the change in viability of trophoblast cells between normotensive, preeclamptic and preeclamptic with *U. urealyticum* infection, with the corresponding change in the expression of HSP70, HSF-1 and JNK.

### [II] MATERIALS AND METHODS

#### 2.1. Selection of subjects

The patients of obstetrics and gynecology department enrolled in a public sector hospital were chosen as subjects. The study was carried out for a period of one year. pre-eclamptic patients undergoing c-section; of age group 22-40 years characterized with a blood pressure greater than 140/90 mm Hg but less than 160/110 mm Hg, a proteinuria levels > 0.3 g/dL found in no less than 2 random specimens and xanthine oxidase activity of approximately 2.6 units/mg protein [20]. Patients with severe Pre-eclampsia and other severe maternal complications were excluded from the study. Normotensive healthy pregnant subjects undergoing c-section who were of similar race, body mass index (BMI) and without maternal and fetal complications during pregnancy were also chosen as control for the study. The clinical characteristics of the pre-eclamptic and normotensive subject selected for the study are given in [Table-I]. All the c-section deliveries were by choice of the patient or due to previous c-section. *U. urealyticum* infection in preeclamptic placenta was confirmed by performing microbial culture in A8 agar and U9 broth.

#### 2.2. Isolation of trophoblast

Third-trimester villous trophoblast cells, which were used for comparison, were isolated from term placentas by the method of Douglas and King [21]. Human term placentas from the preeclampsia and normotensive subjects after delivery were obtained immediately after elective c-section, in accordance with the established guidelines of the institutional ethical committee along with the informed consent of the patient. Briefly, placental villi were cut and thoroughly washed to remove blood. Thereafter, they were incubated four times in a digestion medium composed of HBSS, containing trypsin and deoxyribonuclease for 30 min at 37°C in a water bath with continuous shaking. The dispersed cells were layered on top of a discontinuous 5–70% Percoll gradient, and centrifuged for 25 min at 507 Xg. The intermediate layers (density between 1.048 and 1.062) containing cytotrophoblast cells were removed and washed, and cell viability was determined by trypan blue exclusion. Following trophoblast isolation, cells were seeded at a density of approximately 1.6×10⁶ cells per well in 6-well plate. The complete culture medium, constituted of M199, 2mM glutamine, 10% FBS. All the experiments were performed within a day of trophoblast isolation in order to overrule the influence of cultivation process.

#### 2.3. Isolation of endothelial cells

Endothelial cells were isolated from term human placenta of normotensive and preeclampsia subjects with and without *U. urealyticum* infection according to the method of Herr et al [22] with slight modification. The excised Placental chorionicdida was washed in Hank’s Balanced Salt Solution (HBSS) to remove the blood and visible blood clots, microvessels were removed from the tissue. It was then thoroughly minced in HBSS and was passed through a 90 μm sieve. Collagenase type I (Sigma, St Louis, MO, USA) at 1.4mL per gram of placental tissue was added, and the contents were shaken at 37°C for 80 min. Following several washes with HBSS and centrifugation at 100 Xg for 5 min, the pellets were placed on ice. After re-suspending and incubating the cell pellet in 0.5 mL trypsin-EDTA/g tissue, the suspension was passed through a 250 μm sieve. The filtrate was centrifuged at 100 Xg for 5 min single cell suspension that was obtained was treated with Dynabead CD31 (Invitrogen,Oslo, Norway), and then washed with phosphate buffered saline (PBS) containing 0.1 % BSA. This mixture was incubated at 4°C for 20 minutes with tilting and rotation. The Dynabead endothelial cell complex was collected with a magnetic particle concentrator. The cells were washed twice with PBS and cultured overnight at 1 million cells per culture flask (125 mm²) in M199 medium containing 20% fetal calf serum in a 5% CO₂ atmosphere at 37 °C overnight. The non adherent cells were removed by washing with PBS thrice on the following day.
2.4. Viability assessment

The viability of the isolated endothelial cell and trophoblast were assessed by trypan blue exclusion method [24]. Briefly 10 µL of the isolated cells were mixed with 0.4% trypan blue solution and was allowed to react for 5 minutes in a moist chamber. The viable unstained cells were then counted using a hemocytometer. The results were expressed as % of viability [23].

2.5. Co-immunoblot analysis of HSP70, HSF-1, and JNK

The placental trophoblast protein aliquots containing 50µg proteins were run on two 10 % SDS-polyacrylamide gels that were placed together in the Dual gel electrophoretic system. Both the gels were then blotted on to PVDF membranes (BioTrace PVDF 0.4 m, Pall Corporation, Germany) according to the method of Towbin et al., [24]. The blotted membranes were cut to molecular weight of the respective antibodies used and were then developed for visible band formation. The antibodies used were anti HSP70 (SPA810), anti JNK1/2 ((KAP-SA011), anti HSF1 (SPA 901) antibody) and anti β-actin(CSA-400). Enzyme labelled goat antimouse IgG secondary antibody treatment and colour development was done using BCIP-NBT substrate system. The band intensities were scanned with the HP Scan Imager and quantified using the TotalLab Software, GELS, USA. The results were confirmed by repeating the experiment thrice.

2.6. Statistical analysis

The results of the tests performed were expressed as mean ± standard deviation. The values were compared using one-way ANOVA test. The viability of the isolated trophoblast and endothelial cells were assessed by correlation analysis. Statistical analysis package SPSS version 7.0 was used for performing one-way ANOVA. A p value of <0.05 is considered significant and a p value of < 0.001 is considered highly significant.

[III] RESULTS

Clinical Characteristics of the normotensive and preeclampsia samples analyzed are tabulated in Table-1. The normotensive and preeclamptic subjects selected for analysis had a significant alteration in the systolic and diastolic pressure, xanthine oxidase during pregnancy while an insignificant change in the systolic and diastolic pressure pre pregnancy. There was also a significant decrease in the birth weight of fetus born to preeclamptic mother. The preeclamptic subjects were all primiparous.

The trypan blue dye exclusion method was used to assess the viability of trophoblast and endothelial cells. There was a significant decrease in the viability of trophoblast and endothelial cells isolated from preeclamptic subjects (p<0.05) compared to normotensive subjects. The viability of trophoblast and endothelial cells isolated from preeclamptic subjects with U. urealyticum (p<0.001) were further decreased when compared with normotensive subjects. The viability changes were measured in the cells immediately after isolation. However the isolated cells were also grown for 5 days. The results are represented in Figure–1.

The expression changes of HSP70 and the corresponding change in the expression of JNK and HSF-1 are analyzed using co-immunoblotting. There was a significant increase in the expression of HSP70, HSF-1 and JNK1/2 in preeclamptic trophoblast (p<0.05) when compared with normotensive trophoblast. There was a highly significant increase in

Table: 1. Clinical characteristics of the normotensive and pre-eclamptic subjects selected for analysis: The clinical data of the mother and the fetus was obtained from the hospital. There were no follow-ups for these cases after delivery. Statistical T test was performed among the two groups for comparative analysis and the p value obtained are mentioned in the table.
expression of HSP70, HSF-1 and JNK1/2 in preeclamptic trophoblast with *U. urealyticum* infection (p<0.001) [Figure–2].

**[IV] DISCUSSION**

Preeclampsia represents a major long-term cardiovascular risk factor for mother and child, though the pregnancy specific disorder is thought to end when the placenta is removed [25]. Abundant evidence indicates reduced placental perfusion and superficial implantation in preeclampsia. Trophoblast is one of the essential placental cells with an important role in placenta related complications. Biologically, trophoblast-mediated vascular remodeling within the placental bed allows for marked distensibility of the uteroplacental vessels, thus accommodating the increased blood flow needed during gestation. Abnormalities in this invasive process have been correlated with early and mid-trimester pregnancy loss, preeclampsia and eclampsia, and intrauterine growth retardation [26]. A link between placentation relation disorders and CVD and suggest that placentation related disorders may constitute an early expression of cardiovascular risk factors [27].

Women who develop preeclampsia also run a long-term augmented risk of cardiovascular disease and premature death. The fetus born to preeclamptic mother is also at an equal risk to develop cardiovascular disease. The decrease in viability of trophoblast isolated from preeclamptic placenta when compared to normotensive placenta suggests that preeclamptic stress is associated with trophoblast complication. Further decrease in viability in preeclamptic trophoblast during *U. urealyticum* infection when compared to normotensive placenta shows the role of *U. urealyticum* in altering preeclamptic trophoblast complication. The decrease in viability of endothelial cell in relation to the alteration in trophoblast viability suggests the role of trophoblast in modulating the endothelial cell function during preeclampsia and *U. urealyticum* infection. Studies in our laboratory have also shown a strong relationship between preeclampsia and atherosclerosis (data not shown). Acute atherosis of uterine wall spiral arteries seen in pregnancy complications and the molecular interaction between trophoblast and endothelial cells could add important elements to explain cardiovascular disease during preeclampsia and *U. urealyticum* infection [28]. The alteration in trophoblast function during preeclampsia and an enhanced damage of preeclamptic trophoblast during *U. urealyticum* infection suggests that *U. urealyticum* aggravates the chance of cardiovascular disease in the developing fetus.

![Graph showing viability of trophoblast and endothelial cell](image1)

**Fig: 1.** Comparative analysis of viability of trophoblast cells and endothelial cell from normotensive, preeclampsia and preeclampsia with infection. *N:* Normotensive placenta; *P:* Preeclamptic placenta; *Pi:* Placenta from preeclamptic subjects with *U. urealyticum* infection; *p*<0.05 when compared with normotensive subjects; *#p*<0.001 when compared with normotensive subjects.

![Graph showing expression of proteins](image2)

**Fig: 2.** Western blotting analysis of proteins from normotensive, preeclamptic and preeclamptic subjects with *U. urealyticum* infection. *N:* Normotensive trophoblast; *P:* Preeclamptic trophoblast; *Pi:* Preeclamptic trophoblast with infection. *p*<0.001 when compared to normotensive subjects. *p*<0.05 when compared to normotensive subjects.
As metabolically active tissues vital to the maintenance of pregnancy, placental tissue particularly in preeclampsia experiences stress and are more susceptible to ROS-mediated apoptosis. The increase in HSP70 observed during preeclampsia plays a vital role in maintaining the integrity of the developing fetus. Further increase in HSP70 during U. urealyticum infection suggests the increase in necessity of this cytoprotective protein under such condition. Preeclampsia is found to result in future hypertension in both mother and fetus [31]. The study on trophoblast suggests that trophoblast dysfunction might be a reason for future hypertension. Circulating HSP70 levels predict the development of cardiovascular disease in subjects with established hypertension [32]. Thus the increased HSP70 is found to have a protective role in controlling oxidative stress generated by trophoblast dysfunction and controlling hypertension mediated damage, thereby posing a protective effect against risk of cardiovascular disease.

JNK activation contributes to regulation of essential cellular processes, such as differentiation, apoptosis and direct cell movements [33]. Reports suggest that activation of the JNK pathways functioned to promote trophoblast cell survival [34] and protects the trophoblast from apoptosis induced by growth factor withdrawal [35]. The expression of JNK1/2 and HSF-1 was increased significantly in preeclamptic trophoblast with and without U. urealyticum infection. JNK is a key mediator in the HSP70 synthesis, through phosphorylation of HSF1 [36]. The over-expressed JNK will increase the expression of phospho-HSF-1 to favor the expression of HSP70. This condition is similarly aggravated in preeclampsia during U. urealyticum infection. This suggests that JNK favors the synthesis of HSP70 in-order to restore cellular and protein homeostasis by phosphorylating HSF-1 in trophoblast cells under conditions of preeclampsia and preeclamptic U. urealyticum infection. The cytoprotection established by the interaction of JNK and HSP70 in trophoblast cells may further protect the damaging endothelial cells under conditions of preeclampsia and U. urealyticum infection. These results imply that though there is an increasing risk of cardiovascular disease to both the mother and fetus subject to preeclampsia and preeclamptic U. urealyticum infection, cytoprotection of trophoblast and endothelial cell by HSP70 might be of considerable help in reducing the complication. Thus HSP70 is a natural defensive protein that can be used as a therapeutic target to unmask the cardiovascular complications under conditions of preeclampsia and preeclampsia U. urealyticum infection.

[V] CONCLUSIONS

The alteration in viability of trophoblast isolated from preeclampsia and preeclamptic U. urealyticum infection reflects the severity of trophoblast complication during U. urealyticum infection. Infection is not only associated with pre term birth but also poses the mother and the new born for cardio vascular disease in later life by induction of trophoblast dysfunction. Under such condition, the maintenance of HSP70 level which acts as a protective chaperone is crucial. The increase in the expression of HSP70, HSF-1 and JNK in trophoblast during preeclampsia and preeclampsia U. urealyticum infection analyzed in the present study suggests the defensive role of these proteins (HSP70) under such condition. Thus monitoring and maintaining the level of cytoprotective HSP70 under conditions of preeclampsia and preeclampsia with U. urealyticum infection will aid in reducing the potential risk factors for future cardiovascular disease suggesting its role as therapeutic target under such conditions.

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