Original Article

Vascular Endothelial Growth Factor Alleviates Endoplasmic Reticulum Stress via PERK, IRE1, and ATF6 Pathways in Trophoblast Cells

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Abstract

Introduction: In developing countries, preeclampsia is still a leading cause of maternal mortality and morbidity, affecting nearly 8%–10% of pregnancies and overall death. Apart from various stresses that placenta undergoes while pregnancy, endoplasmic reticulum (ER) stress has been the center of attraction for several researchers all across the globe. Imbalance in circulating pro- and anti-angiogenic agents in maternal serum has also been linked with the upregulation of stress at subcellular level. The present study is an attempt to demonstrate the role of pro-angiogenic factor in mitigating ER stress in trophoblast cells. **Material and Methods:** Evaluation of expression of ER stress markers (eIF2α, X-box binding protein-1, and ATF6) at various time points was done after exposure of varying concentration (s) of pro-angiogenic factor (from preeclamptic mothers) to trophoblast cells (BeWo cells). Expression was also analyzed when BeWo cells were exposed to recombinant vascular endothelial growth factor (VEGF) along with serum from preeclamptic mothers. Molecular techniques used were immunofluorescence staining and Western blot analysis. **Results:** Immunofluorescence staining and Western blot analysis demonstrated upregulated expression of studied ER stress markers in BeWo cells when they were exposed to Preeclampsia (PE) sera. Exogenous addition of recombinant VEGF along with preeclamptic sera significantly reduced the expression of ER stress markers. **Discussion and Conclusion:** In the present study, significantly reduced expression of ER stress markers in BeWo cells indicates an interrelationship of angiogenic factor and molecular transmembrane sensors. Further experimentations thus may provide a strong base for the modulation of ER stress sensors, which could be effective in minimizing ER stress in preeclamptic pregnancies and thus would bring a new hope to numerous women worldwide.

Keywords: *BeWo cells, endoplasmic reticulum stress, preeclampsia, pro-angiogenic marker*

Introduction

Angiogenesis occurs throughout life in both health and disease, beginning *in utero* and continuing through old age.[1] Vascular endothelial growth factor (VEGF) is an important angiogenic factor required for the growth and development of placental vasculature.[2] Its suppression in maternal circulation by anti-angiogenic factors such as sFlt-1 and s-Eng may poorly affect the invasion of trophoblast cells into the maternal decidua, which, in turn, can cause defective spiral artery remodeling, leading to adverse outcomes such as preeclampsia (PE) and intrauterine growth restriction (IUGR) in pregnant women.[3] The trophoblast cells in such patients, recently, have been found to demonstrate high levels of endoplasmic reticulum (ER) stress which is associated with oxidative and immunological stresses.[4] The aforesaid stresses lead to disruption of cellular homeostasis and promote aggregation of misfolded proteins in the lumen of ER, which activates a series of signaling pathways, collectively referred as unfolded protein response (UPR).[5] All the three arms of UPR (PERK, IRE1, and ATF6) primarily tend to alleviate the stress through adaptive response. Failure of adaptive response by individual arm (s) of UPR induces activation of apoptotic pathway (s).[6]

Preeclampsia is a multisystemic disorder, accounting for nearly 8%–10% of overall maternal deaths. It targets several organs, including the kidneys, liver, and brain, and is a leading cause of maternal and perinatal morbidity and mortality.[7] As an important angiogenic player, VEGF influences multiple end points in the pathophysiology of PE.[8] The significant role of VEGF in

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maintaining healthy pregnancy is available across literature; however, its role in alleviating ER stress by affecting the signaling of the three arms of UPR (PERK [eIF2α], IRE1 [X-box binding protein-1 (XBP1)], and ATF6) is yet to be explored. Hence, the present study was designed to analyze if reduced levels of VEGF could have an impact on ER stress of trophoblast cells.

Material and Methods

In-vitro experiments were carried out to analyze the effect of VEGF on the attenuation of ER stress in trophoblastic cells (BeWo cells). The human choriocarcinoma cell line (BeWo) was procured from American Type Culture Collection and maintained in F-12 HAM nutrient medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were passaged with 0.025% trypsin and 0.01% ethylenediaminetetraacetic acid.

The study was divided into two experimental and two control groups depending on various treatments given to BeWo cells as follows: Group A: preeclamptic sera (PS), Group B: PS + recombinant VEGF (PS + re-VEGF), Group C: positive control tunicamycin (PC_{Tun}), and Group D: negative control: untreated (NC). After the various treatments, markers of the three arms of UPR (PERK, IRE 1, and ATF6) were assessed at various time points (8 h, 14 h, and 24 h) using immunofluorescence staining and validated by Western blot analysis [Figure1].

The patients' sera (n = 15) were collected from the enrolled pregnant women from the antenatal clinic and the inpatient ward of the Department of Obstetrics and Gynecology, All India Institute of Medical Sciences, New Delhi, India. Protocol of the study was approved by the institute's Ethical Committee, and written informed consent was obtained from all the enrolled women. PE was defined according to ACOG guidelines: Blood pressure (BP) ≥140 mm Hg systolic and ≥ 90 mm Hg diastolic (mild) or ≥160 mm Hg systolic and ≥110 mm Hg diastolic (severe) on 2 occasions at least 4 h apart after 20 weeks of gestational age in women with a previously normal BP, confirmed within a short interval to facilitate timely antihypertensive therapy; proteinuria >300 mg/24 h urine collection or protein/creatinine ratio >0.3 mg/dl or dipstick reading of >1+ or in the absence of proteinuria, newonset hypertension with new onset of one or more of the following: thrombocytopenia: platelet count <100,000/µl, renal insufficiency: serum creatinine >1.1 mg/dl or doubling of serum creatinine in the absence of other renal disease, impaired liver function: elevated blood levels of liver transaminases to twice normal concentrations, pulmonary edema and cerebral edema. A volume of 5 ml of venous blood was collected and centrifuged at 1200 rpm for 4 min. Serum was separated and stored in aliquots at − 80°C. The serum samples were analyzed for VEGF by ELISA and used for cell culture experiments.

Immunofluorescence microscopy

BeWo cells were trypsinized, seeded, allowed to grow on coverslips in multiple well chambers, and incubated at 37°C in 5% CO_2. They were subjected to various treatments as mentioned above. After 8 h, 14 h, and 24 h, the cells were taken out from the incubator and washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min at room temperature. After fixation, the cells were washed with PBS and permeabilized with PBS + 0.1% Triton-X100 followed by PBS washing. Nonspecific blocking was done using 5% normal goat serum in PBS and Triton X. The cells were incubated for 12 h at 4°C in primary antibodies, namely, anti-eIF2α (1:200), anti-XBP1 (1:200), and anti-ATF6 (1:1000). The cells were washed with PBSTx and thereafter incubated in secondary antibody in 1:500 dilution for 1 h at room temperature in a dark room. The cells were washed in PBS and mounted with flouroshield mounting media with DAPI (4′,6-diamidino-2-phenylindole) on the slide and observed under a fluorescence microscope (Nikon Eclipse Ti-S elements using NiS-AR software) Nikon fluorescence microscope (Nikon Eclipse TiS elements using NiSAR software, Japan).

Western blot

The cells were lysed in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (2% SDS, 60 mMTris-HCl [pH 6.8], 10% glycerol, 0.001% bromophenol blue, and 0.33% mercaptoethanol) and boiled for 5 min. The lysates were analyzed by immunoblotting using 1:500 of anti-eIF2α, anti-XBP1, and 1:1000 of anti-ATF6 for 12 h at 4°C. The blots were then incubated in secondary antibody (HRP conjugated) for 2 h. The blots were visualized by treating the membranes in DAB, tetrahydrochloride, and H_2O_2. β-actin was used as protein loading control. The blots were scanned in a gel documentation system, using Quantity 1 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were analyzed by GraphPad Prism 7, San Diego, CA, USA. Data were presented as mean ± standard deviation. The average level of the variable between the two groups was compared by paired t-test. The comparison between more than two groups was done by ANOVA with Bonferroni's multiple comparison test. P <0.05 was considered statistically significant.

Results

Three ER stress markers (eIF2α, XBP1, and ATF6) from the different arms of UPR pathway were analyzed after various treatments as mentioned in the materials and methods section. The sera from ten preeclamptic mothers with VEGF levels ranging from 100 to 125 pg/ml were pooled to treat the trophoblastic cell lines (Group A).

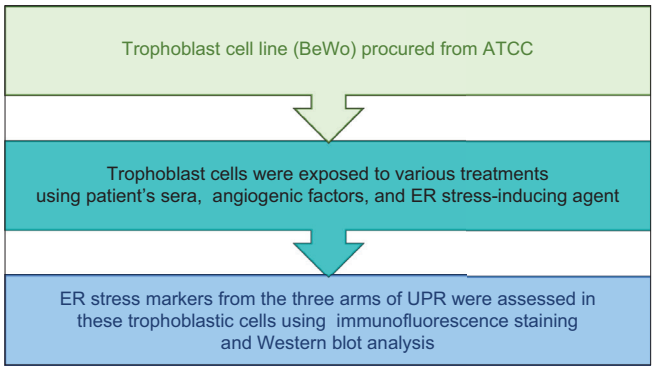

Figure 1: Study design (*in-vitro* study)

The PE sera are known to have lower VEGF and high levels of sFlt-1 as compared to those of normotensive, nonproteinuric pregnant women. The supplementation with recombinant VEGF was done to neutralize the sFlt-1 in patient's sera (Group B).

PERK pathway (eIF2α expression)

eIF2α is a marker of PERK pathway. Patients' sera-treated BeWo cells demonstrated brighter signals of eIF2α. The characteristic ER stress granules were very prominent at 14 h as compared to those at 8 h and 24 h [Figure 2a]. The signal intensity of eIF2α in BeWo cells reduced when recombinant VEGF was added to patients' sera (Group B).

Immunoblot data also demonstrated upregulated eIF2α expression in patient's sera-exposed cells (Group A [PS]) as compared to cells of Group B (PS + re-VEGF) and Group D (NC) [Figure 2b].

IRE1 pathway (X-box binding protein-1 expression)

Immunolocalization revealed enhanced expression of XBP1 at 14 h as compared to that at 8 h and 24 h in BeWo cells treated with preeclamptic patients' sera (Group A) in contrast to Group B. No signal (s) of XBP1, however, were detected in Group D [Figure 3a]. Western blot analysis demonstrated maximum protein expression at 14 h in Group A as compared to Group B and Group D [Figure 3b].

ATF6 pathways (ATF 6 expression)

ATF6 immunolocalization revealed maximum expression at 14 h as compared to that at 8 h and 24 h in BeWo cells of Group A as compared to Group B. No signal (s) of ATF6, however, were detected in Group D [Figure 4a]. Immunoblot data revealed maximum protein expression at 24 h in Group A as compared to 8 h and 14 h [Figure 4b].

Studied endoplasmic reticulum stress markers demonstrated earmarked expression in BeWo cells treated with tunicamycin (PC)

Immunofluorescence staining demonstrated stronger expression of all the markers with 5 μg/ml dose of tunicamycin [Figures 2a, 3a and 4a]. Immunoblot data revealed higher expressions of eIF2α at 14 h and 24 h as compared to those at 8 h. XBP1 and ATF6 expression was found to be higher with 5 μg/ml of tunicamycin [Figures 2b, 3b, and 4b].

The expression of all the three ER stress markers was enhanced in BeWo cells when they were treated with PE sera. However, the increased ER stress of BeWo cells could be reversed when they were treated with patients' sera whose VEGF levels were enhanced by adding the recombinant VEGF [Figures 2c, 3c and 4c].

The expression of the three markers also showed time-dependent variation in all these groups.

Comparison of normalized protein values of various ER stress markers between PE sera (PS) and PE sera along with recombinant VEGF (PS + re-VEGF)-treated BeWo cells is depicted in Figures 2c, 3c and 4c.

The expression of only eIF2α and XBP1 was found to be lower in BeWo cells from Group B as compared to Group A at 8 h and 14 h, and the difference was found to be statistically significant [Figures 2c and 3c]. However, at 24 h, the expressions of all the three markers (eIF2α, XBP1, and ATF6) were found to be lower in BeWo cells from Group B as compared to those from Group A, and the difference was statistically significant: eIF2α ($P < 0.0001$), XBP1 ($P < 0.0001$), and ATF6 ($P < 0.0001$) [Figures 2c, 3c, and 4c].

Discussion

The present study demonstrated that reduced levels of VEGF could upregulate ER stress in trophoblast cells. Supplementation with recombinant VEGF could reverse the ER stress in these cells. The human choriocarcinoma cell line (BeWo) of placental origin was used because it mimics *in-vivo* syncytialization of placental villous trophoblasts. We analyzed the signaling response of ER stress markers (eIF2α, XBP1, and ATF6) using immunofluorescence staining and Western blot analysis at different time points (8 h, 14 h, and 24 h). The BeWo cells of Group A were exposed to sera from preeclamptic pregnant women who have lower levels of VEGF and also higher amounts of sFlt-1.[9] The BeWo cells of Group B, on the other hand, were exposed to PE sera whose VEGF levels were enhanced and sFlt-1 concentration was reduced by the addition of exogenous VEGF (recombinant VEGF). Under normal physiological conditions, VEGF exerts its biological effects through two high-affinity tyrosine kinase receptors, namely, VEGFR1 or fms-like protein kinase-1/Flt-1 and VEGFR2 or kinase insert domain receptor/KDR.[10,11] The soluble form of Flt-1 is sFlt-1, which acts as a decoy receptor and lowers the bioavailability of circulating VEGF by capturing it. Many studies globally and in India have shown that in PE patients, the levels of sFlt-1 are higher and the levels of VEGF are reduced as compared to normal pregnant women.[12-14] Therefore, these low levels of VEGF could not only cause disturbances in the endothelial cell homeostasis, neovascularization, vascular tone, and blood pressure, but

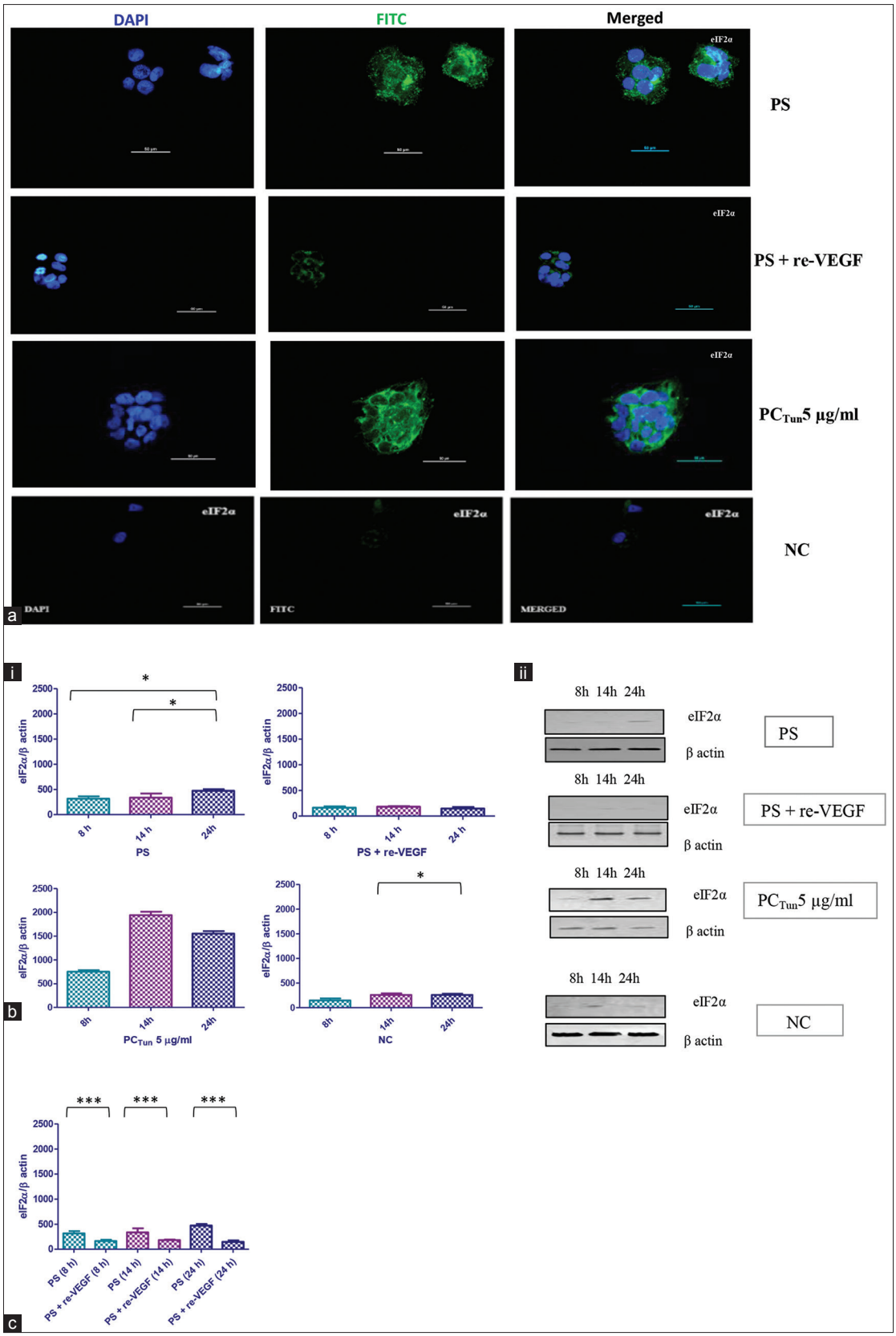

Figure 2: (a) eIF2α immunolocalization in BeWo cells. Representative photomicrographs of BeWo cells showing eIF2 alpha expression following various treatments: PE sera, PE sera + re- vascular endothelial growth factor (PS + re-vascular endothelial growth factor), tunicamycin (positive control/PC$_{Tun}$ 5µg/ml), and no treatment (negative control/NC). (b) (i) Bar diagrams represent the normalized values of eIF2α, (ii) Representative images of immunoblot showing the expression of eIF2α in BeWo cells. β-actin was used as protein loading control. (c) Comparison of normalized protein values of eIF2α between PS and PS + re-vascular endothelial growth factor-treated BeWo cells

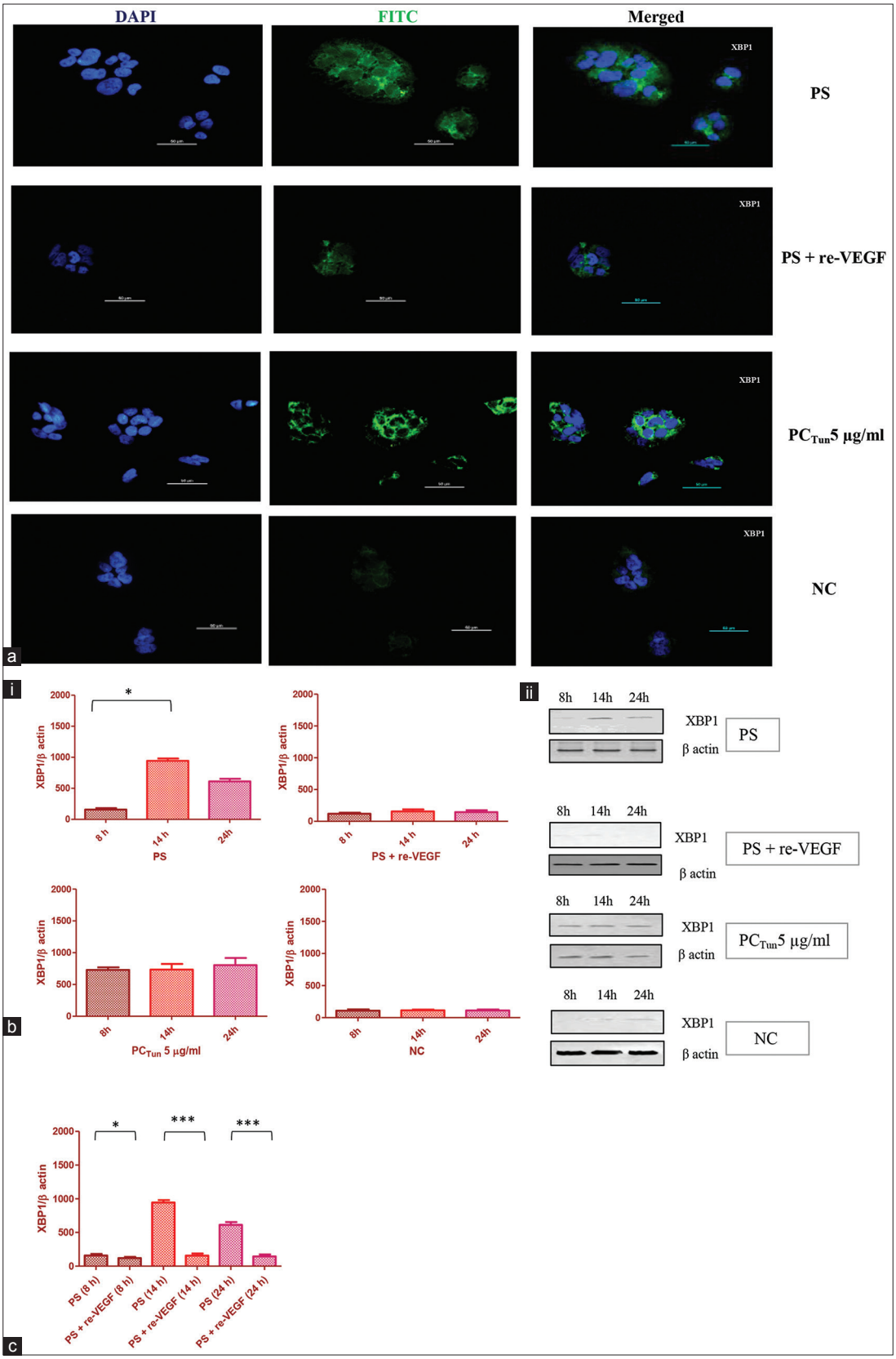

Figure 3: (a) XBP-1 immunolocalization in BeWo cells. Representative photomicrographs of BeWo cells showing eIF2 alpha expression following various treatments: PE sera (PS), vascular endothelial growth factor (PS + re-vascular endothelial growth factor), tunicamycin (positive control/PC$_{Tun}$ 5 µg/ml), and no treatment (negative control/NC). (b) (i) Bar diagrams represent normalized values of XBP1, (ii) Representative images of immunoblot showing the expression of XBP1 in BeWo cells. β-actin was used as protein loading control. (c) Comparison of normalized protein values of XBP1 between PS and PS + re-vascular endothelial growth factor-treated BeWo cells

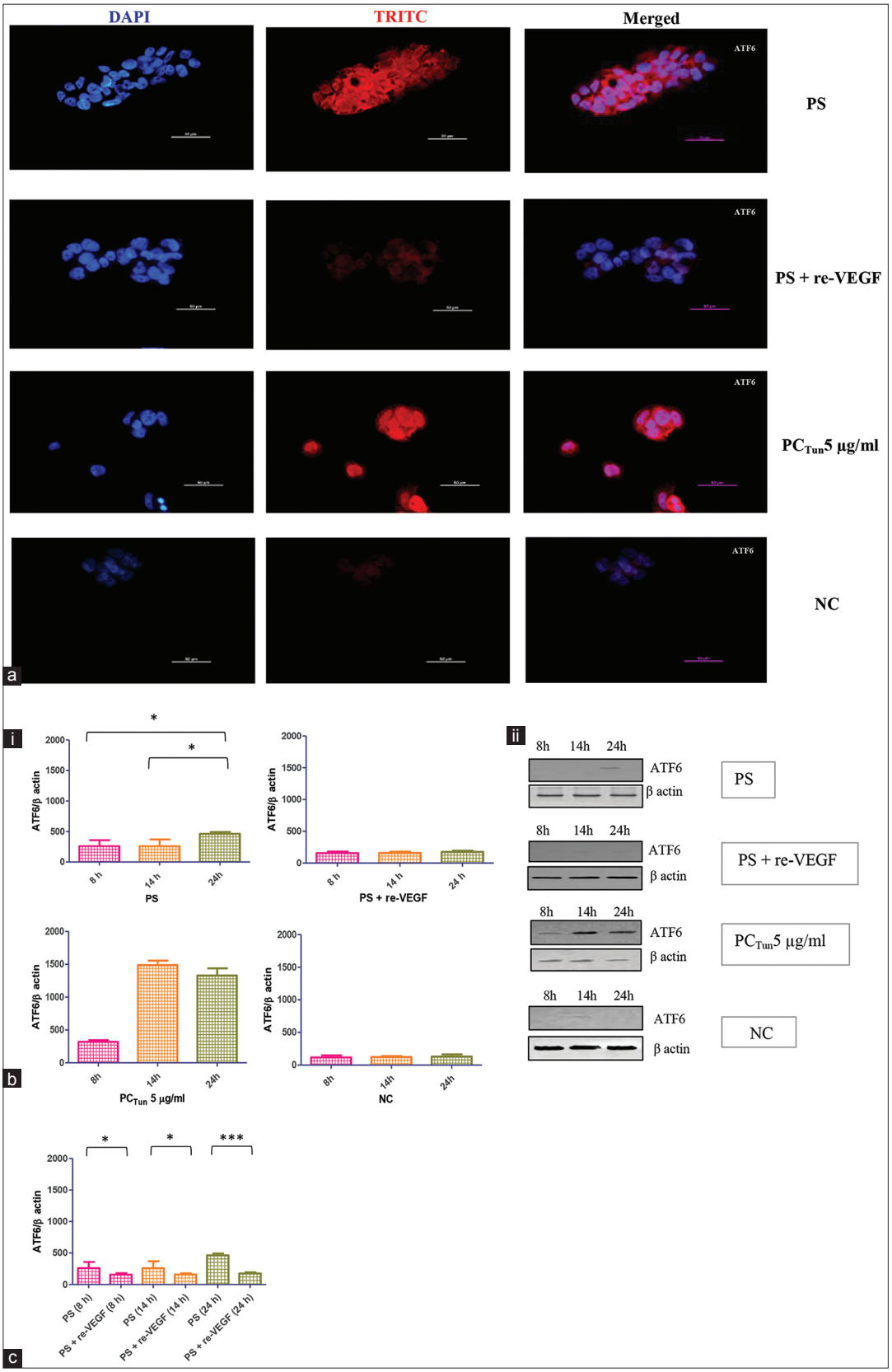

Figure 4: (a) ATF6 immunolocalization in BeWo cells. Representative photomicrographs of BeWo cells showing eIF2 alpha expression following various treatments: PE sera, PE sera + re-vascular endothelial growth factor (PS + re-vascular endothelial growth factor), tunicamycin (positive control/PC_{Tun} 5µg/ml), and no treatment (negative control/NC). (b) (i) Bar diagrams represent normalized values of ATF6, (ii) representative images of immunoblot showing the expression of ATF6 in BeWo cells. β-actin was used as protein loading control. (c) Comparison of normalized protein values of ATF6 between PS and PS + re-vascular endothelial growth factor-treated BeWo cells

also affect placental development by enhancing the stresses on the various cells constituting the placenta.

The maintenance of the placental vasculature is of critical importance as it ensures establishment of an effective maternal circulation, a process that is inextricably linked with the physiological conversion of the spiral arteries from highly tortuous and thick-walled vessels to flaccid sinusoidal conduits of low resistance.[15] Failure in the aforesaid would place the placenta at an increased risk of ischemia–reperfusion-type insult, a fierce event for the induction of oxidative stress,[9] which, in turn, leads to adverse obstetric outcomes such as preeclampsia and/or IUGR.[16] This failure of spiral artery remodeling in the placental bed of pregnancies affected by preeclampsia was first demonstrated by Pijnenborg *et al.* and later associated with a partial failure of placental trophoblast invasion.[15,17] Hypoxic placenta in PE has been shown to have higher amounts of oxidative stress. The ER stress generally is said to co-exist with oxidative stress. We have shown upregulation of one of the markers of ER stress, i.e., GRP78 in PE placenta (unpublished results). However, the cause behind this ER stress has not been explored. Thus, we hypothesized that low levels of VEGF could increase the ER stress. We observed that the expression of the three ER stress markers from the three pathways of UPR (PERK, IRE1, and ATF-6) was enhanced in BeWo cells when they were treated with PE sera. However, the increased ER stress of BeWo cells could be reversed when they were treated with patients' sera whose VEGF levels were enhanced by adding the recombinant VEGF.

Activation of PERK results in phosphorylation of eukaryotic initiation factor 2 subunit α (eIF2 α), blocking protein translation and reducing the protein burden within ER. Adaptive response goes as long as the level of stress is below threshold; however, in case of prolonged stress, eIF2α-ATF4-C/EBP homologous protein (CHOP) pathway gets activated, and it drives the cells toward apoptosis. In the present study, the expression of eIF2α was maximum at 24 h in cells treated with lower VEGF concentration (PS: group A), indicating the delayed attenuation time of PERK arm, which is consistent with the study carried out by Ron and Walter in 2007 where they observed eIF2α signal till 30 h,[18] whereas addition of re-VEGF to preeclamptic sera (PS + re-VEGF, Group B) reduced the eIF2α expression at all the time points.

In the present study, the expression of XBP1 was maximum at 14 h in cells treated with low VEGF concentration (preeclamptic sera: group A). Addition of re-VEGF to PE sera (Group B) reduced the XBP1 expression. No trigger for ER stress has been identified that selectively elicits only protective responses or only apoptosis. Instead, ER stress activates all UPR signaling pathways, thereby simultaneously producing antagonistic outputs.[19] Thus, in order to address this paradox, we examined the molecular signaling of all the three cell fate regulators of UPR (IRE1, PERK, and ATF6). The opposing effects of IRE1 and PERK determine whether ER-stressed cells will live or die.[20] Our study demonstrated that although all the three branches were activated upon induction of ER stress, yet the behavior of individual signaling pathways varied markedly with time after the onset of stress.

The mammalian genome encodes two isoforms of IRE1, IRE1α, and IRE1β. IRE1α was identified as a positive regulator for cell survival. It was believed that IRE1α signaling was terminated during irremediable ER stress to enable apoptosis.[21-23] A dissociation of IRE1α from GRP78 (BiP) due to an elevated level of unfolded proteins in the ER leads to the activation of IRE1α. As an ER transmembrane protein, it monitors ER homeostasis through an ER luminal stress-sensing domain and triggers UPR through a cytoplasmic kinase domain and an RNase domain.[24] It initiates diverse downstream signaling of the UPR either through unconventional splicing of the transcription factor XBP1 or and through posttranscriptional modifications via Regulated IRE1-Dependent Decay of multiple substrates.[25-28] The spliced XBP1 enters into the nucleus to transcriptionally reprogram UPR target genes.

In the present study, ATF6 expression was maximum at 24 h in Group A (preeclamptic sera-treated BeWo cells). Addition of re-VEGF to PE sera (Group B) reduced the ATF6 protein levels. The outcome of the present study is harmonious with that by Karali *et al.* where they demonstrated the protective effect of VEGF on induction of ATF6 and PERK arms of UPR in the context of governing of mTORC2-mediated phosphorylation of AKT on Ser473.[29] In addition, in coherence of our results, Karali *et al.* found that ATF6, or eIF2α, dramatically inhibited VEGF-induced vascularization in mouse matrigel plugs, suggesting that the ER and the UPR machinery constitute components of the VEGF signaling circuit that regulates EC survival and angiogenesis, extending their role beyond adaptation to ER stress.[29]

Mammals possess two homologous ATF6 proteins, ATF6α and ATF6β.[30] In response to ER stress, ATF6 translocates from the ER to the Golgi where it is first cleaved by site-1 protease in its luminal domain, separating ATF6 into two halves. The NH2-terminal half remains anchored to the membrane and is subsequently cleaved by site-2 protease. The cytosolic domain of ATF6 is then liberated from the membrane and translocates to the nucleus and induces the expression of genes with ER stress response element in their promoter such as the ER-chaperone protein Bip and the transcriptional factors CHOP and XBP1.[31] Since long time, ATF6 has been considered to fulfill merely adaptive functions during ER stress, and its sole intersection with ER stress-induced apoptosis consists of a possible role in the regulation of CHOP expression.[32] In future, experiments

can be planned to further understand the interaction of various angiogenic and ER stress markers and the role of ER stress sensors in the pathophysiology of preeclampsia.

Conclusion

Thus, in the present study, markers of ER stress were upregulated though at different time points when BeWo cells were treated with low VEGF PE sera. However, the increased ER stress of BeWo cells could be reversed when they were treated with patients' sera whose VEGF levels were enhanced and sFlt-1 was reduced by adding the recombinant VEGF. Thus, further studies are required to identify various pharmacological agents to increase pro-angiogenic factors such as VEGF and simultaneously reduce anti-angiogenic factors such as sFlt-1, which would alleviate the various stresses not only on placenta but also on the mother and fetus and later may provide therapeutic options for preeclampsia. If such agents are effective in reducing the manifestations of the disease, the delivery could then be safely postponed for even a few weeks, which could have a significant impact on neonatal morbidity and mortality.

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Conflicts of interest

There are no conflicts of interest.

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