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Original Article

Role of sVEGFR (sFlt-1) in inducing endoplasmic reticulum stress in trophoblast cells and its status in preeclampsia

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ABSTRACT

Introduction: Preeclampsia (PE) and its subtypes (early and late onset) are serious concerns all across the globe affecting about 8% of overall pregnancies and accounts for approximately 60,000 deaths annually with a predominance in developing countries. The two-stage model, deficient spiral artery remodelling (stage I) and an imbalance between angiogenic and anti-angiogenic factor(s) (stage II) are well established facts so far. Increased sFlt-1 along with high oxidative stress and endoplasmic reticulum stress (ER stress) have recently been suggested in pregnancies with PE. The second decade of 21st century highlighted a new window to explore further the role of endoplasmic reticulum stress in the onset of the variant forms of PE. In our previous studies, we reported apoptosis and oxidative stress, induced by sFlt-1 in trophoblast cells. However, the role of sFlt-1 in inducing ER stress is still an unrevealed hugger-mugger till date.

Methods: Study was divided into two parts (1) Serum Analysis of sFlt-1 and GRP78 was done using ELISA (2) *In Vitro* experiments: Activation of ER stress markers (GRP78, eIF2α and CHOP) were assessed at various time points (8 h, 14 h, 24 h) at protein (Immunofluorescence, Western blot) and transcript level (qRT-PCR).

Results: Significant raised levels of sFlt-1 and GRP78 in preeclamptic sera was found. We observed significant ER stress in the placental cells (BeWo Cells) (*in vitro*) when exposed to normotensive sera with recombinant sFlt-1 and also when treated with recombinant sFlt-1 alone.

Discussion: We reported significant ER stress in the placental cells (BeWo Cells) (*in vitro*) when exposed to normotensive sera with recombinant sFlt-1 and also when treated with recombinant sFlt-1 alone.

1. Introduction

Hypertensive disorders of pregnancy are an important cause of severe morbidity, long term disability and death among both mothers and their babies. *inter alia*, Preeclampsia (PE) stands out as a major subset. The entity for the underlying pathophysiology of the disease preeclampsia is the placenta, as delivery of the same is the only effective intervention to abolish the symptoms. Inasmuch as pathophysiological changes exist from very early stages of the pregnancy, hypertension and proteinuria usually become ostensible in the second half of pregnancy (WHO report, 2011). Modest placental development is governed by censorial equilibrium of vascular factors (pro-angiogenic and anti-angiogenic). One such protein-factor is the soluble form of the receptor, vascular endothelial growth factor receptor-1 (sVEGFR-1/sFlt1-1), which is produced in excess by the placenta of women with PE in first trimester.[1] The binding of sFlt1-1 to VEGF and PlGF scale down the concentrations of free VEGF and PlGF in prelusive gestation which are essential for nervy vasculogenesis, angiogenesis, trophoblast cell proliferation and thereby affecting the normal placental development.[2] The resultant imbalance amongst molecular-vascular factors (PlGF,VEGF and sFlt-1) and subsequent shortfall in bioavailability of VEGF during preeclampsia may also result in higher vulnerability to placental oxidative cell damage.[3] Apart from imbalance in angiogenic factors, other factors such as inflammation, immunological interactions and oxidative stress

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etc. also play a vital role in pathophysiology of the disease. Production of reactive oxygen species (ROS) (oxidative stress) has been linked to endoplasmic reticulum (ER) stress and the unfolded protein response (UPR)[4] (Fig. 1).

ROS plays a critical role in several cellular processes which is generated in the cytosol and several organelles, including the ER and mitochondria.[5] ER stress represents one component of a set of integrated cellular responses to stress in cellular premises. Perturbations of ER homeostasis may result in misfolding or abnormal glycosylation of these proteins which have the potential to reduce their biological activity. Stimuli such as accumulation of unfolded proteins, nutrient deprivation and the oxidative stress can induce ER stress and activate unfolded protein response (UPR) which is a cellular self-defense mechanism, aiming to alleviate ER stress and reestablish homeostasis. The UPR attempts to restore ER functions by attenuating protein translation, increasing folding capacity and facilitating degradation of misfolded proteins. Under conditions of elevated ER stress, there is an increase in transcription of specific subset of genes that lead to increase in ER protein folding capacity.[6] The three arms (PERK, IER1 and ATF6) of ER stress pathways primarily tend to maintain the proteostasis of the cellular ambience however beyond certain threshold, death signaling cascade come into role. PERK and IRE1 arms mediate translational control. Phosphorylation of eIF2α eventuates in case of PERK pathway to alleviate protein overload; IRE1 pathway induces mRNA decay, it also minify translation load and hence is the necessity for folding capacity within ER.[7,8]

The circulating levels of sFlt-1 may influence multiple endpoints in pathophysiology of PE, many of which have been previously shown to be dysregulated in preeclampsia but its effect on endoplasmic reticulum stress markers at different time points (*in vitro*) however is yet to be studied. Thus a baseline study was planned to find out whether the sFlt-1 can induce ER stress and how does it affect the poise amidst adaptive and apoptotic signaling during the ER stress *in vitro*.

2. Materials and methods

2.1. Study subjects

60 pregnant women were enrolled from the antenatal clinic and the inpatient ward of the Department of Obstetrics and Gynaecology, All India Institute of Medical Science, New Delhi, India. The preeclamptic women (n = 30) after clinical diagnosis were enrolled as cases and normotensive, non-proteinuric pregnant women (n = 30) (maternal and gestational age matched) without any other medical complications were enrolled as controls. Protocol of the study was approved by the institute ethics committee and written informed consent was obtained from all the enrolled women. Preeclampsia was defined according to ACOG guidelines: Blood Pressure- 140mm Hg systolic or >90 mm Hg diastolic on 2 occasions at least 4 h apart after 20 weeks of gestational age in women with a previously normal BP and 160 mm Hg systolic or >110 mm Hg diastolic, confirmed within a short interval (minutes) to facilitate timely antihypertensive therapy; Proteinuria >300 mg per 24-hour urine collection or protein/ creatinine ratio > 0.3 mg/dl or dipstick reading of >1+ or in the absence of proteinuria, new-onset 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.

Pregnant women with chronic hypertension, chorioamnionitis, diabetes, renal disease, cardiac disease were excluded from the study. 5 ml of venous blood was collected, centrifuged at 1200 RPM for 4 min, serum was separated and stored in aliquots at −80 °C. The serum samples were stored for the ELISA and cell culture experiments. The experimental procedure was done in two parts.

2.2. Part I

2.2.1. Serum analysis of sFlt-1 and GRP78 using ELISA

The levels of sFlt1-1 and GRP-78 were estimated in the serum of preeclamptic and control groups by sandwich ELISA (sFlt-1 ELISA kit: R&D Systems Inc., Minneapolis, MN, U.S.A., GRP 78: Enzo Life Sciences, Inc.).

2.3. Part II

In vitro experiments were carried out to analyze the effect of sFlt1-1 on induction of ER stress in trophoblastic cells (BeWo cells). The human choriocarcinoma cell line (BeWo) was procured from American Type Culture Collection (ATCC) and maintained in F-12 HAM nutrient medium supplemented with 10% fetal bovine serum, 100 U/ml pencillin, 100 μg/ml Streptomycin. Cells were passaged with 0.025% trypsin and 0.01% EDTA (Fig. 2).

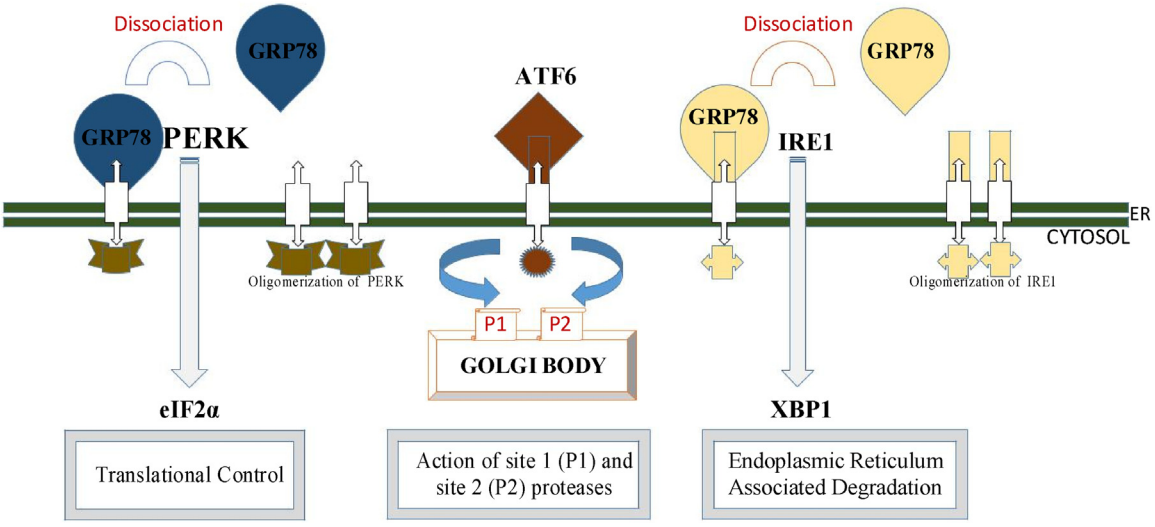

Fig. 1. Unfolded Protein Response and Endoplasmic Reticulum stress Pathway.

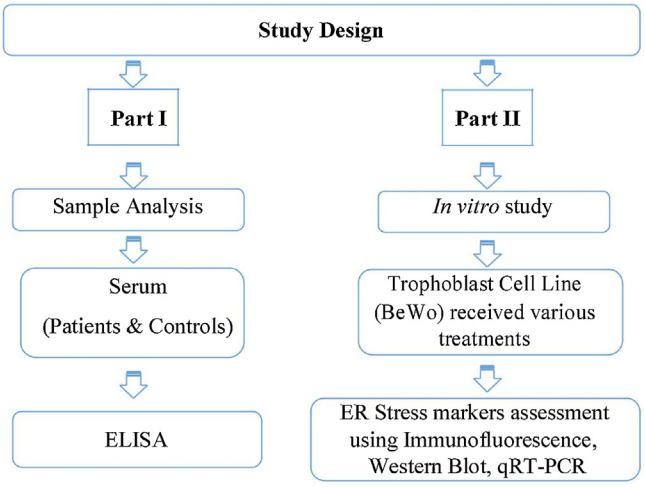

Fig. 2. Plan of Study.

The study was divided into five experimental groups depending on various treatments given to BeWo cells (Fig. 3). After the various treatments, activation of ER stress markers (GRP78, eIF2α and CHOP) were assessed at various time points (8 h, 14 h, 24 h) at protein level (Immunofluorescence, Western blot) and gene level (qRT-PCR).

2.3.1. Immunofluorescence microscopy

BeWo Cells were trypsinized, seeded, allowed to grow on coverslips in multiple well chamber and incubated at 37°C in 5% CO2. After 8 h, 14 h and 24 h, cells were taken out from incubator and washed with PBS, fixed in 4% PFA for 15 min at room temperature. After fixation, cells were washed with PBS and permeabilized with PBS + 0.1% Triton X-100 followed by PBS washing. Nonspecific blocking was done using 5% normal goat serum in PBS and Triton X. Cells were incubated for 12 h at 4°C in primary antibodies (anti GRP78 antibody (1:1000), anti eIF2α antibody (1:200), anti DDIT3/CHOP antibody (1:500). Cells were washed with PBSTx and thereafter incubated in secondary antibody in 1:500 dilution for 1 h at room temperature in dark room. Cells were washed in PBS and mounted with flouroshield mounting media with DAPI on the slide and observed under the fluorescence microscope (Nikon Eclipse Ti-S elements using NiS-AR software).

2.3.2. Western blot analysis

Cells were lysed in SDS-PAGE sample buffer [2% SDS, 60 mM Tris–HCl (pH 6.8), 10% glycerol, 0.001% bromphenol blue, 0.33% mercaptoethanol] and boiled for 5 min. The lysates were analyzed by immunoblotting using 1:1000 of anti GRP78, 1:500 of anti eIF2α and 1:1000 of anti DDIT3/CHOP (Abcam) 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 H2O2. β-actin was used as protein loading control. For densitometric analysis, the blots were scanned in a gel

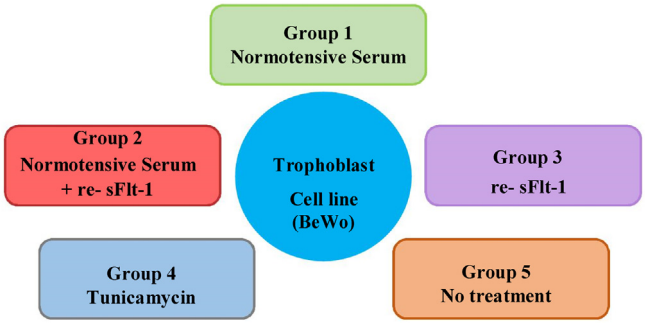

Fig. 3. Experimental groups.

documentation system, using Quantity 1 software (Bio-Rad, Hercules, CA, USA).

2.3.3. qRT-PCR (quantitative real time-polymerase chain reaction)

RNA extraction was done from treated cells *via* Ambion (Invitrogen) kit followed by c-DNA conversion by Thermo revert aid H-minus reverse transcriptase kit. cDNA was amplified by quantitative RT-PCR for determining mRNA expression of GRP78, eIF2α and CHOP against gene of interest with an internal control (β actin and GAPDH).

2.4. Statistical analysis

Data was analyzed by Microsoft Office Excel Version 2013 and Graph Pad Prism. For mRNA expression by qRT-PCR, relative quantification cycles of gene of interest (ΔCq) was calculated by ΔCq = Cq (target) - Cq (reference). Relative mRNA expression with respect to internal control gene was calculated by $2^{-\Delta Cq}$. Data was presented in mean ± SD/median (Range) as appropriate. Average level of the variable between the two groups was compared by paired t-test/Wilcoxon signed rank test. For comparing more than two groups, ANOVA with Bonferroni's multiple comparison test/ Kruskal Wallis with Dunn's multiple comparison test were used. p value<0.05 was considered statistically significant (Table 1).

3. Results

Maternal serum of 60 pregnant women were analyzed. Of these, 30 were preeclamptic as per ACOG guidelines and 30 were age (gestational and maternal) matched normotensive, non-proteinuric pregnant women, who served as control. The mean systolic and diastolic blood pressures in preeclamptic group were 158.9 ± 11.88 mm Hg (mean ± SD) and 101.43 ± 8.39 mm Hg (mean ± SD) respectively where as in control group, the systolic and diastolic pressures were 117.8 ± 7.34 mm Hg (mean ± SD) and 74.2 ± 6.39 mm Hg (mean ± SD) respectively. The difference in the systolic and diastolic pressures between the groups were statistically significant ($p < 0.0001$). The body mass index in preeclamptic patients was 27.83 ± 5.61 (mean ± SD) and in control group was 23.67 ± 3.59 (mean ± SD). The difference was statistically significant ($p < 0.0001$). Urine protein in preeclamptic and control group

Table 1
Primers: Designed by NCBI.

Gene	Forward primer	Reverse primer
GRP78	5′-TGTTCAACCAATTATCAGCAAACTC - 3′	5′ - TTCTGCTGTATCCTCTTCACCAGT - 3′
eIF2α	5′ - AAGCATGCAGTCTCAGACCC - 3′	
CHOP	5′ - AGAACCAGGAAACGGAAACAGA - 3′	5′ - TCTCCTTCATGCGCTGCTTT - 3′
GAPDH	5′ - AGCCGAGCCACATC - 3′	5′ - TGAGGCTGTTGTCATACTTCTC - 3′
β-Actin	5′ - GAGCACAGAGCCTCGCCTTT - 3′	5′ - TCATCATCCATGGTGAGCTGG - 3′

women were analysed by urine dipstick method. 10% of preeclamptic women (3/30) showed 1+ urine protein, 43.33% showed 2+ urine protein (13/30) and 46.66% showed 3+ urine protein (14/30) (Table 2)

3.1. sFlt-1 and GRP-78 levels

The level of sFlt-1 in the maternal serum of preeclamptic patients was 11,295.25 (2936.2–37818) pg/ml and in control group was 2936.2 (1180.43–6706.6) pg/ml. The serum sFlt-1 levels were significantly higher in women with preeclampsia than in control group and the difference was statistically significant. ($p = 0.0001$) (Table 3). The level of GRP78 in the maternal serum of preeclamptic patients was 1103.26 ± 104.27 ng/ml and in control group was 1018.61 ± 125.51 ng/ml. The serum GRP 78 levels were higher in sera of women with preeclampsia than in control group and the difference was statistically significant. ($p = 0.012$) (Table 4).

3.2. In vitro *experiments*

3.2.1. Expression of ER stress markers (GRP78, eIF2α and CHOP) in normotensive sera (NT) treated BeWo cells [Fig. 4a–c]

Immunofluorescence microscopy revealed no significant expression of ER stress markers at different time points [Fig. 4a] nevertheless immunoblot manifested higher expression of GRP78 and eIF2α at 14 h as compared to 8 h and 24 h. CHOP expression was found more at 24 h as compared to 8 h and 14 h [Fig. 4b]. mRNA levels of GRP78, eIF2α, and CHOP were found to be higher at 14 h as compared to 8 h and 24 h [Fig. 4c].

3.2.2. Expression of ER stress markers (GRP78, eIF2α and CHOP) in normotensive sera along with re-sFlt-1 treated BeWo cells [Fig. 5a–c]

Immunofluorescence microscopy revealed significant expression of GRP 78 at 8 h, eIF2α at 14 h and CHOP at 24 h. Characteristic stress granules in case of eIF2α were observed at 14 h [Fig. 5a]. The expression of GRP78 and eIF2α proteins were found to be higher at 14 h as compared to 8 h and 24 h time points. Maximum expression of CHOP was found at 24 h [Fig. 5b]. mRNA levels of GRP78 and eIF2α were found to be higher at 14 h whereas CHOP mRNA levels were found to be higher at 24 h [Fig. 5c].

3.2.3. Expression of ER stress markers (GRP78, eIF2α and CHOP) in re-sFlt-1 (9 and 12 ng/ml) treated BeWo cells [Fig. 6a–c]

Significant expression of CHOP was observed at 14 h with 12 ng/ml concentration of re-sFlt-1 as compared to 9 ng/ml. Characteristic stress granules in case of eIF2α were also seen at 14 h [Fig. 6a]. Immunoblot revealed higher expressions of GRP78 and eIF2α at 14 h with 12 ng/ml concentration (c2) of recombinant sFlt-1 as compared to 9 ng/ml concentration (c1). CHOP expression was observed maximum at 24 h with 9 ng/ml (c1) concentration of recombinant sFlt-1 [Fig. 6b]. GRP78 mRNA levels were found maximum at 14 h (c2) whereas mRNA levels of eIF2α, and CHOP were found maximum at 24 h (c2) [Fig. 6c].

3.2.4. Expression of ER stress markers (GRP78, eIF2α and CHOP) in Tunicamycin treated BeWo cells [Fig. 7a–c]

The expression of all the markers were observed more so with 5 μg/ml dose of tunicamycin as compared to its lower dose [Fig. 7a]. Immunoblot data revealed higher expressions of GRP 78 and eIF2α at 14 h and 24 h as compared to 8 h. Expression was even more when its concentration was increased. CHOP expression was found to be higher with 5 μg/ml of tunicamycin as compared to 2.5 μg/ml of tunicamycin [Fig. 7b]. GRP78 and eIF2α mRNA levels were found to be higher at 14 h as compared to 8 h and 24 h. CHOP mRNA levels were found maximum at 24 h [Fig. 7c].

3.2.5. Expression of ER stress markers (GRP78, eIF2α and CHOP on untreated BeWo cells [Fig. 8a–c]

Mild expression of GRP78 at 8 h and eIF2α at 14 h was observed. However, no expression of CHOP was observed at different time points [Fig. 8a]. Western blot analysis revealed higher expression of GRP78 and eIF2α at 14 h as compared to 8 h and 24 h. CHOP expression was found maximum at 24 h [Fig. 8b]. GRP78 and eIF2α mRNA levels were found maximum at 14 h however CHOP mRNA levels were found to be higher at 24 h [Fig. 8c]

3.2.6. Comparison of normalized protein values and mRNA levels of various ER stress markers between NT sera and NT sera + re- sFlt-1 treated BeWo cells[Fig. 9a–f]

At 8 h, expressions of all ER stress markers were found to be higher in NT + re-sFlt-1 treated BeWo cells as compared to NT sera and the difference was found to be statistically significant [GRP78 ($p < 0.0001$), eIF2α ($p < 0.0001$) and CHOP ($p < 0.0001$)] [Fig. 9a–c]. At 14 h, GRP78, and eIF2α expressions were also higher in NT + re-sFlt-1 treated BeWo cells as compared to NT sera and the difference was statistically significant [GRP78 ($p < 0.0001$), eIF2α ($p < 0.0001$) and CHOP ($p < 0.0001$)] [Fig. 9a–c]. At 24 h, expressions of all ER stress markers were found to be higher in NT + re-sFlt-1 treated BeWo cells as compared to NT sera treated BeWo cells and the difference was statistically significant. GRP78 ($p < 0.0001$), eIF2α ($p = 0.0002$) and CHOP ($p < 0.0001$) [Fig. 9a–c]. At 8, 14 and 24 h, GRP 78, eIF2α, and CHOP mRNA levels were found to be higher in NT + re-sFlt-1 treated BeWo cells as compared to NT sera treated cells and the difference was found to be statistically significant [GRP78 ($p < 0.0001$), eIF2α ($p < 0.0001$) and CHOP ($p < 0.0001$)] [Fig. 9d–f].

3.2.7. Results summary

When BeWo cells were treated with normotensive sera (NT sera) whose sFlt-1 concentration was increased by addition of recombinant sFlt-1 exogenously, the expression of ER stress markers (at both protein and mRNA levels) increased as compared to normotensive sera alone treated cells. The sFlt-1 may be one of the antiangiogenic factors present in patients's sera which not only contributes to oxidative stress but also may cause endoplasmic reticulum stress. The GRP78 expression was observed in the beginning (usually 8 h time point) and started reducing with time in all the groups. However, CHOP expression appeared usually late (maximum at 24 h) as seen in cells of group2 (NT + re-sFlt-1), group3 (re-sFlt-1) and group4 (tunicamycin treatment). However signals of CHOP protein were either feeble or undetectable in cells of group1 (NT sera), and group5 (untreated). The protein and mRNA levels of eIF2α, could be visualised between 8 h and 24 h, *i.e.* the time point between GRP78 induction and appearance of CHOP signals.

Table 2
Clinical Characteristics of Preeclamptic and normotensive, non proteinuric pregnant women (controls).

Study Groups					
Clinical characteristics	Preeclampsia (n = 30)		Normotensive, Non proteinuric (Control) (n = 30)		Statistical significance (p value)*
Systolic blood pressure (mmHg)	158.9	11.88	117.8	7.34	$p < 0.0001$
Diastolic blood pressure (mmHg)	101.43	8.39	74.2	6.39	$p < 0.0001$
Body Mass Index	27.83	5.61	23.67	3.59	$p < 0.0001$
Protein (gm/day)	4.9	1.6	0.7	0.2	$p < 0.0001$

n = number of subjects, Data presented as mean ± SD, Paired *t*-test, *statistical significance, $p < 0.05$.

Table 3
Maternal serum levels of sFlt-1 in preeclamptic and normotensive, non proteinuric pregnant women (controls).

Study Groups			
Serum levels of sFlt-1	Preeclampsia Median (Range) n = 30	Normotensive non proteinuric (Control) Median (Range) n = 30	Statistical significance (p Value)*
sFlt-1	11,295.25 2936.2– 37,818	2936.2 1180.43–6706.6	0.0001

n = number of subjects, Data presented as median (range), Paired *t*-test, *statistical significance, $p < 0.05$.

Table 4
Maternal serum levels of GRP78 in preeclamptic and normotensive, non proteinuric pregnant women (controls).

Study Groups			
Serum levels of GRP78	Preeclampsia Mean SD n = 30	Normotensive non proteinuric (Control) Mean SD n = 30	Statistical significance (p Value)*
GRP78	1103 104.27	1018.61 125.51	0.012

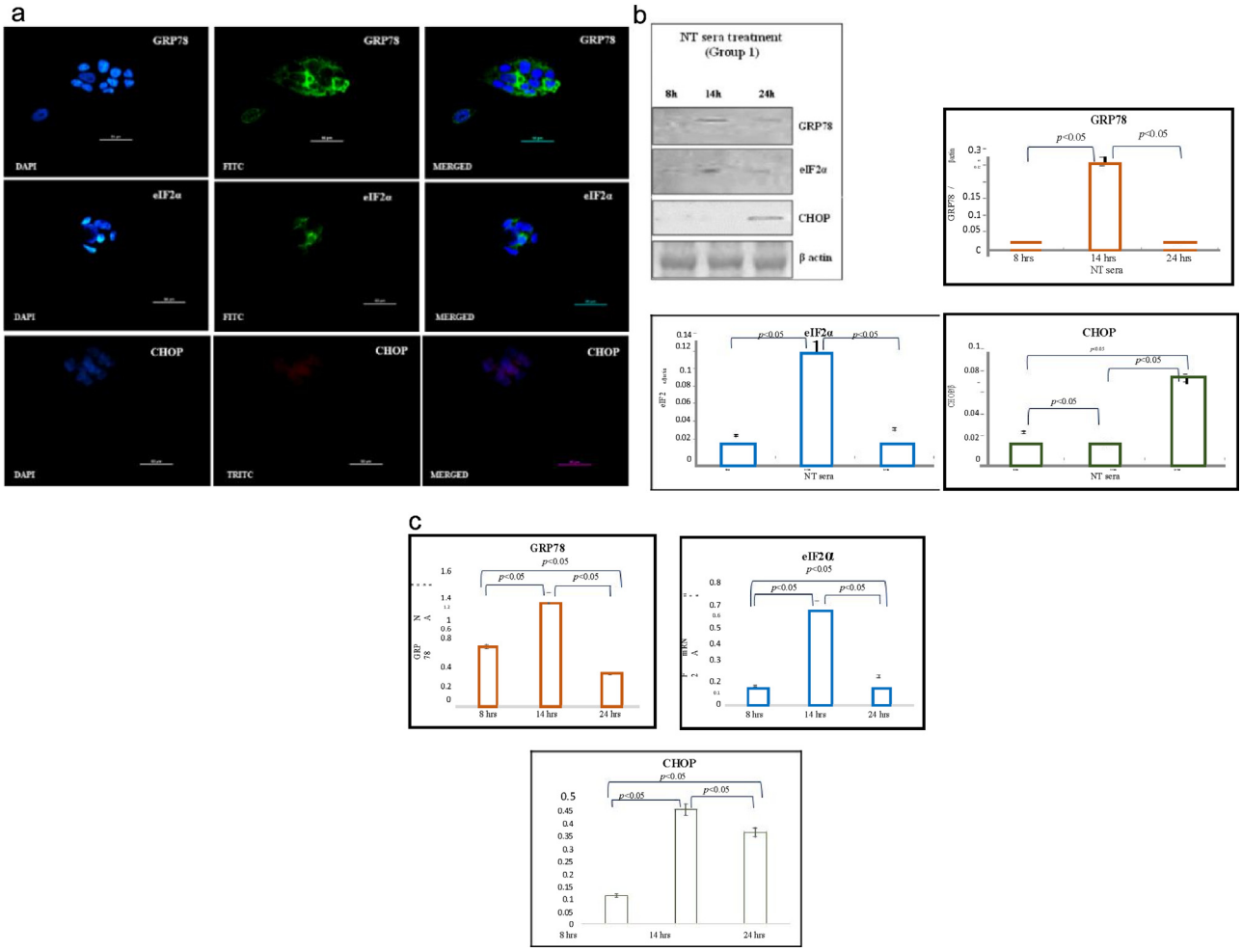

Fig. 4. (a) Representative immunofluorescence staining pattern of anti-GRP78 antibody positive BeWo cells following 8 h, anti-eIF2α antibody positive BeWo cells following 14 h and anti- DDIT3 (CHOP) antibody positive BeWo cells following 24 h treatment of NT sera; Figure 4b: Representative images of immunoblot showing the expression of ER stress markers, GRP78, eIF2α and CHOP in BeWo cells. β-Actin was used as protein loading control. The Bar diagrams represent the normalized values of the markers. Results are representative of 7 independent experiments. Data presented as mean ± SD. Statistical analysis was done using one way ANOVA with Bonferroni's post hoc; Fig. 4c: Bar diagrams represent the relative mRNA expression of GRP78, eIF2α, and CHOP and was found maximum at 14 h. GAPDH was used as positive control. Data presented as mean ± SD. One way ANOVA with Bonferroni's post hoc test was applied (*p* values indicated on graph itself).

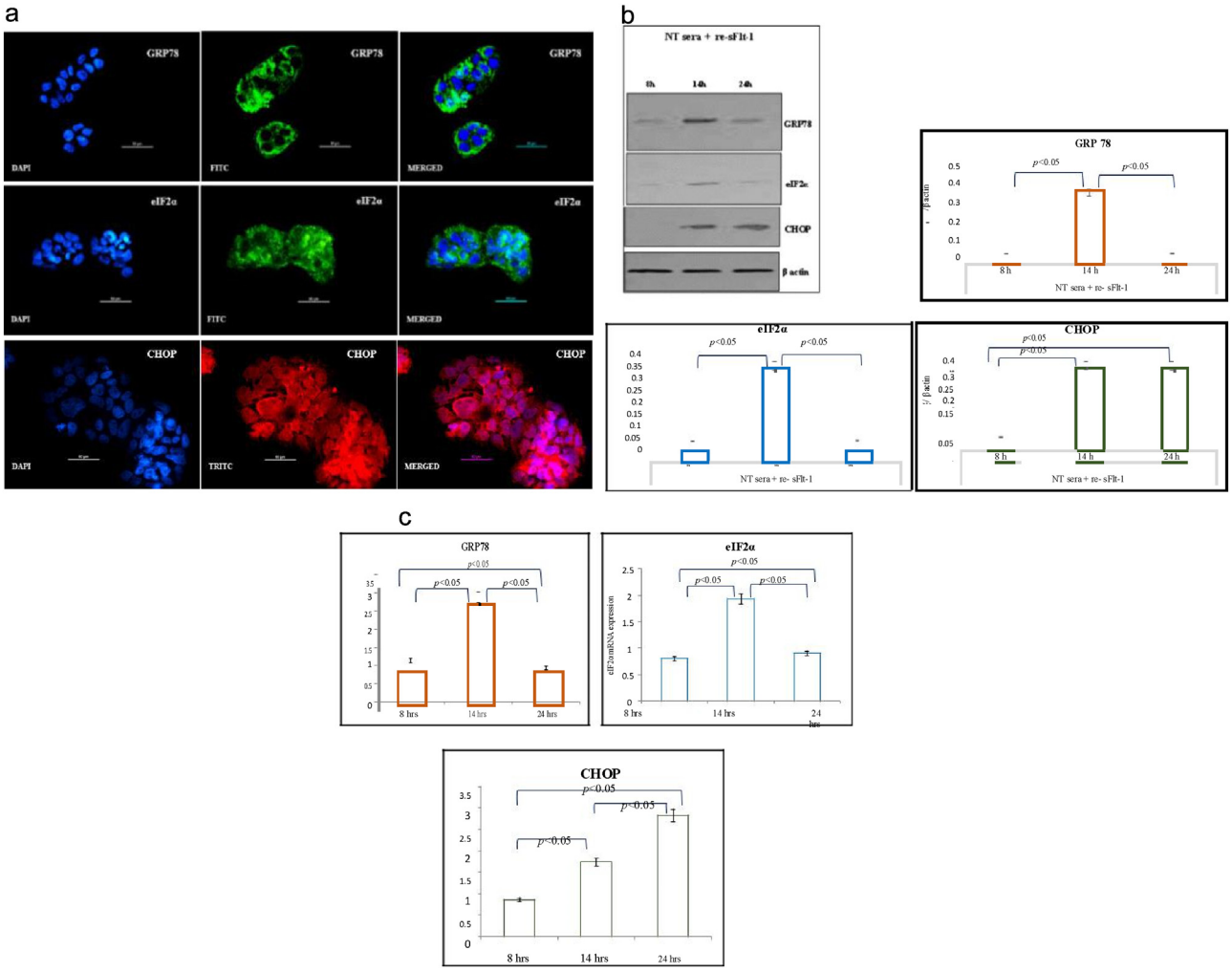

Fig. 5. (a) Representative immunofluorescence staining pattern of anti-GRP78 antibody positive BeWo cells following 8 h, anti-eIF2α antibody positive BeWo cells following 14 h and anti- DDIT3 (CHOP) antibody positive BeWo cells following 24 h treatment of NT sera + re- sFlt-1; Fig. 5b: Representative images of immunoblot showing the expression of ER stress markers, GRP78, eIF2α and CHOP in BeWo cells. β-Actin was used as protein loading control. The Bar diagrams represent the normalized values of the markers. Results are representative of 7 independent experiments. Data presented as mean ± SD. Statistical analysis was done using one way ANOVA with Bonferroni's post hoc; Fig. 5c: Bar diagrams represent the relative mRNA expression of GRP78 and eIF2α was found maximum at 14 h whereas at 24 h for CHOP. GAPDH was used as positive control. Data presented as mean ± SD. One way ANOVA with Bonferroni's post hoc test was applied (p values indicated on graph itself).

4. Discussion

The poise and propinquity amongst vascular factors (angiogenic and anti-angiogenic) play vital role in successful pregnancy. VEGF, an angiogenic growth factor is a key survival agent as this promotes neovascularization, contributes to maintenance of vascular tone by influencing NO production, reduces blood pressure and is important for maintenance of normal glomerular filtration[9] and stabilizes endothelial cells in mature blood vessels. It is therefore, low levels of VEGF could cause disturbances in the placental development during pregnancy. Under normal physiological conditions, VEGF exerts its biological effects through VEGFR1 or fms like protein kinase-1/Flt-1 and VEGFR2 or kinase insert domain receptor/KDR.[10] VEGFR1 has two isoforms: a transmembrane and soluble isoform. The soluble isoform of VEGFR1 (Flt-1) known as s-VEGFR1(sFlt-1), binds with free VEGF in circulation with higher affinity thereby preventing its interaction with its endogenous receptors (VEGFR1 and VEGFR2) and thus reducing its biological effects like placental development and maintenance of maternal vasculature. Therefore, whenever serum levels of sFlt-1 increase, their binding with VEGF may reduce the circulating (free) VEGF levels below a critical threshold and thus adversely affecting the placental development and in turn pregnancy because VEGFs are essential for vasculogenesis and angiogenesis.

Our group for the first time reported the alterations in the levels of anti-angiogenic (sFlt-1) and pro-angiogenic (VEGF, PlGF) factors in blood sera and PlGF in the urine of PE patients in the patients of Indian origin.[11] In the present study we aimed to explore the role of sFlt-1 in inciting endoplasmic reticulum (ER) stress, in trophoblast cells (choriocarcinoma of placental origin-BeWo cells). We estimated the levels of sFlt-1 (Table3) and GRP78 (Table4) (central regulator of ER stress) in blood serum of 30 each of healthy pregnant women (normotensive, non proteinuric) and preeclamptic women. These sera were later used for *in vitro* experiments. The serum sFlt-1 levels found more in preeclamptic women [11,295.25 (2936.2–37818)] pg/ml than in gestational and maternal age matched normotensive and non proteinuric pregnant women [2893.20 (1180.43–6706.6)] ($p = 0.0001$) suggested that an anti-angiogenic state may be involved in the pathogenesis of PE.[12] Multiple reports, globally also provide persuasive evidence to support the concept that sVEGFR-1 has a role in the pathogenesis of preeclampsia: the plasma and/or serum levels ofsVEGFR-1/sFlt-1 are higher in women with prececlampsia than in women with normal pregnancies, however the reason for increased

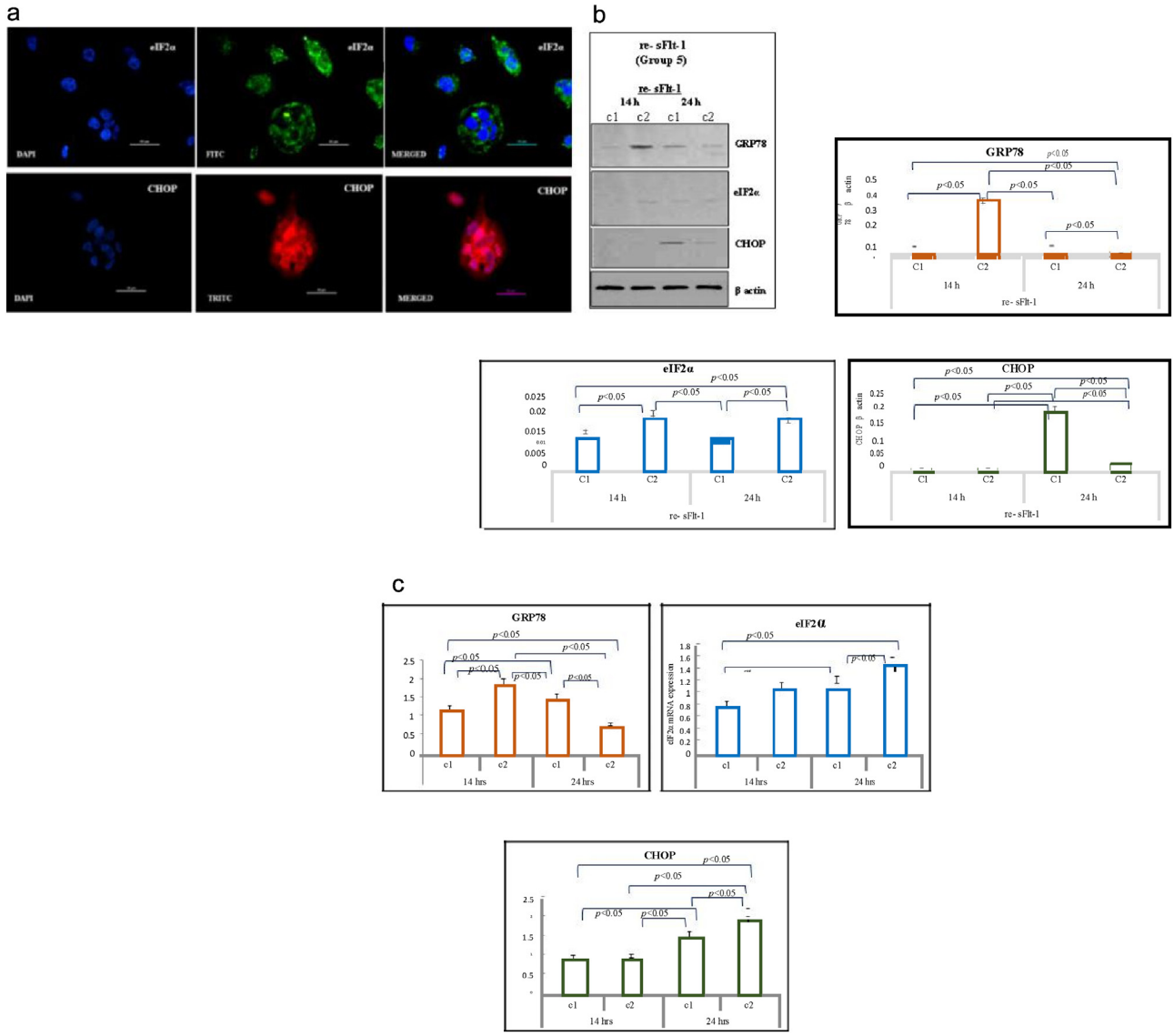

Fig. 6. (a) Representative immunofluorescence staining pattern of anti-eIF2α and anti- DDIT3 (CHOP) antibody positive BeWo cells following 14 treatment of re- sFlt-1 (12 ng/ml); Fig. 6b: Representative images of immunoblot showing the expression of ER stress markers, GRP78, eIF2α and CHOP in BeWo cells. β-Actin was used as protein loading control. The Bar diagrams represent the normalized values of the markers. Results are representative of 7 independent experiments. Data presented as mean ± SD. Statistical analysis was done using one way ANOVA with Bonferroni's post hoc; Fig. 6c: Bar diagrams represent the relative mRNA expression of GRP78 was found maximum at 14 h (12 ng/ml)) whereas mRNA levels of eIF2α and CHOP were found maximum at 24 h (12 ng/ml). GAPDH was used as positive control. Data presented as mean ± SD. One way ANOVA with Bonferroni's post hoc test was applied (p values indicated on graph itself).

placental sFlt-1 release from trophoblast is still unknown. On the other hand, decreased plasma and/or serum levels of free VEGF and placental growth factor (PlGF) were also found associated with PE.[13] Sera from women with preeclampsia demonstrated anti angiogenic effects on endothelial tube formation and this effect could be reversed by addition of VEGF and PlGF.[12]. The altered balance between pro and anti angiogenic factors (VEGF & sFlt-1) and increased oxidative stress contribute to endothelial dysfunction in preeclampsia.[14] Also sFlt-1 plays a role in oxidative stress, resulting in activation of apoptosis in trophoblast cells.[15] Thus a vicious cycle between trophoblast dysfunction and oxidative stress exists in the PE placenta that is enhanced by sFlt-1. This could be of great importance depicting mechanism of progression of PE.

Apart from oxidative stress, placental endoplasmic reticulum stress has been reported in preeclamptic women.[16] The extensive secretory activity of the syncytiotrophoblast renders it

vulnerable to ER stress, and molecular and morphological evidence confirms high levels of stress in placentas from case of early onset of preeclampsia as compared to late onset.[17] Ischemia–reperfusion type injury at maternal fetal interface is a strong possibility of ER stress in preeclampsia.[17]

The central regulator of UPR, GRP78, after dissociation form trans-membrane sensors (PERK, IRE1 and ATF6) activates signaling cascade from the ER lumen to cytosol and nucleus. The present study aimed to explore whether the increased levels of sFlt-1 in the preeclamptic patients had any correlation with the central regulator of UPR and thus in turn to ER stress. Therefore we screened the GRP78 levels in maternal sera of preeclamptic patients and corresponding gestational age matched controls. The GRP78 levels were found to be higher in PE patients [1103.26 ± 104.27 {mean (SD)] as compared to normotensive, non-proteinuric [1018.61 ± 125.51 {mean (SD)}] pregnant women and the difference was statistically significant (p=0.023) (Table 4). Thus the higher levels of circulating GRP78 in patient's sera suggest the dissociation of GRP78 from the transmembrane sensors of ER and their subsequent release in the maternal serum thereby initiating/

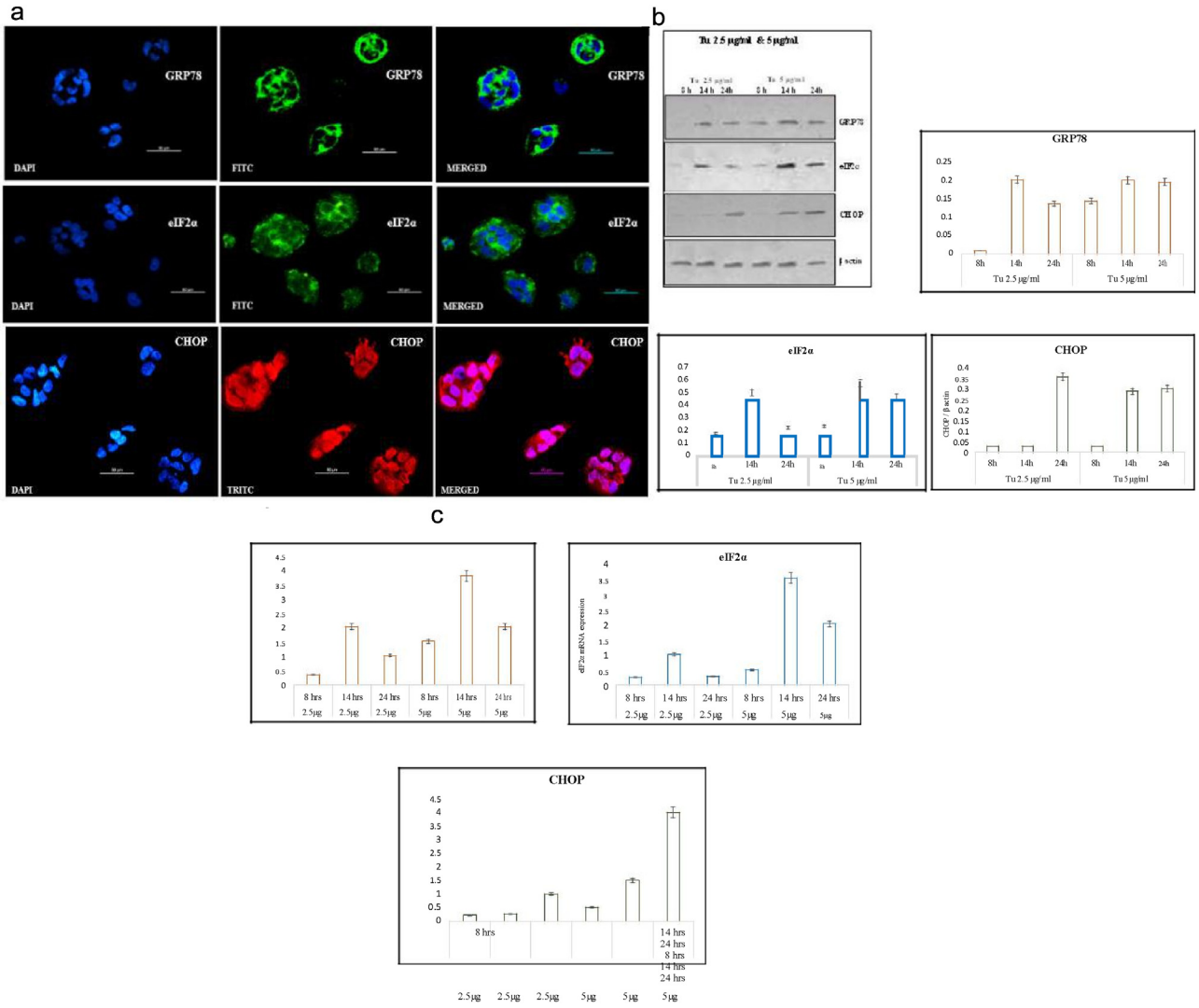

Fig. 7. (a) Representative immunofluorescence staining pattern of anti-GRP78 antibody positive BeWo cells following 8 h, anti-eIF2α antibody positive BeWo cells following 14 h and anti- DDIT3 (CHOP) antibody positive BeWo cells following tunicamycin treatment (5 µg/ml). Fig. 7b: Representative images of immunoblot showing the expression of ER stress markers, GRP78, eIF2α and CHOP in BeWo cells. β-Actin was used as protein loading control. The Bar diagrams represent the normalized values of the markers. Results are representative of 7 independent experiments. Data presented as mean ± SD. Statistical analysis was done using one way ANOVA with Bonferroni's post hoc; Fig. 7c: Bar diagrams represent the relative mRNA expression of GRP78 and eIF2α was found maximum at 14 h whereas at 24 h for CHOP. GAPDH was used as positive control. Data presented as mean ± SD. One way ANOVA with Bonferroni's post hoc test was applied (p values indicated on graph itself).

activating the ER stress. We also found that patients with increased sFlt-1 in their sera had higher GRP78 levels suggesting a positive correlation (r = 0.2439). However these results did not establish a cause and effect relationship *i.e.* whether the sFlt-1 is the causative factor for increased GRP78 found in PE patients. In order to answer this question, *in-vitro* experiments were performed. The human choriocarcinoma cell line (BeWo) was given various treatments with varying sFlt-1 concentrations and the expression of ER stress markers were analyzed using immunofluorescence, western blot and quantitative real time PCR at different time points. In the present study, the signaling response of ER stress markers was observed at different time points (8 h, 14 h and 24 h) when the BeWo cells were exposed to low levels of sFlt-1 (normotensive sera). Also the response of various ER stress markers were analyzed after adding recombinant sFlt-1 (re-sFlt-1) to normotensive sera. The prime regulator of UPR, the GRP78, normally binds to N-terminus of ER stress sensors in resting condition and in the absence of stress, but withdrawal of this molecular chaperone by competitive binding to accumulating misfolded proteins causes dimerization, autophosphorylation and activation of PERK, IRE1 and ATF6. Expression of this chaperone was observed as early as 8 h, reached the peak at 14 h and it started diminishing after 14 h when BeWo cells were exposed to normotensive sera (Fig. 4a,b). GRP78 expression was further upregulated when BeWo cells were treated with normotensive sera along with re-sFlt-1 (Fig. 5a,b) as compared to NT sera alone (p<0.0001) at all the time points (8 h, 14 h, 24 h) (Fig. 9a). Similar pattern of expression was observed at mRNA levels at all time points 8h, 14h and 24h (p<0.0001) (Figs. Fig. 44c, 5c, 9d). Activation of PERK results in the phosphorylation of eukaryotic initiation factor 2 subunit α (eIF2 α), blocking protein translation and reducing the protein burden within the ER.[18] Adaptive response goes as long as the level of stress is below threshold, however in case of prolonged stress, eIF2α-ATF4−CHOP pathway gets activated and it drives the cells toward apoptosis. In the present study, expression of eIF2α was observed maximum at 14 h when BeWo cells were exposed to low sFlt-1 concentration (normotensive sera) (Fig. 4a,b). Addition of re-sFlt-1 to NT sera further enhanced the expression of eIF2α (Fig. 5a,b) and the difference between NT sera and NT sera + re-sFlt-1 treated cells was statistically significant at all

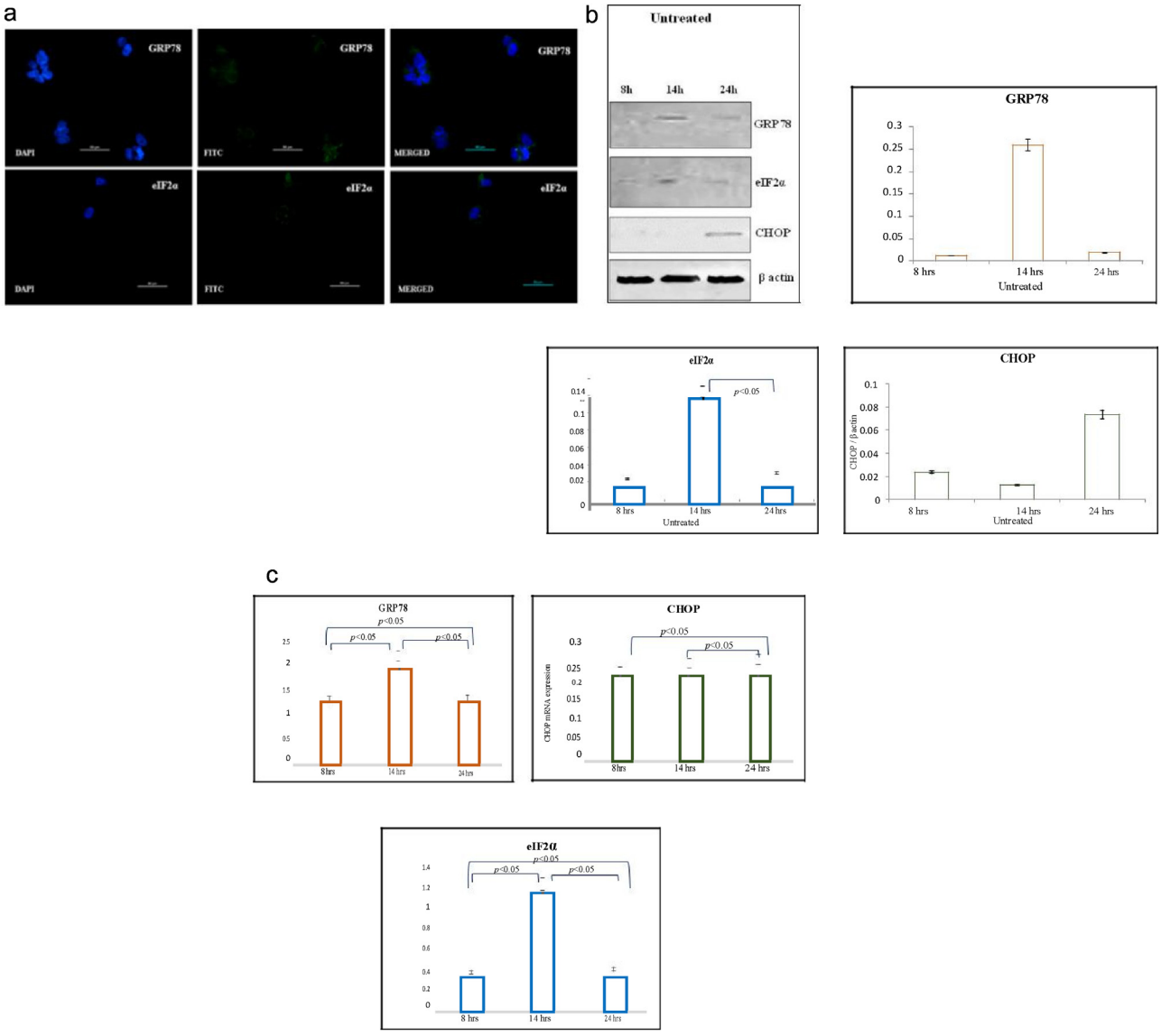

Fig. 8. (a) Representative immunofluorescence staining pattern of anti-GRP78 antibody positive BeWo cells following 8 h and anti-eIF2α antibody positive BeWo cells following no treatment. Fig. 8b: Representative images of immunoblot showing the expression of ER stress markers, GRP78, eIF2α and CHOP in BeWo cells. β-Actin was used as protein loading control. The Bar diagrams represent the normalized values of the markers. Results are representative of 7 independent experiments. Data presented as mean ± SD. Statistical analysis was done using one way ANOVA with Bonferroni's post hoc; Fig. 8c: Bar diagrams represent the relative mRNA expression of GRP78 and eIF2α found maximum at 14 h and at 24 h for CHOP. GAPDH was used as positive control. Data presented as mean ± SD. One way ANOVA with Bonferroni's post hoc test was applied (p values indicated on graph itself).

the time points (8 h, p=0.0001; 14 h, p<0.0001; 24 h, p= 0.0002) (Fig. 9b). The expression of eIF2α at mRNA levels delineated similar pattern as of protein expression when BeWo cells were treated with NT + re-sFlt-1 as compared to NT sera at all time points 8h, 14h and 24h (p<0.0001) (Fig. 9e). PERK/eIF2α-dependent induction of the pro-apoptotic transcriptional factor CHOP (CCAAT-enhancer-binding protein homologous protein) has been shown to be involved in ER stress induced apoptosis both *in vitro* and *in vivo*.[19] CHOP-deficient mouse embryonic fibroblast cells (MEFs) were partially protected against ER stress-induced apoptosis, and kidneys of CHOP−/− mice showed less apoptosis after treatment with the ER stressor tunicamycin, an inhibitor of glycosylation.[20] Several CHOP target genes have been implicated in ER stress-induced apoptosis: members of the BCL-2 family, GADD34, the pseudokinasetribbles-related 3 (TRB3), and ER oxidase 1α (ERO1α). CHOP was shown to downregulate transcription of anti-apoptotic BCL-2.[21] CHOP can transcriptionally upregulate expression of pro-apoptotic BH3-only protein Bim which is a mediator of ER stress-induced apoptosis in several cell types,[22]. [23] However, the upregulation of transcription factor CHOP is evidenced by pro-apoptotic consequence of eIF2α phosphorylation.[24] In the present study, CHOP expression was maximum at 24 h in BeWo cells exposed to NT sera (Fig. 4a,b). Addition of re-sFlt-1 to NT sera further enhanced the expression of CHOP (Figure 5a,b) and the difference between NT sera and NT + re-sFlt-1 treated cells was statistically significant at all the time points (8 h, p<0.0001; 14 h, p<0.0001; 24 h, p<0.0001) (Fig. 9c). We observed similar expression pattern of CHOP at mRNA levels as that of protein expression at all time points 8h, 14h and 24h (p<0.0001) (Figs. 4c, 5c, 9f). In the present study, BeWo cells, when treated with two independent concentrations of recombinant sFlt-1, no significant expression of ER stress markers were seen either at protein or mRNA levels at 8 h (Fig. 6a–c). At 14 h, upregulation of GRP78 and eIF2α noticed with 12ng/ml (Fig. 6a–c). On the other hand, expression of CHOP was maximum at 24 h (Fig. 6a–c) indicating increased apoptosis reflecting prolonged stress with progression of

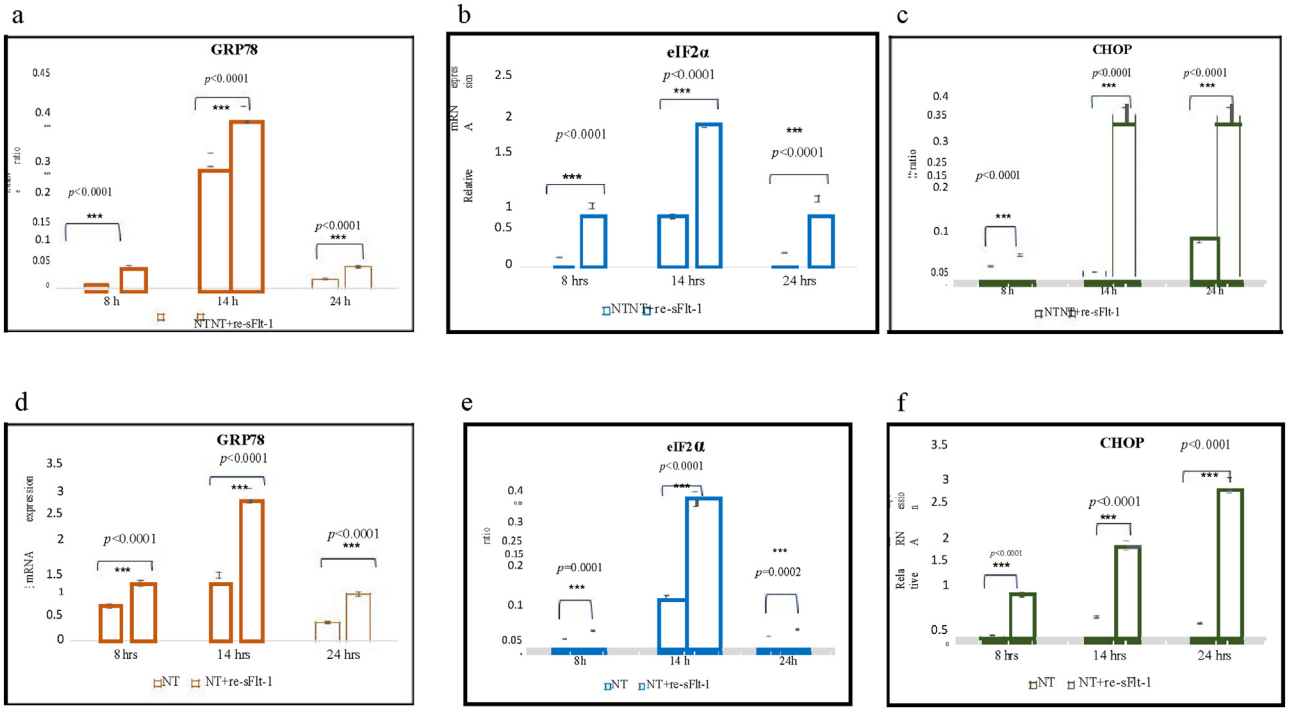

Fig. 9. (a, b, c) Comparison of normalized protein values of various ER stress markers between NT sera treated BeWo cells and cells which received both NT sera + re-sFlt-1; Fig. 9d, e, f: Comparison of relative mRNA expression of ER stress markers between NT sera treated BeWo cells and cells which received both NT sera + re-sFlt-1. Statistical analysis was done using paired *t*-test at all the indicated time points for all markers.

time. Our results are in concurrence with that of Miyake *et al.*, 2016 who examined the direct effect of sFlt-1 on two ovarian cancer cell lines, one colorectal cancer cell line (*in vitro study*) and nude mice with ovarian tumor model (*in vivo study*) in order to explore the mechanism underlying the anti-angiogenic or anti-tumor effects of sFlt-1. They studied the mechanism of cell injury induced by two different types of sFlt-1 administrations *i.e.* by exogenous administration of re-sFlt-1 to culture media in four cell lines and by transfection of LV-sFlt-1 into the cells. It was observed that both approaches effectively damaged the tumor cells and also had anti-angiogenic effects. The reduction in tumor volume in mice model was correlated positively to the dose of re-sFlt-1.[25] Thus in the present study, we contemplated that markers of ER stress (evaluated by immunofluorescence, western blot and qRT-PCR) were upregulated though at different time points when BeWo cells were treated with high sFlt-1 concentration and minified when BeWo cells were administered with lower dose of recombinant sFlt-1.

5. Conclusion

The sVEGFR1/sFlt-1 may be regarded as one of the several putative factors present in patients's sera which not only contributes to apoptosis, oxidative stress but also may cause endoplasmic reticulum stress thereby playing an important role in maternal signs and symptoms of preeclampsia. The above study can serve as an experimental and therapeutic template to investigate newer therapeutic regime in the management of preeclampsia. Further studies are required to identify various pharmacologic agents to counteract the effects of sFlt-1 as therapeutic options for preeclampsia. This could go a long way in decreasing the maternal mortality in India if sFlt-1 is used adequately for screening of suspecting pregnant mothers for preeclampsia at the community level comprehensively.

Authors Contributions

SM and RD conceived and designed the study. SM did all the experiments. NB provided the clinical inputs and critical suggestions. SM and SDD did statistical analysis. SM and RD wrote first draft. SM, RK, KL and AS did compilation of the final draft.

Conflict of Interest

None.

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