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Defective Trophoblast Invasion in Early Onset Preeclampsia: Role of Matrix Metalloproteinase-9 and Tissue Inhibitor of Metalloproteinase-1

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Abstract

Objectives: To investigate the expression/levels of Matrix Metalloproteinase-9 (MMP-9) and its inhibitor Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) in the placentae of early-onset preeclamptic patients and their maternal age-matched normotensive, non-proteinuric controls at both mRNA and protein levels.

Methods: In the present study, a total of 60 caesarean delivered placentae (30 each of early-onset preeclamptic patients and controls) were collected from Department of Obstetrics and Gynecology, AIIMS, New Delhi. Protein expression of MMP-9 and TIMP-1 was observed by immunohistochemistry and immunofluorescence stainings. Gelatin Gel Zymography was done to evaluate MMP-9 activity and Western Blot was done for the quantification of MMP- 9 and TIMP-1 proteins. The mRNA levels of MMP-9 and TIMP-1 were determined by qRT-PCR.

Results: Significantly down-regulated expression/levels of MMP-9 whereas up-regulated for that of TIMP-1 in the placentae obtained from early-onset preeclamptic patients in comparison to normotensive, non-proteinuric control placentae were observed.

Conclusions: The current research implies that aberrant functioning of MMP-9 and TIMP-1 in early-onset preeclamptic patients contribute to impaired placentation which might be relevant for possible future screening programs in order to predict and to design therapies for early onset preeclamptic patients.

Keywords: Pregnancy, Early-onset preeclampsia, Trophoblast Invasion, Matrix metalloproteinase, Tissue inhibitor of metalloproteinase

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Introduction

Normal pregnancy is associated with hemodynamic and uterine changes that allow adequate uteroplacental blood flow and uterine expansion for the growing fetus. These pregnancy-associated changes require significant uteroplacental and vascular remodeling [1]. Matrix Metalloproteinases (MMPs) are regulators of vascular and uterine remodeling [1]. Matrix Metalloproteinase-9 (MMP-9) is the only effective hydrolase secreted by trophoblast cells, involved in extracellular matrix (ECM) remodeling and placental angiogenesis with structural spiral arteries transformation, which precedes proper trophoblastic invasion [2]. Tissue Inhibitor of Metalloproteinase-1 (TIMP-1), corresponding to MMP-9 inhibits latter's activity to degrade collagen [3]. The balanced expression of MMP-9 and TIMP-1 has profound implications for regulating trophoblast invasion [3,4]. Increase in MMP-9 has been implicated in vasodilation, placentation and uterine expansion during normal pregnancy [1] however, reduced MMP-9 could impede uterine growth and expansion and lead to decreased vasodilation, hypertensive pregnancy, premature labour and Preeclampsia (PE) [1]. Previous studies reported that, for the specific inhibitor TIMP-1, no corresponding change was observed but the state of equilibrium between MMP-9 and TIMP-1 was altered in PE [3]. PE is a multisystem disorder of pregnancy defined by high blood pressure and proteinuria [5] and has been classified into 2 different

disease entities: Early-onset Preeclampsia (EOPE) and Late-onset Preeclampsia (LOPE) [6,7]. EOPE develops before 34 weeks of gestation, whereas late-onset PE develops at or after 34 weeks of gestation [6,7]. EOPE is associated with placental dysfunction, reduction in placental volume, intrauterine growth restriction, abnormal uterine and umbilical artery Doppler evaluation, low birth weight, multi-organ dysfunction, perinatal death and adverse maternal and neonatal outcomes [6]. The results of previous research into the role and expression pattern of MMP-9 and TIMP-1 in pregnancies complicated by early-onset preeclampsia are uncertain. Therefore, the aim of this study was to discover whether placentae from early onset preeclamptic pregnancies were associated with altered expression of MMP-9 and TIMP-1 as compared to placentae from normotensive pregnancies.

Materials and Methods

Sample size

Thirty each of EOPE patients and maternal age-matched normotensive, non-proteinuric controls placentae (caesarean delivered) were collected from Department of Obstetrics and Gynaecology, AIIMS, New Delhi after taking ethical clearance from Institute Ethics Committee (IECPG-247) and written informed consent was obtained from all the participants enrolled in the study. EOPE patients were recruited as per ACOG (American College of Obstetrics and Gynaecology) guidelines. Patients with chronic hypertension, chorioamnionitis, diabetes, renal disease and cardiac disease were excluded from the study.

Protein expression of MMP-9 and TIMP-1 was observed by Immunohistochemistry (IHC) and Immunofluorescence (IF). Gelatin gel zymography was done to determine the proteolytic activity of MMP-9. Western Blot was carried out to determine MMP-9 and TIMP-1 protein levels. MMP-9 and TIMP-1 mRNA expression/levels were analyzed using qRT-PCR. Relative normalized expression (Fold change) was calculated by $2^{-\Delta\Delta Cq}$ method.

Immunohistochemistry

Paraffin tissue blocks were sectioned on microtome (Thermo Scientific™ HM 325) (5µm sections) and were taken on Poly-L-lysine (Sigma) coated slides. UltraVision™ Quanto Detection System HRP DAB (Thermo, TL-125-QHD) was used to determine the protein expression of MMP- 9 and TIMP-1. Primary antibodies to MMP-9 (Abcam) at a dilution of 1:500 and TIMP-1 (Thermo) at a dilution of 1:100 were used. Slides were counterstained with hematoxylin, dehydrated in graded ethanol, cleared and cover slips applied. Stained slides were observed under Nikon Eclipse Ti-S elements microscope using NiS-AR software (version 5.1). Other chemicals were procured from Fischer Scientific.

Immunofluorescence

Paraffin tissue blocks were sectioned on microtome (Thermo Scientific™ HM 325) and were taken on Poly-L-lysine (Sigma) coated slides. Two changes of xylene (each for 5 minutes) followed by two changes of absolute alcohol (each for 3 minutes) and subsequently one change of 90% alcohol (1 minute) were given. Slides were rinsed with distilled water. Antigen retrieval was done with sodium citrate buffer (15 minutes at 95°C-100°C) followed by treatment with TBSTx (2 minutes) (2 times). BSA (blocking agent) was applied on slides for 35 minutes followed by overnight incubation with primary antibodies [MMP-9 (Abcam) at a dilution of 1:50 and TIMP-1 (Thermo) at a dilution of 1:10] at 4°C. Slides were then rinsed with PBST followed by incubation with secondary antibodies [MMP-9 (FITC conjugated, Abcam) at a dilution of 1:500 and TIMP 1 (TRITC conjugated, Thermo) at a dilution of 1:500] for 2.5 hours and then washed with PBS. Mounting was done with fluoroshield mounting media with DAPI (Abcam). Stained slides were observed under Confocal microscope.

Gelatin gel zymography

Protein extraction (from placental tissues of both patients and controls) was done with RIPA buffer (Thermo) and protease inhibitor cocktail. 7.5% acrylamide gel was prepared containing gelatin. 5x non-reducing sample buffer was added to the isolated protein samples. 10 µl protein sample was loaded to each well. Protein molecular weight marker (Thermo) was also loaded followed by running the gel at 90 V (vertical electrophoresis apparatus, BioRad) in electrophoresis buffer until good band separation is achieved. Gel was washed (2 x 30 min) with washing buffer then rinsing in incubation buffer for 5–10 min at 37°C with agitation. Fresh incubation buffer was added to the gel followed by incubation for 24 h at 37°C. Gel was stained with Coomassie blue for 30 min and rinsed with water. Incubation was done with destaining solution to visualize the bands showing pro and active forms of MMP-9 in the study groups.

Western blot

Protein extraction (from placental tissues of both patients and controls) was done with RIPA buffer (Thermo) and protease inhibitor cocktail. Separating and stacking gels were prepared. 3x non-reducing sample buffer was added to the isolated protein samples followed by denaturation at 95°C for 5 minutes and the samples were loaded along with protein molecular marker to the wells. Subsequently, running the gel at 50 V (vertical electrophoresis apparatus, BioRad) in electrophoresis buffer for 3-3.5 hours until good band separation is achieved. Nitrocellulose membrane was used for the transfer of gel products on to the membrane in transfer buffer for 90 minutes. Membrane was washed with TBST followed by blocking in 5% BSA TBST for 90 minutes. Overnight incubation was done with primary antibodies [MMP-9 (Abcam) at a dilution of 1:1000 and TIMP 1 (Thermo) at a dilution of 1:500] at 4°C. Washing was done with TBST followed by incubation with secondary antibodies [MMP- 9 (Abcam) at a dilution of 1:1000 and TIMP 1 (Thermo) at a dilution

of 1:1000] for 3 hours and then washed with TBST. Enhanced Chemiluminescence (ECL) kit (Thermo) was used for visualization of bands in Densitometer (Protein Simple).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Placental tissue (kept in RNA later) was blotted on absorbent paper and 100 mg was taken, washed in 1x 0.1 M PBS. RNA isolation was done using Ambion, Invitrogen. The quality of RNA was examined by denaturing gel and quantity was measured on Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific- NanoDrop ™ 2000); c-DNA synthesis was done using Thermo revert aid H-minus reverse transcriptase kit (Thermo). Quality of cDNA was checked on 0.8% agarose gel visualized by Ethidium Bromide (EtBr) stain under UV and quantity was measured on Microvolume UV-Vis Spectrophotometer; cDNA was subsequently used for Quantitative RT-PCR. Quantitative RT-PCR reactions were carried out in 20 µl volume, including SYBR Green (Thermo), forward and reverse primers [MMP-9, TIMP-1 (Sigma)], cDNA (template) and nuclease free water (CFX96 Touch™ Real-Time PCR Detection System, BioRad). Initial denaturation was performed at 95°C for 3 minutes, final at 95°C for 15 seconds, annealing at 60°C and extension at 70°C-72°C. Primers were designed by NCBI and confirmed by *in silico* PCR (Table 1). GAPDH was used as reference gene.

Statistical analysis

Data was analyzed by STATA 14.1 and GraphPad Prism 9.4.1. For qRT-PCR data analysis, relative quantification cycles of gene of interest (ΔCq) were calculated by ΔCq=Cq(target)-Cq(reference). Relative normalized expression (Fold change) was calculated by $2^{-\Delta\Delta Cq}$ method. $p<0.05$ was considered statistically significant.

Results

Clinical characteristics of early onset preeclamptic patients and maternal age matched normotensive, non-proteinuric controls are mentioned in Table 2.

Weak expression of MMP-9 and enhanced signaling of TIMP-1 was observed in the early onset preeclamptic placentae (Figures 1-4).

The immunolocalization of MMP-9 as visualized by immunohistochemistry and immunofluorescence revealed reduced staining intensity in syncytiotrophoblasts, stroma and blood vessels in the placentae from pregnancies complicated by early onset preeclampsia as compared to healthy controls whereas stronger localization of TIMP-1 was observed in the stromal component and syncytium in the placentae from early onset preeclamptic patients as compared to normotensive, non-proteinuric control placentae.

Gelatinolytic activity of MMP-9 was reduced in the early onset preeclamptic patients (Figure 5). MMP-9 activity (both pro and active forms) was significantly lower in the placentae from early onset preeclamptic patients as compared to normotensive, non-proteinuric controls.

Table 1. *Primers were designed by National Centre for Biotechnology Information (NCBI) and confirmed by in silico PCR.*

MMP-9	FP- CGCCAGTCCACCCTTGT	RP- CAGCTGCCTGTCGGTGAGA
TIMP-1	FP- CCCTGGAACAGCCTGAGCTT	RP- TGGATAAACAGGGAAACACTGTGC
GAPDH	FP- AGCCGAGCCACATC	RP- TGAGGCTGTTGTCATACTTCTC
Note: GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, FP: Forward Primer, RP: Reverse Primer		

Table 2. *Maternal study population-clinical characteristics.*

Clinical features	EOPE patients (n=30)	Controls (n=30)	p-value (paired-t-test)
Maternal age (years)	29.9 ± 4.09	30.1 ± 3.69	0.76
Systolic BP (mm Hg)	162.1 ± 11.58	117.5 ± 6.32	<0.0001
Diastolic BP (mm Hg)	101.4 ± 9.25	76.1 ± 6.27	<0.0001
Proteinuria (Dipstick)	3+ = 10 (33.33%), 2+ = 14 (46.66%), 1+ = 6 (20%)	nil or in traces	Not applicable
Intrauterine Growth Restriction (IUGR)	6 (20%)	0 (0%)	Not applicable
Note: Data presented as mean ± SD, paired t-test evaluates p-values between groups, $p\leq0.05$ was considered statistically significant, *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$			

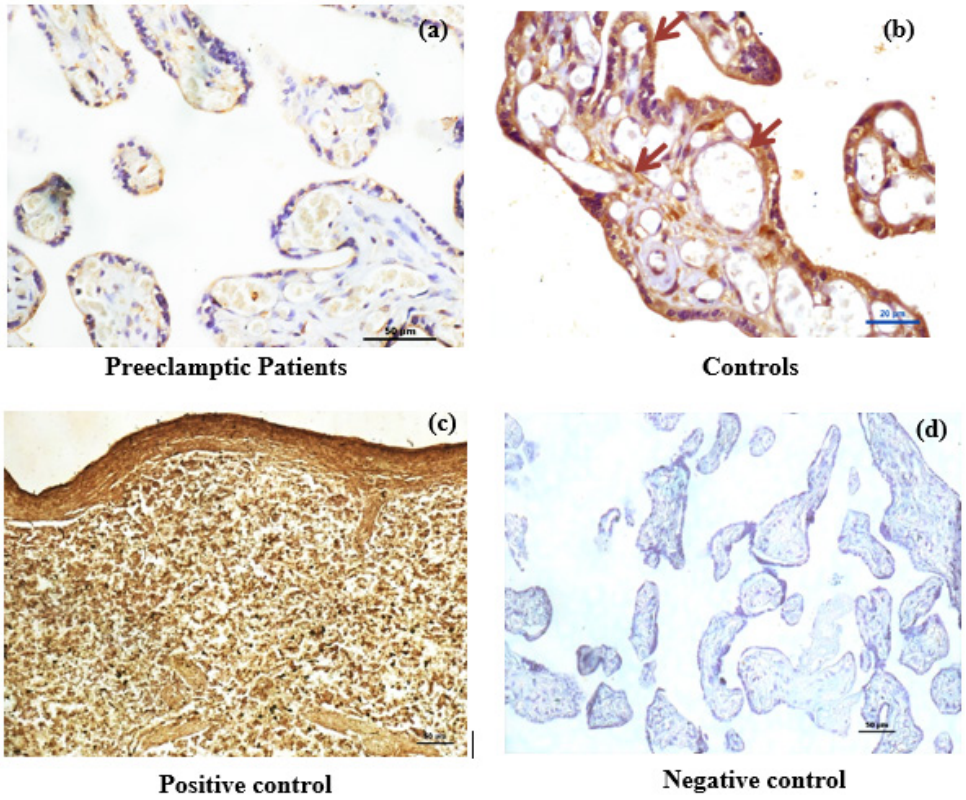

Figure 1. *Representative Immunohistochemistry images of placentae from EOPE patients (a) and normotensive, non-proteinuric controls (b) showing MMP-9 localization;* ***Note:*** *Arrows refer to MMP-9 positive cells. Human spleen serves as positive control for MMP-9 expression (c); (d): negative control.*

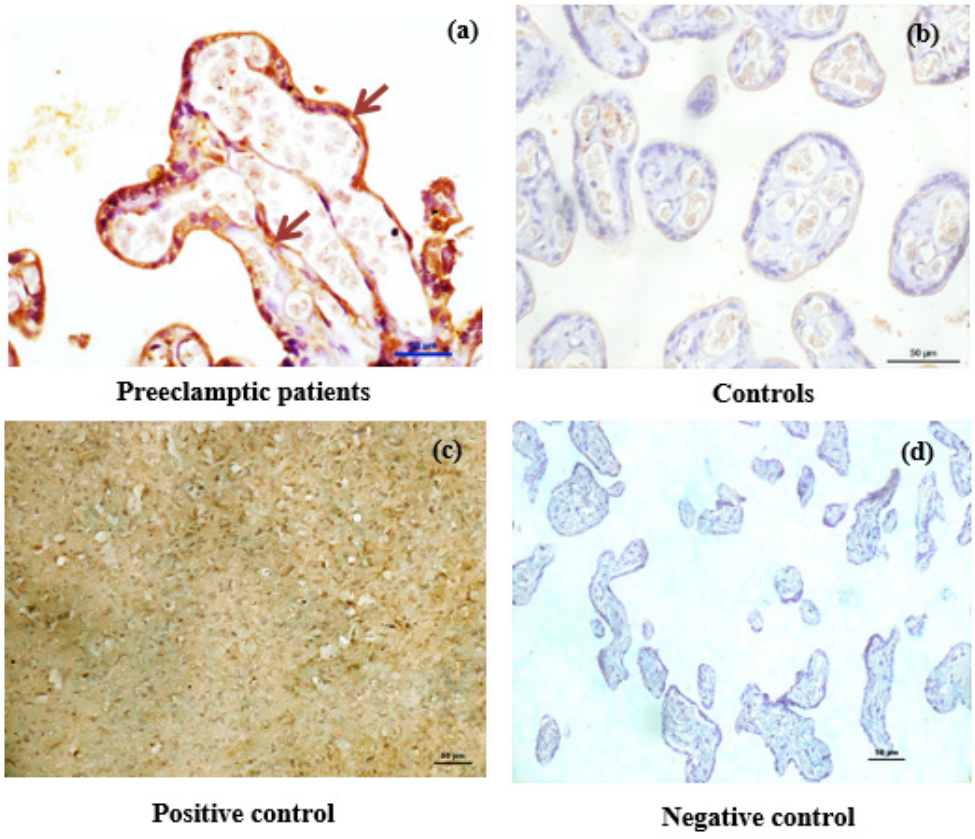

Figure 2. *Representative Immunohistochemistry images of placentae from EOPE patients (a) and normotensive, non-proteinuric controls (b) showing TIMP-1 localization;* ***Note:*** *Arrows refer to TIMP-1 positive cells. Rat brain tissue serves as positive control for TIMP-1 expression (c); (d): negative control.*

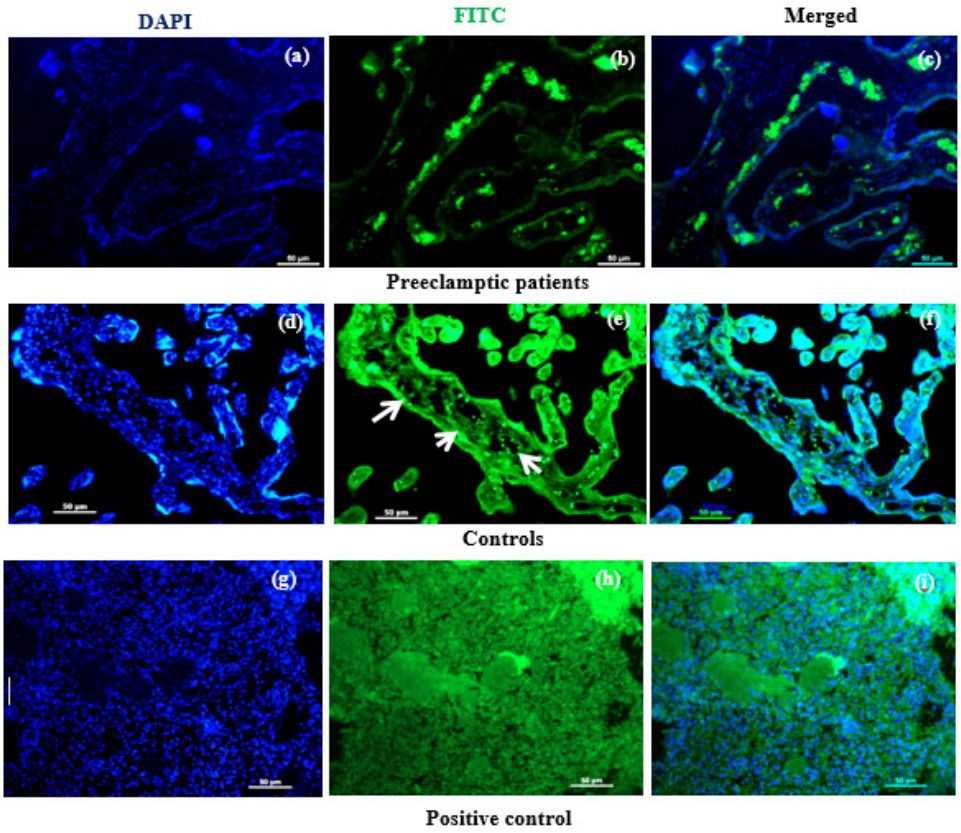

Figure 3. *Representative immunofluorescence images of placentae from EOPE patients (a-c) and normotensive, non-proteinuric controls (d-f);* ***Note:*** *Arrows refer to FITC stained MMP-9 positive cells. Nuclei stained by DAPI. Human spleen serves as positive control (g-i).*

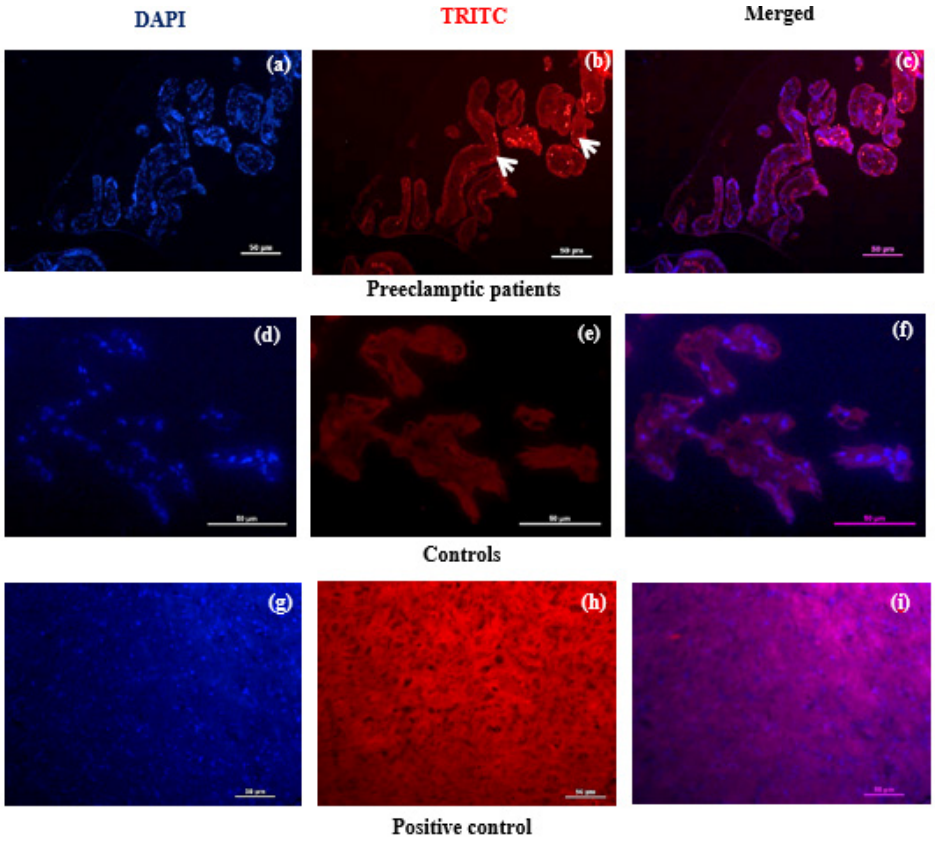

Figure 4. *Representative immunofluorescence images of placentae from EOPE patients (a-c) and normotensive, non-proteinuric controls (d-f);* ***Note:*** *Arrows refer to TRITC stained TIMP-1 positive cells. Nuclei stained by DAPI. Rat brain tissue serves as positive control (g-i).*

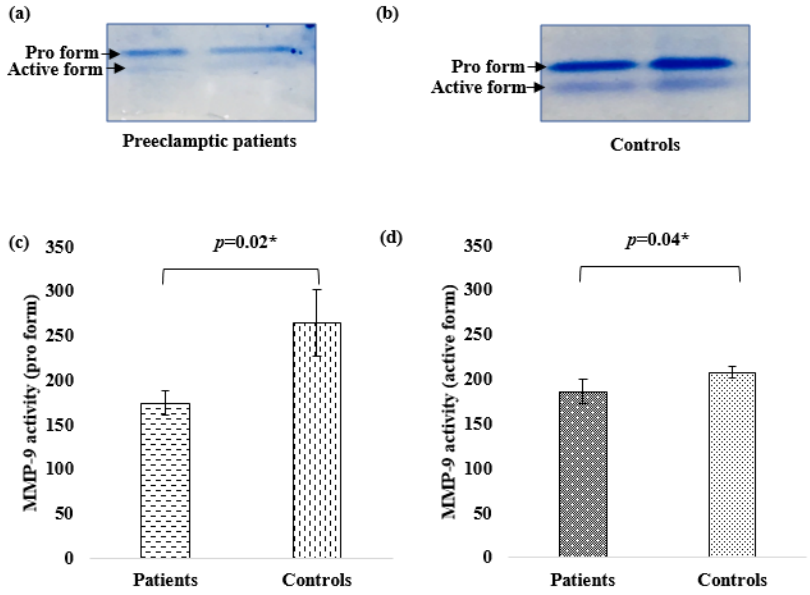

Figure 5. *Representative zymograms depicting gelatinase activity on maternal side of placentae from EOPE patients (a) and normotensive, non-proteinuric controls (b). Comparison of EOPE patients and controls using the Paired t-test for the Pro (92 KDa) (c) and active (86 KDa) (d) forms of MMP-9;* ***Note****: Data presented as mean ± SD; p≤0.05 was considered statistically significant, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001*

Reduced MMP-9 and elevated TIMP-1 protein expression was observed in the early onset preeclamptic patients (Figure 6). Immunoblot data revealed significant decrease in the protein expression of MMP-9 and increase for that of TIMP-1 in the placentae from early onset preeclamptic patients as compared to normotensive, non-proteinuric controls.

Downregulated gene expression of MMP-9 whereas upregulated for that of TIMP-1 was seen in the early onset preeclamptic patients (Figure 7) (Tables 3,4). qRT-PCR data showed 6.28 folds reduction of the mRNA expression of MMP-9 in the placentae from early onset preeclamptic patients as compared to those of normotensive non-proteinuric controls, whereas 8.32 folds increase in the mRNA expression of TIMP-1 was observed in the placentae from early onset preeclamptic patients.

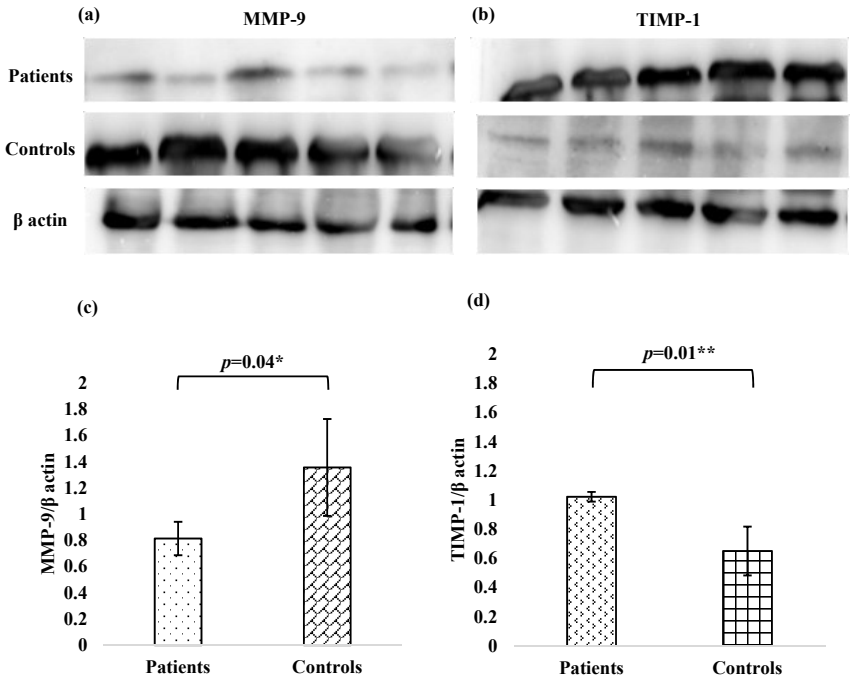

Figure 6. *Immunoblots showing MMP-9 (a) and TIMP-1 (b) protein expression in the placental tissues of EOPE patients and normotensive, non-proteinuric controls. Comparison of normalized protein expression of MMP-9 (c) and TIMP-1 (d) between the EOPE patients and Controls was done using Paired t-test; Note: β actin: protein loading control; data presented as mean ± SD; p≤0.05 was considered statistically significant, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001*

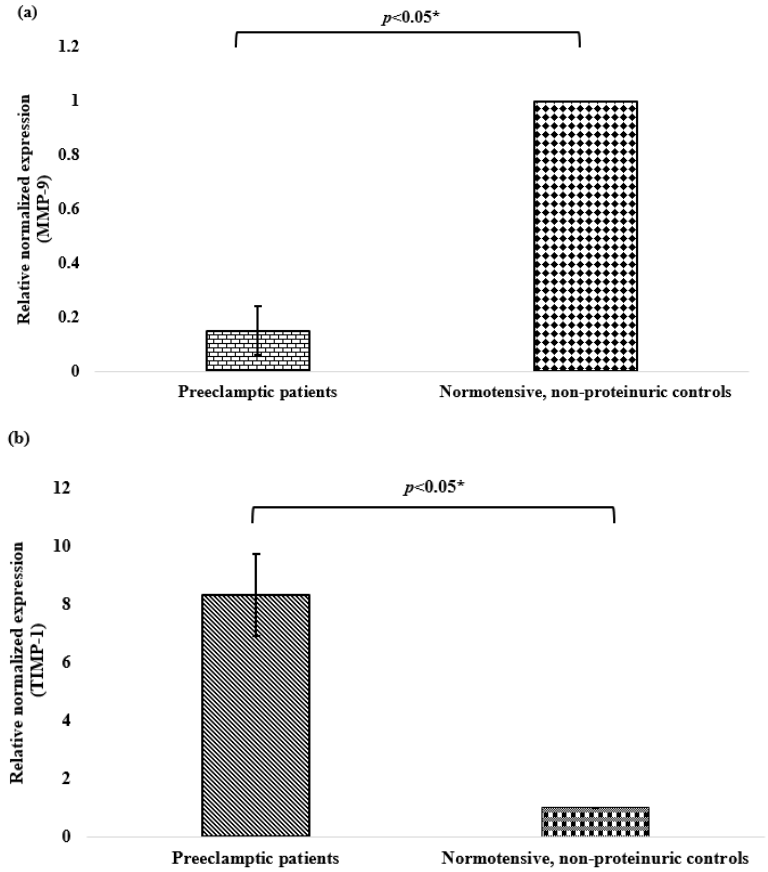

Figure 7. *Relative normalized expression (Fold change) was calculated and compared for MMP-9 (a) and TIMP-1 (b) genes between the EOPE patients and normotensive, non-proteinuric controls;* ***Note:*** *$2^{-\Delta\Delta Cq}$ method was used.*

Table 3. *Relative Normalized expression (Fold change) was calculated for MMP-9 gene in the EOPE patients and normotensive, non-proteinuric controls by $2^{-\Delta\Delta Cq}$ method.*

Gene expression	EOPE placentae (n=30)	Controls placentae (n=30)
Cq (MMP-9)	29.16	27.39
Cq (GAPDH)	23.38	24.25
ΔCq	5.78	3.14
ΔCq Expression	0.018	0.113
ΔΔCq	0.15	1

Table 4. *Relative Normalized expression (Fold change) was calculated for TIMP-1 gene in the EOPE patients and normotensive, non-proteinuric controls by $2^{-\Delta\Delta Cq}$ method.*

Gene expression	EOPE placentae (n=30)	Controls placentae (n=30)
Cq (TIMP-1)	27.81	31.17
Cq (GAPDH)	21.04	21.35
ΔCq	6.77	9.82
ΔCq Expression	0.0091	0.0011
ΔΔCq	8.3297	1

Discussion

In the present study, we have analysed the expression/levels of MMP-9 and TIMP-1 in the placentae from EOPE patients (n=30) and their maternal age-matched normotensive, non-proteinuric controls (n=30). MMPs are essential biochemical mediators to facilitate trophoblast invasion and adequate spiral artery remodeling [8,9]. MMPs, also called matrixins, are a family of 17 zinc-dependent endopeptidases, which participate in many biological processes [8,9]. The regulation of MMPs activity at the maternal-fetal interface appears to be critical for successful placentation [8,9]. MMP-9 is the only effective hydrolase secreted by the trophoblast cells which can digest Extracellular matrix (ECM) proteins [3]. MMP-9 is strongly localized in the placental bed (primarily to extravillous cytotrophoblasts) and appears to regulate trophoblast invasion [10]. Appropriate trophoblast invasion and vascularization require a functional synergism between MMP-9 and its regulating factor TIMP-1 [3]. We observed reduced expression of MMP-9 in syncytiotrophoblasts, stroma and around blood vessels in the placentae from early onset preeclamptic patients as compared to control placentae. The reduced expression of MMP-9 in trophoblast cells could cause shallow invasion, disturbed and inadequate remodeling of the maternal spiral arteries, thus reducing blood flow to the intervillous space [3,11]. Insufficient conversion of the spiral arteries into low-resistance, high-capacity vessels in early pregnancy can lead to systemic hypertension and fetal hypoxia in later pregnancy as the fetus and placenta outgrow their blood supply, often observed in preeclampsia (PE) especially the early onset type (EOPE) [11].

EOPE is the most severe clinical variant of disease occurring in 5%-20% of all cases of PE and is associated with impaired fetal growth, fetal pathology and uterine blood circulation, small size of the placenta, preterm delivery, neonatal morbidity and mortality [12]. EOPE developments are associated with impaired trophoblast invasion, immune maladaptation and increased markers of endothelial dysfunction [13], however, Late Onset type (LOPE) is occurring in about 75%-80% of all cases of preeclampsia, associated with maternal morbidity (metabolic syndrome, impaired glucose tolerance, obesity, dyslipidemia and chronic hypertension) but normal birth weight and normal placental volume [14]. We have observed strong signaling of TIMP-1 in the syncytium and stroma in the EOPE placentae. Our results corroborate with the previous study by Zhang *et al*., 2019 where they observed enhanced intensity of MMP-9 in cytoplasm of trophoblast cells, vascular endothelial cells and villous mesenchymal cells in the healthy placentae [3], however, they also observed that the specific inhibitor of MMP-9 i.e. TIMP-1 had no corresponding change and the equilibrium state between MMP-9 and TIMP-1 changed [3].

Another study proposed that MMP-9 ablation in MMP-9 knockout mice shows a phenotype that imitate PE due to impaired trophoblast differentiation and invasion [15]. Shokry *et al*., in 2009 and Omran *et al*., in 2011 both reported decreased MMP-9 levels in the preeclamptic patients as compared to controls [16,17]. Dang *et al*., in 2013 confirmed that MMP-9 was abundantly expressed in the tissues of normal pregnant rats by immunohistochemistry, gelatin gel zymography and western blot analysis supporting a role of MMP-9 in the uteroplacental and vascular remodeling during normal pregnancy [18].

In the present study, gelatin gel zymography revealed that both the pro and active forms of MMP-9 were significantly reduced in the placentae from EOPE patients as compared to their maternal age matched normotensive, non-proteinuric controls. The quantitative estimation of MMP-9 and TIMP-1 proteins by western blot found significant decrease in the levels of MMP-9 and increase for that of TIMP-1 in the EOPE placentae thereby validating the immunostaining (immunohistochemistry and immunofluorescence) results. Gene expression pattern of MMP-9 and TIMP-1 was also altered in the EOPE patients.

In this study, we postulated that the altered levels of matrix metalloproteinase-9 and its regulator, tissue inhibitor of metalloproteinase-1, could have an impact on the pathogenesis of EOPE.

Conclusion

In contrast to normotensive, non-proteinuric controls, the placentae of early-onset preeclamptic patients showed decreased expression of MMP-9 and elevated expression of TIMP-1. We have shown altered protein and mRNA expression/levels of these two markers in the EOPE placentae. Further research is required to determine the significance of MMP-9 and TIMP-1 as important mediators of the pathophysiology of early-onset preeclampsia. Longitudinal prospective studies can be designed to analyze their use as potential biomarkers and biological therapeutic targets.

Acknowledgment

We are deeply grateful to all the pregnant women enrolled from the Department of Obstetrics and Gynaecology, All India Institute of Medical Sciences, New Delhi, for donating their placentae following parturition. They patiently gave their signed consent for the study.

Availability of Data and Materials

Most of the data are contained within the manuscript. Data described in the manuscript are located with the first author (Dr. Pallavi Arora) and the corresponding author (Professor Renu Dhingra) which can be shared upon request.

Authors' Contributions

Pallavi Arora and Renu Dhingra conceived and designed the study. Pallavi Arora did all the experiments. Neerja Bhatla provided the clinical inputs and critical suggestions.

Sadanand Dwivedi assisted in statistical analysis. Renu Dhingra and Pallavi Kshetrapal confirm the authenticity of all the raw data. Pallavi Arora wrote the first draft. Pallavi Arora, Renu Dhingra and Pallavi Kshetrapal did subsequent proofreading of the manuscript and compiled the final draft. The final manuscript was read and approved by all the authors.

Ethical Committee Consent

Ethical clearance was obtained from Institute Ethics Committee, All India Institute of Medical Sciences, New Delhi (IECPG-247) and written informed consent was obtained from all the participants enrolled in the study.

Patient Consent for Publication

Written informed consent for publication was obtained from all the participants of the current study.

Appendix

ACOG guidelines

Blood Pressure- 140 mm Hg systolic or >90mm Hg diastolic on two occasions at least 4 hours 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 > 0.3mg/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 > 1.1mg/dl or doubling of serum creatinine in the absence of another renal disease, impaired liver function: elevated blood levels of liver transaminases to twice normal concentrations, pulmonary edema, and cerebral edema.

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