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Relevance of placental pathological changes of maternal inflammatory syndrome along with the obstetric and clinical parameters in preeclampsia

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Abstract- Preeclampsia is a disorder of pregnancy occurring after midgestation with clinical manifestations of hypertension and proteinuria and has been linked with fetal and maternal morbidity and mortality. Maternal inflammatory syndrome has been associated with preeclampsia. The pathogenetic mechanism proposed behind this syndrome is the maternal endothelial dysfunction caused by atleast of subset of various placental derived factors released into maternal circulation. These are syncytiotrophoblastic microparticles (STBM) or syncytial knots, exosomes or the soluble endoglins. The STBM or the syncytial knots are the end products of trophoblastic life cycle which have been proposed to be altered in the disorder of preeclampsia. So, the aim of the present study was to assess and compare the apoptosis of trophoblastic cells and the syncytial knots in the preeclamptic (20 placentas) and normal (20 placentas) by using TUNEL assay and M30 immunostaining. We also aim to compare various obstetric and clinical parameters affected in preeclampsia (blood pressure, urinary protein, gestational age, birth wt of the babies) between the two groups. In our study, TUNEL Assay as well as M30 immunostaining shows higher trophoblastic as well as syncytial knot apoptotic indices in preeclamptic placentas as compared to normal placentas. The results of our study also show that the clinical parameters (blood pressure, urinary protein) were higher whereas obstetric parameters (gestational age, birth wt of the babies) were lower in preeclamptic patients as compared to controls.

Index Terms- Apoptosis, Immunohistochemistry, DNA fragmentation, Preeclampsia, Placenta

I. INTRODUCTION

Preeclampsia is a heterogenic multisystem disorder, well known for its sudden occurence during the second half of pregnancy, the rooted cause of which has been suggested to be the abnormal placentation[1]. This assumption is based on the studies suggesting that the definite cure for preeclampsia is the delivery of placenta. Placenta is a remarkable organ continuously in dynamic state of cell division and demise and is the maternofetal contact zone which serves to maintain an interface for the exchange of gases and nutrients between mother and the growing fetus[2]. Mother must adequately be adaptive and responsive to the placental and fetal demands. From the implantation to the maternal adaptation to the increasing demands of growing fetus, every process has an impact on the developing fetus as well as on the maternal endometrium. Fetal survival in maternal womb as well as in the external environment after birth depends on inutero perfect implantation and hence placental development. The normal placental development requires continuous process of trophoblastic invasion of maternal endometrium as well as of vasculature. The interstitial trophoblastic invasion causes proper anchorage to the endometrium whereas endovascular invasion leads to uterine spiral artery remodeling ultimately causing increased maternal blood flow and hence proper uteroplacental perfusion[3]. The process of trophoblastic invasion requires a continuous process of apoptosis cascade[4]. Any alteration in the process of trophoblastic invasion can disturb the normal placental physiology and placentation and ultimately affects maternal homeostasis and fetal growth.

The trophoblastic cell undergoes apoptosis during normal placental development[5]. As pregnancy progresses, the placental apoptosis increases suggesting that it could play a role in the normal development and aging of placenta[6]. During placenta formation, the trophoblasts differentiate and form a double layered covering around the fetus, outer being the multinucleated syncytiotrophoblastic layer whereas inner is the mononucleated cytotrophoblastic stem cells[7]. Both the layers are vital as all the cells divide and ultimately undergo cell demise or apoptosis. The initiator phase of apoptotic cascade occurs in the cytotrophoblasts cells whereas execution phase proceeds in the syncytiotrophoblastic cells[8]. Towards the end of the trophoblastic apoptosis pathway, old apoptotic nuclei accumulate and form membrane sealed bodies termed as syncytial knots corresponding to apoptotic bodies of the apoptotic pathway[9]. Finally, these knots are extruded from syncytiotrophoblast layer into maternal circulation. These get trapped into lung capillaries and phagocytosed by activated cells such as macrophages[10]. The liberated knots are also known as syncytiotrophoblast membrane microparticles (STBM). As these knots are membrane sealed bodies, they prevent an inflammatory response by maternal blood vessels and organs. The pathophysiology of preeclampsia has been linked with various causative factors and altered trophoblastic apoptosis is one of them[11]. Out of the adverse clinical manifestations of preeclampsia, the most severe is the maternal inflammatory syndrome caused by the proposed role of released STBM[12]. These findings move our thoughts to a path that lead us towards constructing our research design to aim for finding the pathophysiology behind STBM mediated maternal inflammatory response. Therefore, our first aim is to study the trophoblastic apoptosis process by estimating and comparing the trophoblastic apoptotic index and syncytial knot index in normal and preeclamptic placentas by using M30 immunostaining and

TUNEL Assay. M30 antibody detects cytoplasmic cytokeratin cleavage products produced during early phase of apoptotic cascade. This phase of cytokeratin breakdown persist for a longer span of time whereas TUNEL assay identifies endonuclease mediated DNA breakdown in the nuclei of apoptotic cells occurring late during apoptosis pathway[13,14]. A combination of two or more assays may be more appropriate to detect the majority of cells undergoing apoptosis at one time. Secondly, we aim to compare various obstetric and clinical parameters (blood pressure, urinary protein, gestational age, birth wt of the babies) affected in preeclampsia as it has been confirmed that preeclampsia is the disorder of abnormal placentation and poor perfusion which may lead to intrauterine growth restriction and premature births[15]. The appropriate assessment of apoptotic activity may find its clinical relevance in predicting the progression of the disease in case of preeclampsia.

II. Materials and Method

A total of 40 placentas were collected from the labour room out of which 20 were from normal pregnant women and 20 were from preeclamptic pregnant women with variable gestational age groups (30-42weeks). The protocol of the study was approved by the ethics committee and consent was obtained from each individual for the study group. Patients with chorioamnionitis, chronic hypertension, pregestational diabetes, renal disease, cardiac disease, active asthma, thyroid disease and pre-existing seizure disorder were excluded from this study.

The samples were taken randomly from the different areas of placental disc and areas of visible infarcts, calcification or hematomas were excluded. The samples then fixed in 10% formaldehyde and processed for TUNEL assay and M30 immunostaining. All the techniques were standardised according to ambient conditions of our laboratory.

In TUNEL assay, tissue sections were deparaffinized and rehydrated according to standard protocols. The slides were permeabilized by using 0.1% sodium citrate and quenching was done by 3% H_2O_2 in methanol. TUNEL reaction mixture was prepared by adding 50µl of enzyme solution (terminal deoxynuleotidyl transferase enzyme isolated from calf serum) to 450µl of label solution (Biotinylated nucleotide mixture in reaction buffer) and incubated for 60 minutes at 37ºC in a humidified chamber. Then, the samples were incubated in 50-100µl of POD substrate containing streptavidin conjugated to horseradish peroxidase for 10 minutes at 15-25ºC. After each step, the slides were washed with PBS. The slides were counterstained in hematoxylin and finally mounted with permanent mounting media (DPX). An adjacent serial section pretreated with DNAse I was used as a positive control. The tissue sections not treated with Tdt enzyme were used as negative control. All 40 samples of placenta were analyzed for expression of apoptotic nuclei and digital images of 10 randomly selected high- power fields were obtained using a Nikon microscope (Nikon Corp, Tokyo, Japan). The sections were examined by two independent observers. Approximately 1000-3000 nuclei were counted for each placenta in preeclamptic and control groups. Apoptotic nuclei were easily differentiated from non-apoptotic by their brown labeling and condensed heterochromatic nucleus. The total number of nuclei (apoptotic nuclei + live nuclei) and total number of apoptotic nuclei were counted. The TUNEL apoptotic index (Total number of apoptotic nuclei per total number of nuclei multiply by 100) was calculated for the trophoblastic cells and syncytial knots.

In M30 immunostaining, tissue sections were prepared as for the TUNEL assay. All subsequent steps were carried out at room temperature in a humidified chamber. The peroxidase activity was blocked by using 3% H_2O_2 prepared in methanol for 10 minutes. The specimens were then incubated in 1-3 drops of serum block for 2 hours to prevent non-specific binding to collagen and connective tissue. The specimens were incubated for 24 hrs with M30 antibody (1:50) followed by 1-3 drops of biotinylated secondary antibody (anti mouse IgG) for 30 minutes. The specimens were thereafter incubated in 1-3 drops of HRP-streptavidin complex for 30 minutes. The slides were then counterstained with hematoxylin and dehydrated by passing through ascending grades of ethanol and slides were mounted with DPX and were observed under light microscope. The negative tissue control included eliminating the primary antiserum and replacing species-specific antiserum with normal horse serum. Sections of colon adenocarcinoma served as positive control. All 40 samples of placenta were analyzed for expression of cytokeratin neoepitope in the apoptotic cells using M30 antibody staining in serially sectioned slides. Cells with the brown cytoplasmic stain were considered M30 immunostaining positive. M30 apoptotic index was calculated for trophoblastic cells as well as for syncytial knots.

The clinical characteristics (blood pressure, urinary protein, gestational age, birth wt of babies) were compared among the two groups. The comparison between apoptotic indices of trophoblastic cells and syncytial knots in normal and preeclamptic placentas was done by using both the techniques.

Statistical analysis

Statistical analysis was carried out using Stata 9.0 / Data analysis software (college Station, Texas, USA). Data were presented as number (%) or mean±SD/ Median (range) as appropriate. Quantitative variables between the groups were compared using student "t" test for independent samples/ Wilcoxon rank sum test. Categorical variables were compared between the groups using Chi square test. The p value less than 0.05 was considered statistically significant.

III. Results

Clinical characteristics of preeclamptic and control group (Table I, II)

The mean systolic and diastolic pressures in preeclamptic group were 142.5±8.6mmHg and 96.3±8mmHg respectively whereas in control group, the systolic and diastolic pressures were 112±8.6mmHg and 79.7±6.1mmHg respectively. There was a significant difference in the systolic and diastolic pressures in both the groups (p- 0.0001). Urinary protein was determined in 24-hour urine samples of preeclamptic and control groups. The levels of protein in preeclamptic group was 5.3(1.8-8) g/day whereas the level was 0.35(0.1-1.6)g/day in control group. There was a significant difference in urinary protein between preeclamptic and control groups (p - 0.001). Thus, there was

increase in blood pressure and urinary protein in preeclamptic patients as compared to control group.

Obstetric characteristics of preeclamptic and control group (Table III)

The average gestational age in preeclamptic and normal patients was 35.1 ± 2.7 wks and 38.25± 1.4 wks respectively. Therefore, the gestational age was low in preeclamptic group as compared to control (p -0.0001). The baby's birth weight was 2343.6 ± 237 gms in preeclamptic group as compared to 2924.9 ± 260 gms in control group at a statistically significant value of p-0.0001. Thus, the gestational age and birth weight of babies were low in preeclamptic group as compared to control (p - 0.001).

Comparison of obstetric characteristics of preeclamptic and control group (Table IV)

The gestational age was less than 37 weeks in 80% of preeclamptic and 10% of control groups. The birth weight of the baby was less than 2500gms in 70% of preeclamptic and 5% of control groups. Thus, the higher percentage of preeclamptic group had reduced gestational period and decreased birth weight of the baby.

Apoptotic indices by TUNEL Assay and M30 immunostaining (Table V, VI, Figure 1)

TUNEL assay shows higher trophoblastic and syncytial knot indices in preeclamptic placentas (71.4±6.7, 89.3±13.1 respectively) as compared to normal placentas (45±10.7, 34.5±9.5 respectively). Similarly, M30 immunostaining also shows higher trophoblastic and syncytial knot indices in preeclamptic placentas (92±6.3, 73.6±20.5 respectively) as compared to normal placentas (61.9±14.4, 36.9±20 respectively) (p-0.0001)

Table I: Clinical characteristics of preeclamptic and control groups.

Clinical characteristics	**Mean ± SD (range)**		**Difference in means 95%C.I.**	**p value**
	Preeclampsia (n=20)	**Control (n=20)**		
Blood Pressure **Systolic (mm Hg)** **Diastolic (mm Hg)**	 142.5±8.7(120-160) 96.3±9.5(82-118)	 112±8.6(100-124) 79.7 ±6.1(72-90)	 11.09(107.9-138.3) 6.54(76.8-91.8)	 0.0001 0.0001

n = number of subjects.
Data is presented in Mean ±SD (range)
Student t test for independent samples
C.I.= Confidence Interval
$p < 0.05$, statistically significant

Table II: Clinical characteristics of preeclamptic and control groups.

Clinical characteristics	**Median (range)**		**p value**
	Preeclampsia (n=20)	**Control (n=20)**	
Urine Protein (gm/day)	5.3(1.8-8)	0.35(0.1-1.6)	0.0001

n = number of subjects.
Data is presented in Median (range)
Two samples Wilcoxon rank sum (Mann Whitney Test)
$p < 0.05$, statistically significant

Table III: Obstetric characteristics of preeclamptic and control groups.

Obstetric characteristics	**Mean ± SD (range)**		**Difference in means 95%C.I.**	**p value**
	Preeclampsia (n=20)	**Control (n=20)**		
Gestational age (weeks)	35.1±2.7(31-40)	38.25 ±1.4(36-41)	4.55(37.5-33.8)	0.0001
Birth weight (gms)	2343.6±237 (1800-2700)	2924.35±260 (2500-3500)	7.37(2802-2232)	0.0001

n = number of subjects.
Data is presented in Mean ±SD (range)
Student t test for independent samples
C.I.= Confidence Interval

Table IV: Comparison of obstetric characteristics in preeclamptic and control groups.

Obstetric characteristics	**Number of subjects (%)**		**OR (95% C.I.)**	**p-value**
	Preeclampsia (n=20)	**Control (n=20)**		
Gestational age (wks)				
≤ 37	16(80)	2(10)	16.71(2.97-93.88)	0.001
>37	4(20)	18(90)		
Birth weight (gms)				
≤ 2500	14(70)	1(5)	44.33(4.78-410.85)	0.001
> 2500	6(30)	19(95)		

n = number of subjects.
Chi square or Fisher's exact test was applied
OR= Odds ratioC.I. = Confidence Interval
Percentage (%) indicated in parenthesis.
$p< 0.05$, statistically significant.

Table V: Apoptotic Index (Percentage of apoptotic nuclei) with TUNEL Assay in preeclamptic and control groups.

Cells used for counting	Apoptotic Index [Mean ±SD(range)]		p value
	Preeclampsia (n= 20)	**Control (n=20)**	
Trophoblastic cells	71.4±6.7(55.7-83.3)	45±10.7(27-68.9)	0.0001
Syncytial Knots	89.3±13.1(25-60)	34.5±9.5(12-51.7)	0.0001

n = number of subjects.
Data is presented in mean ± SD (range).
Student t test for independent sample
p< 0.05, statistically significant

Table VI: Apoptotic Index (Percentage of apoptotic nuclei) with M30 antibody staining in preeclamptic and control groups.

Cells used for counting	Apoptotic index[Mean ±SD(range)]		p value
	Preeclampsia (n= 20)	**Control (n=20)**	
Trophoblastic cells	92±6.3(76.3-99)	61.9±14.4(37.3-87.9)	0.0001
Syncytial Knots	73.6±20.5(25-100)	36.9±20(0-66.6)	0.0001

n = number of subjects.
Data is presented in mean± SD (range).
Student t test for independent sample
p< 0.05, statistically significant

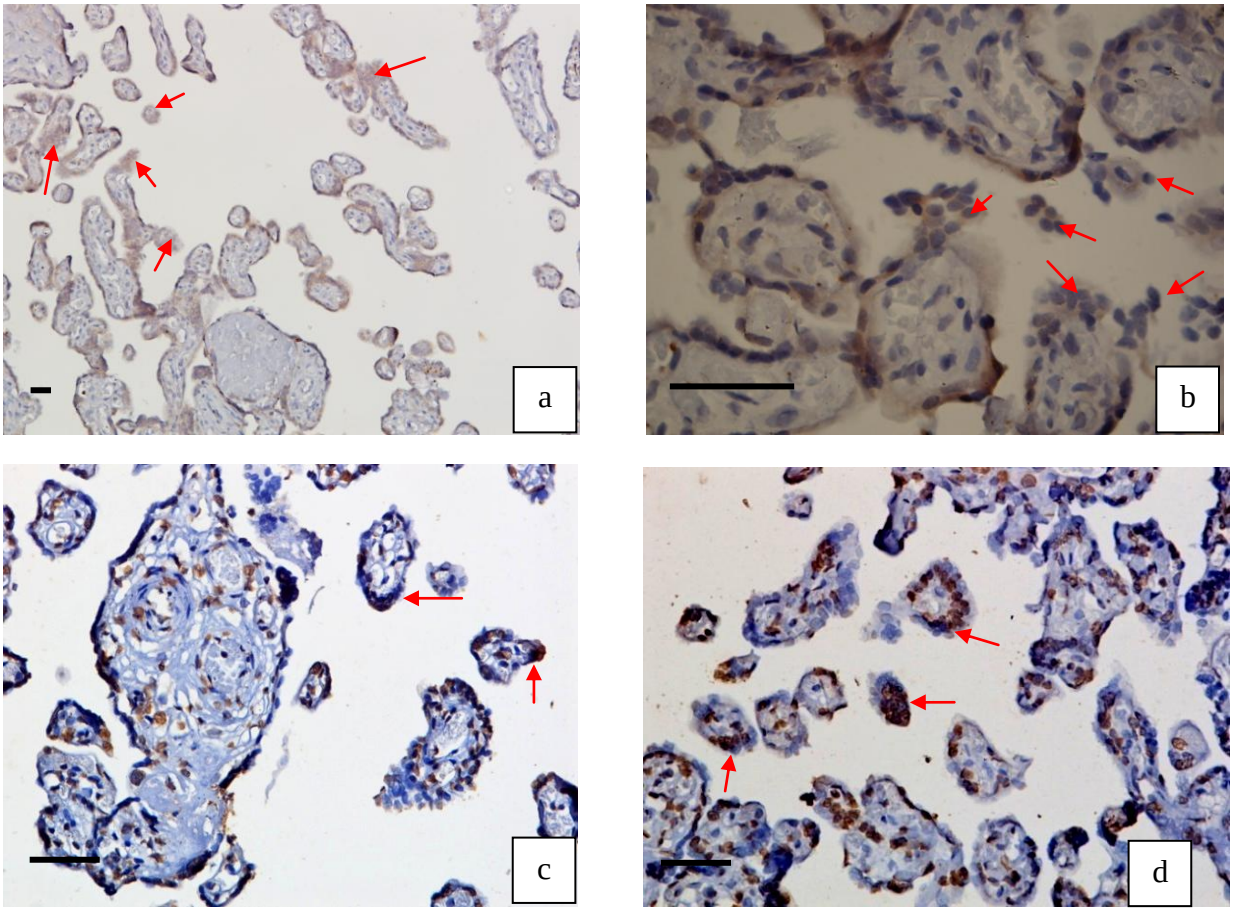

Figure 1: Preeclamptic placentas showing M30 immunostaining and TUNEL positive cells

Figure 1: Preeclamptic placentas showing M30 immunstaining and TUNEL assay positive cells
a and b. M30 immunostaining positive syncytial knots (brown stained) (red arrows)
c and d. TUNEL Assay positive syncytial knots (brown stained) (red arrows)

Scale bar 100µ

IV. Discussion

Preeclampsia has been very well described as "disease of theories" because of multiple hypothesis proposed to describe its complex pathophysiology. After tracing the path to achieve our aims designed to construct our research, we can now pendown a chain of pathological events proposedly structured during the pathogenesis of preeclampsia.

The exaggerated trophoblastic apoptosis burdens the preeclamptic placentas by forming increased syncytial knots as shown with our results. This actually brings about rapid destruction of maternalfetal interface and excessive liberation of syncytial knots in the maternal circulation. These syncytial fragments may divert their path to either aponecrotic or necrotic route[16].

Aponecrotic route- Excessive apoptotic process in preeclampsia is continued with the necrotic events damaging plasma membranes, causing water influx and release of cytoplasmic contents. This results in secondary necrosis or aponecrosis.

Necrotic route- This is seen in severe preeclampsia showing no signs of apoptosis with lack of chromatin condensation within syncytial nuclei. There is continuous incorporation of trophoblastic nuclei without syncytial shedding thereby accumulating aged trophoblastic debris within, which soon breaks into maternal circulation.

The ongoing apoptosis and necrotic events can be picked up by the use of various immunohistochemical techniques. M30 antibody stains the cytoplasm of cells specifically undergoing cytokeratin breakdown during apoptosis. Whereas TUNEL assay identifies DNA breakdown occurring in apoptotic as well as in

necrotic cells[17]. During our study, we could demonstrate following subsets of syncytial knots based on their pattern of staining.

1. Syncytial knots which were M30 immunostaining positive and TUNEL assay negative: these were still attached on to the villous surfaces, indicating the early phase of apoptosis.
2. Syncytial knots which were TUNEL assay positive but M30 immunostaining negative: these were the extruded syncytial knots showing the late apoptotic phase and can also be the sign of aponecrotic or necrotic shedding occurring in severe preeclampsia.
3. Syncytial knots which were both M30 immunostaining and TUNEL assay positive: these syncytial knots were still attached on the villous surface and might be soon extruded out.
4. Syncytial knots which were both M30 immunostaining and TUNEL assay negative: these might be syncytial sprouts which were attached on the villous surface and these never show signs of apoptosis.

Therefore, in our study, higher trophoblastic and syncytial knot apoptotic indices as shown with results of M30 immunostaining reveals exaggerated apoptotic pathway and with TUNEL Assay, the ongoing trophoblastic cell demise and necrotic events can be suggested. Both the aponecrotic and necrotic release of syncytial fragments may lead to stimulation of maternal immune system thereby releasing inflammatory cytokines (TNF α) produced by maternal immune cells[18]. These act by further promoting apoptosis by upregulating Fas/Fas ligand pathway and endothelial dysfunction by causing oxidative stress, vasoconstrictor release and stimulating coagulation cascade[19]. The endothelial dysfunction in preeclampsia have been confirmed by studying the markers of endothelial cell activation (von willebrand factor, fibronectin, endothelin)[20,21,22]. These reflect failure of endothelial dependent dilator function causing hypertension and generalized vasoconstriction leading to hypoperfusion of various organs seen as in the form of liver, brain, adrenal necrosis and infarction . The endothelial defect in kidney is responsible for proteinuria in preeclamptic patients[23]. Our study also reflect the above pathogenetic manifestations of hypertension and proteinuria in preeclamptic patients. We also confirm through our study that the interaction of two factors- the reduced placental perfusion and deficient trophoblastic invasion brings about the worst obstetric outcome of intrauterine growth restriction and prematurity which is seen clinically as preterm deliveries of low birth wt babies.

V. Conclusion

With the results of our study, we confirm that not only the pathological changes (increased trophoblastic apoptotic and syncytial knot indices, necrotic release of syncytial fragments) but also the clinical (hypertension, proteinuria) and obstetric (low gestational age, low birthwt babies) parameters were affected in preeclamptic patients. The clinical definition of hypertension and proteinuria is not proportional to the diverse complexity of this disorder. Therefore, the intricate pathological changes should be further unfolded to reveal the underlying etiology of this disease of pregnancy which mostly gets complicated with the maternal inflammatory syndrome. On achieving this step, further molecular studies can be done to reach the therapeutic targets.

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