MATERIALS AND METHODS

SOURCE OF DATA:
1. This study will be conducted at the department of anatomy, Rama Medical College, Hospital, Rama University, Kanpur.
2. The material for study consists of placenta of pre-eclampsia and eclampsia mothers from the Department of Obstetrics and Gynaecology at Rama Hospital.
3. Before the study permission will be taken from the institution ethical committee and professor and head, Department of Obstetrics and Gynaecology.
4. Placentas will be collected immediately after delivery and will be cleaned by washing under running tap water.
5. Any abnormality of cord and membranes will be recorded.
6. The placenta along with cord will be coded and preserved in 10% formalin solution.
7. Venous blood will be collected from the subject to evaluate the platelet cont.
8. Venous blood will be collected from the subject after the 32 week of gestation to evaluate the platelet cont.
9. EDTA mixed venous blood is needed to evaluate the ACE gene I/d polymorphism by simple PCR technique.

MATERIALS:
- Placentae
- Venous blood from mother
- Plastic containers
- Weighing machine
- Measuring tape
- Measuring scale
- Forceps: Blunt and toothed
- Spreading caliper
- EDTA vacuumtainers
- Pcr machine
- Electrophoresis unit
- Primers
Study also required a detailed history of mother regarding the socio demographic profile, present and past obstetric history will be recorded on pre- designed pre-tested proforma: a standard questionnaire was prepared and data will be collected on clinical variables like,

- I.P number
- Age
- Weight
- Height
- B.P
- LMP.
- Gravida: prime gravid/ multigravida
- Antenatal care: no/irregular/ regular

**Socio-economic status:**

- Area of mother: rural or urban
- Literacy: illiterate/primary/ secondary/ graduate
- Economy: below poverty line/ above poverty line
- Mode: House wife/ working women

- Addictions: Tobacco chewing; yes or no
- Alcohol consumption;yes or no
- Food style: Veg or Non-Veg
- Others: consanguineous marriage or not.

**Examination of placenta as under morphological parameters:**

1. Shape of placenta
2. The site of umbilical cord insertion
3. Vascular pattern: Disperse/Magistral
4. Presence of calcification
5. Presence of infarction
6. Hemorrhagic spots
7. Accessory placental lobe

**Examination of placenta as under morphometrical parameters:**

1. Weight of placenta in grams
2. Diameter of placenta in centimeters
3. Placental thickness
4. Surface area of placenta
5. Placental volume
6. Number of cotyledons
7. Cord insertion ratio
8. Foeto placental weight ratio

All these morphometric parameters of the placenta will be recorded using standard procedures.

**The processing of tissue of placenta for the histological examination:**
The sections shall be processed and stained with hematoxylin and eosin (H & E) as follows:

Fixation will be done in 10% buffered formalin and processed by routine paraffin embedding. Multiple 3-5um sections shall be cut by rotary microtome and mounted on clean glass slide coated with egg albumin. Sections will be stained by H & E stain and will be analyzed by light microscopy.

**H & E STAINING**
1. Dewax section, hydrate through graded alcohols to water.
2. Remove fixation pigments if necessary.
3. Stain in Harri’s hematoxylin for 5-15 min.
4. Wash well in running tap water until sections blue for 5 min or less.
5. Differentiate in 1% alcohol for 5-10 sec.
6. Wash well in tap water until section are again blue (10-15min)
7. Blue by dipping in alkaline solution.
8. Stain in 1% eosin and for 30 sec-1 min.
9. Wash in running water for 1-5 min.
10. Dehydrate through alcohols, clean and mount in DPX.

Examination of placenta as under following histological parameters.

1. Syncytial knots.
2. Vasculosyncytial membrane.
3. Intervillous haemorrhage.
4. Perivillous fibrinoid necrosis.
5. Intravillous fibrinoid necrosis.
7. Cytotrophoblast proliferation.
10. Hyperplasia of muscular media of foetal vessels

After completion of examination of placenta, rest of the placenta will be incinerated as government guideline.

All the activities with placenta (collection, preservation, etc) will be recorded in Record book
HAEMATOLOGICAL EXAMINATION
Platelet count, CBC, ABO AND Rh grouping.

MOLECULAR ANALYSIS:
Sampling: 5ml of venous blood will be drawn from peripheral vein using Di sodium EDTA vaccutainers. All the samples will be aliquoted and stored at -80°C until tested.
The laboratory work will be carried out in the central research laboratory, Rama Medical College, Hospital & Research Centre.
All the groups have their ACE gene will be analyzed for the I/D sequence of intron 16 of chromosome 17q23.3
Methodology:
1) Isolation of DNA:

DNA will be extracted from whole blood containing EDTA by Ponez et al., standard salting out procedure. The reagents required for DNA isolation are:

Chemicals used for DNA isolation
1) 1 M tris buffer(ph-7.5)
2) 1 M Mgcl2
3) 1 MM Kcl
4) 0.5 M EDTA
5) 10% SDS
6) 5 M Nacl
7) Triton X 100
8) Ethanol

Reagents
1) RBC LYSIS BUFFER:

10 mM of tris HCl
10 mM of KCL
10 mM of MgCl₂
2 mM of EDTA

2) NUCLEUS LYSIS BUFFER:

10 mM of tris HCL
10 mM of KCL
10 mM of MgCl₂
2 mM of EDTA
400 mM of NaCl

All the components will be mixed with sterile double distilled water and the prepared reagents will be stored at 25°C.

Protocol for DNA isolation (salting out Method)

1. Thawed blood samples (5 ml) from EDTA vactuainers will be transferred into clean and sterile centrifuge tubes.

2. 10 ml of RBC lysis buffer will be added and the volume will make up to 15 ml.

3. 0.1% of triton X will be added.

4. The centrifuge tubes will be incubated at 37°C for 5 mins.

5. The samples will be spun at 2000 rpm for 15 mins in a cold centrifuge at 4°C.

6. After centrifugation the supernatant will be discarded off taking care of the pellet.

7. 10 ml of RBC lysis buffer will be again added and vortex mixed.

8. The samples will be spun at 2000 rpm for 15 mins.

9. The supernatant will be discarded off and a white pellet will be obtained.

10. 1 ml of nucleus lysis buffer will be added then vortex mixed.

11. About 20 µl of 10% SDS will be added and mixed properly.

12. The mixture will be incubated in a water bath at 55°C for 1 hour.
13. After incubation, the contents will be transferred into 2ml eppendorf tubes.

14. 400 µl of 5M Nacl will be added

15. The contents will be spun at 10,000 rpm for 15mins

16. After spinning the supernatant will be transferred into a new centrifuge tube leaving behind the pellet in the eppendorf tubes

17. About double the volume if cold absolute ethanol will be added

18. Large clumps of DNA will be obtained by gentle swirling of the tubes

19. The DNA will be scooped with the help of a sterile spatula or a sterile loop from the centrifuge tubes and transferred into eppendorf tubes

20. 200µl of 70% ethanol will be added

21. The contents were centrifuged will be decanted and the eppendorf tubes with the DNA will keep for air drying

22. After drying, about150µl of TE buffer will be added and the DNA stored at \(-20^\circ\text{C}\) for later use.

**Concentration and quality assurance of DNA:**

- The quality of the DNA was analyzed by 0.8% agarose gel electrophoresis and the quality assessed by standard spectrophotometer at 260 nm and 280 nm.

2. **Molecular characterization of polymorphism of ACE gene by Polymerase chain reaction:**

   To determine the ACE genotype of cases and the control groups, the genomic DNA fragments will be amplified by PCR (T100 Biorad). The conditions required for amplification are mentioned below:

   The flanking primer sequences as reported by Rigat et al,1992 were;

   **Forward primer:** 5’CTG GAG ACC ACT CCC ATC TCT TCT 3’
Rewarse Primer: 5’GAT GTG GCC ATC ACA TTC GTC AGAT 3’

Primers will be obtained from Bangalore Genei and will be reconstituted with sterile double distilled water based on the manufacturer’s instruction.

PCR reaction mix (working concentration)
The working conditions for PCR reaction mixture are consumed from Bangalore Genei, India:

1. 10 x PCR Buffer:1X
2. dNTPs:200µM
3. Forward primer:30pM
4. Reverse primer:30pM
5. Taq polymerase:1.5U
6. Template DNA:50µg
7. Distilled water :to make up the volume

PCR conditions:

1) Initial denaturation:94°C-5mins
2) Denaturation: 94°C-30secs
3) Annealing:58°C-45secs
4) Extension :72°C-45secs
5) Cycling condition :30 cycles
6) Final extension:72°C-7mins
7) Hold at 4°C

3. Agarose Gel Electrophoresis
Once the amplification will be obtained, they will be subjected to 2% agarose gel electrophoresis with Ethidium bromide and the bands will visualize under UV light in gel documentation system (Biorad). The requirements for electrophoresis are:

**Requirements for electrophoresis**

1. Purified Agarose
2. 50x TAE buffer
3. Ethidium bromide
4. Bromophenol Blue

**Composition of 50 X TAE Buffer (1000Ml)**

1. Tris Base :242 g
2. Acetic acid :57.5ml
3. EDTA:18.6g
4. pH:7.2

**Procedure:**

1. 2% agarose gel Himedia will be prepared ,by mixing 2 g of agarose in 100ml of 1% buffer
2. The contents were boiled completely till a clear solution was obtained
3. About 100 µg of Ethidium bromide will be added and mixed properly
4. Agarose will be casted into specific trays and allowed to solidify
5. Specific combs will be used to make sample wells on the gel
6. The PCR products will be mixed with Bromophenol blue
7. 2µl of DNA ladder will be loaded into the first well
8. Then, samples will be loaded into the gels and run at 100v
9. After electrophoresis, The PCR products will be visualized under UV light in gel documentation system (Biorad). In the case of Deletion (D allele) and Insertion(I
allele), a 190bp and a 490 bp fragment were obtained respectively and comparison was done with a DNA ladder on the first well.

**From the department of O.G the following data of maternal complications will be collected.**

- Headache
- Epigastric pain
- Pedal edema
- Isolated thrombocytopenia
- Antepartum hemorrhage
- Jaundice
- Pulmonary edema
- Cardio respiratory arrest
- HELLP syndrome
- Postpartum hemorrhage
- Infection
- Leaking of liquor
- Seizures
- Renal failure
- Acute respiratory distress syndrome
- Maternal death

**From the department of O.G the following data foetal complications will be collected.**

- Size and weight of the baby
- Intrauterine growth retardation (IUGR)
- Intrauterine death
- Preterm delivery
- Low birth weight
- Jaundice
- APGAR score at 1 and 5 mints
- Congenital abnormalities if any
- NICU assistance
- Neonatal death
METHOD OF COLLECTION OF DATA:

SAMPLE SIZE CALCULATION AND STATISTICAL ANALYSIS:

✓ Sample size has been calculated in order to control type I & type II error. Assuming a minimum power 80% and 95% significance level the sample size has been calculated using this formula:

\[
 n = \frac{2(P)(1 - P) (Z_\beta + Z_\alpha/2)^2}{d^2}
\]

✓ \(p\) - Incidence of the disease (toxaemia of pregnancy)
✓ \(q = (1-p)\)
✓ \((P_1 - p_2)^2\) or \(d^2\) – Is the difference which we want to detect at a specified power & level of confidence.
✓ \(Z_\beta\) - power of statistical test we want to be minimum 80% for which is \(Z_\beta\) is 0.84.
✓ \(Z_{\alpha/2}\) –is the level of confidence we have chosen 95% confidence in this \(Z_{\alpha/2}=1.96\).
✓ When \(P\) indicates the incidence of the clinical condition e.g.: toxaemia of pregnancy.
✓ Following the literature the incidence of toxaemia of pregnancy has been assumed between 6-10%.
✓ The calculated minimum sample size for our study is 115.
✓ The calculated minimum sample size for control group is 115.
✓ In order to control loss of follow up and manual errors, which we rounded the sample size of 120 for each group.
✓ Data will be collected and entered in MS excel worksheets and results will be analysed with appropriate statistical tools like, tests of significance, logistic regression analysis etc using SPSS software.
METHOD OF SAMPLING:
Placenta with umbilical cord will be collected immediately after delivery. These are identified by I.P number of mother and stored in 10% formalin.
4 groups are made
Group-I: Control group
Group-II: Mild pre-eclampsia
Group-IV: Severe pre-eclampsia
Group-IV: Eclampsia

INCLUSION CRITERIA:
1. Antenatal mothers who are willing to participate in the study.
2. Antenatal mothers who don’t have hypertension at the time of pregnancy with any other abnormality will be taken in to control group.
3. Antenatal mothers diagnosed with pregnancy induced hypertension with their blood pressure of 140/90mmHg or more in to test group.

EXCLUSION CRITERIA:
1. Antenatal mothers who are not willing to participate in the study.
2. Antenatal mothers with the history of renal, liver failure, seizures and other medical problems.
3. Antenatal mother who has the hypertensive disorder before the pregnancy.