6. MATERIALS AND METHODS

6.1 STUDY DESIGN: Cross sectional study was conducted in Department of Anatomy of Dr. D.Y. Patil Medical College, Pimpri, Pune.

6.2 METHODS OF DATA COLLECTION: Consecutive convenient sampling method was used. 50 normotensive and 50 preeclamptic placentae were collected immediately after delivery from women who delivered either vaginally or by cesarean section from Department of Obstetrics and Gynecology of Dr.D.Y.Patil hospital and Yashwantrao Chawan memorial hospital, Pimpri, Pune. Institutional ethical committee clearance was obtained. Written informed consent was obtained from all mothers participating in the study.

Inclusion criteria:

- Age from 20-35 years.
- Primigravida.
- Gestational age between 28-38 weeks.
- Willing to participate and signing consent form after explanation of aim and purpose of study.

Exclusion criteria:

- Preexisting hypertension, diabetes mellitus or any other complication.
- Gestational diabetes or any other gestational complications.
- History of maternal diseases such as autoimmune disease or thrombophilic conditions.
- Evidence of any fetal anomalies or intrauterine death.
Samples were divided into two groups as **group A** and **group B**:

**Group A: Control group (Normotensive):** Placentae were obtained from pregnant women who did not have any clinically detectable abnormalities. These women had normal blood pressure, no proteinuria and no oedema.

**Group B: Study group (Preeclampsia):** Placentae were obtained from known preeclamptic cases who had no history of hypertension before pregnancy or during first 20 weeks of gestation, who had consistently recorded systolic and diastolic blood pressure of 140 / 90 mm of Hg or above and proteinuria ≥300 mg per day. The alterations in blood pressure were observed on at least two different occasions, at least six hours apart. Detailed menstrual and obstetric history and past history was obtained to exclude preexisting hypertension and other complications. Fetal weight, sex, any congenital anomaly and APGAR score at 1 and 5 minutes after delivery were recorded as parameters of fetal outcome.

### 6.3 CLASSIFICATION OF SEVERITY OF PREECLAMPSIA

Classification of severity of preeclampsia was done based on the criteria of American College of Obstetricians and Gynecologists. (ACOG)\textsuperscript{143}

**Mild-moderate**

BP is 140 to 159 mmHg systolic and/or 90 to 109 mmHg diastolic that occurs after 20 weeks of gestation (on 2 occasions at least 6 hours apart) and proteinuria is ≥300 mg/24 hours.

**Severe**

BP is ≥160 mmHg systolic and/or ≥110 mmHg diastolic (on 2 occasions at least 6 hours apart, while the patient is on bed rest) and proteinuria is ≥500 mg/24 hours.
6.4 MORPHOLOGICAL STUDY:

Morphology of placenta was studied under the following:

- Weight
- Diameter
- Thickness
- Number of cotyledons
- Study of maternal and fetal sides
- Presence of necrotic patches

6.4.1 PLACENTAL WEIGHT:
- Placental weight was measured by directly placing the placenta on standardized weighing scale.

6.4.2 PLACENTAL DIAMETER:
- The placenta was placed on a flat surface after trimming and mopping.
- At first the maximum diameter was measured with a metallic scale graduated in centimeters.
- Then second maximum diameter was recorded at right angles to the first one.
- The mean of two diameters was considered as the diameter of placenta.

6.4.3 PLACENTAL THICKNESS:
- Placenta was placed on a flat surface.
- Two circles were drawn from the center of the placenta.
- Thick point needle was inserted to the full thickness of placenta at five points.
- Centre of the inner circle, two different points between inner and outer circle and two different points outside the outer circle.
- Mean of all five points was taken as the thickness of placenta.

6.4.4 NUMBER OF COTYLEDONS:
- Gentle pressure was applied on center of the fetal surface of placenta.
- As a result the cotyledons on the maternal surface became prominent.
- The placenta was then placed on a flat surface with maternal side facing upwards and total number of cotyledons was recorded.
6.4.5 STUDY OF MATERNAL AND FETAL SIDES:

- Maternal and fetal sides of placentae were looked for carefully for any necrotic patches, calcifications and attachment of umbilical cord was noted.
6.5 HISTOPATHOLOGICAL STUDIES

From each placenta whole thickness tissue blocks were taken from center and periphery. Tissue samples from placentae were processed and stained and were observed under light microscope. 100 villi were studied from each of central and peripheral section of placentae for each category of stain. Sections were then photographed by microphotography and transferred to the computer.

6.5.1 HAEMATOXYLIN AND EOSIN STAIN: HISTOPATHOLOGY\textsuperscript{144,145}

Tissues were processed for paraffin blocks as follows:

1. **Fixation**: Each tissue was cut into a small fragment of about 1 cm before fixation. This was to facilitate penetration of fixative and preservation of tissue. Fixative used was 10 % cold formal saline for 48 hours.

2. **Dehydration**: The tissue to be embedded was dehydrated by bathing them in into grades of ascending alcohol (50 -95%)
   - 50% alcohol – 2 hours
   - 70% alcohol – overnight
   - 80% alcohol – 2 hours
   - 95% alcohol – 2 hours
   - Absolute alcohol I – 1 hour
   - Absolute alcohol II – 1 hour

3. **Clearing**: Alcohol was then replaced by xylene.
   - Xylene I – 1 hour
   - Xylene II – 1 hour

4. **Embedding**: Tissue was then placed in melted paraffin at 58 to 60 degree Celsius. Blocks were prepared. Section of 5 micron thickness was taken and was transferred to the glass slide for staining.
6.5.2 PREPARATION OF STAINS:

1% EOSIN STOCK SOLUTION:

Solution was prepared as follows:

- Water soluble eosin Y – 1gm
- Distilled water – 20 ml
- 95% alcohol – 80 ml

i. 1 gm of water soluble eosin Y was added to 20 ml of distilled water.

ii. Eosin was dissolved in water then 80 ml of 95% alcohol was added.

WORKING EOSIN SOLUTION:

Solution was prepared as follows:

- 1% Eosin stock solution – 25 ml.
- 80% alcohol – 75 ml.
- Glacial acetic acid – 0.5 ml

i. 25 ml of 1% Eosin stock solution was added to 75 ml of 80% alcohol.

ii. 0.5 ml of glacial acetic acid was added to 100 ml of stain.

HARRIS’S HAEMATOXYLIN:

Stain was prepared as follows:

- Haematoxylin – 1 gm
- Absolute alcohol – 10 ml
- Ammonium or Potassium alum – 20 gms
- Distilled water – 200 ml
- Mercuric oxide (red) – 0.5 gms

i. Haematoxylin was dissolved in alcohol.

ii. Alum previously dissolved in hot water was added to it.
iii. The solution was quickly boiled and mercuric oxide was added to it till the solution turned dark purple.

iv. It was cooled rapidly under tap water and filtered before use.

6.5.3 STAINING:

Staining was done routinely with Haematoxylin and Eosin. Haematoxylin is a basic dye which stains the nucleus of the cell and eosin is an acidic dye which stains the components of the cytoplasm.

Technique:

1. Deparafinisation: The slides were treated with two changes of xylene.
   - Xylene I – 15 minutes
   - Xylene II – 15 minutes

2. Graded hydration: Slides were passed through following series of alcohol to hydrate the sections.
   - Absolute alcohol I – 2 dips
   - Absolute alcohol II – 2 dips
   - 95 % alcohol – 2 dips
   - 80 % alcohol – 2 dips
   - 70% alcohol – 2 dips
   - 50 % alcohol – 2 dips
   - Rinsed with distilled water.

3. Stained with Haematoxylin for 10 minutes.

4. Washed in running tap water for 10 minutes.

5. Slides were observed under microscope for proper staining. If stained excessively, a dip in acid alcohol was given.

6. Washed in running tap water for 10 minutes.

7. Stained with eosin for 3 minutes.
8. **Dehydration**: The sections were dehydrated in following series of alcohol.

- 50% alcohol – 2 dips
- 70% alcohol – 2 dips
- 80% alcohol – 2 dips
- 95% alcohol – 2 dips
- Absolute alcohol – 2 dips

9. **Clearing and Mounting**: The slides were dried, cleared in xylene and mounted using DPX.

Positive sites stained – Cytoplasm stained pink

Nuclei stained violet
6.6 PERIODIC ACID SCHIFF’S REACTION (PAS): GLYCOGEN^{144,145,146}

Tissues were processed for staining as follows: (as explained in H & E staining)

1. Fixation
2. Dehydration
3. Clearing
4. Embedding
5. Blocks were prepared.

Thin sections of 5 microns were taken.

6.6.1 SCHIFF’S REAGENT:

Reagent was prepared as follows:

i. 1gm of basic fucshin was dissolved in 200 ml of boiling distilled water in a stoppered 1 liter flask.

ii. Shaken for 5 minutes

iii. Cooled exactly at 50 degree Celsius, filtered and 20 ml of N/1 HCl was added to the filtrate.

iv. Cooled further to 25 degree Celsius and 1 gm of sodium metabisulfite was added to it.


vi. 2gms of activated charcoal was added and the mixture was shaken for 1 minute.

vii. Charcoal was removed by filtration and the solution was stored in dark at 0-4 degrees.
6.6.2 STAINING:

Periodic acid is an oxidising agent that has the ability to oxidise 1, 2- glycols to formaldehydes. PAS reaction is a commonly used histological technique to localize glycoproteins and glycogen.

**Technique:**

1. Sections were brought to water.

2. Oxidised in 1 % periodic acid for 10 minutes.

3. Washed in running water for 5 minutes and rinse in distilled water for 1 minute.

4. Treated with Schiff’s reagent for 30 minutes.

5. Washed in running tap water for 10 minutes.

6. Counterstained with Harris haematoxylin for 6 minutes.

7. Washed in running tap water and rinsed in distilled water.

8. Dehydrated, cleared and mounted in DPX.

PAS positive substances stained – Magenta

Nuclei stained Blue
6.7 ALCIAN BLUE STAIN (pH 2.5): GLYCOSAMINOGLYCANS (GAGs)

**Tissues were processed for staining as follows.**

1. Fixation

2. Dehydration

3. Clearing

4. Embedding

5. Blocks were prepared.

6.7.1 **PREPARATION OF ALCIAN BLUE STAIN:**

**Stain was prepared as follows:**

- Alcian blue : 0.5gms
- Glacial acetic acid – 3 ml
- Distilled water - 100 ml
  - i. 0.5 gms of alcian blue and 3ml of glacial acetic acid was added to 100 ml distilled water and dissolved.
  - ii. Filtered and crystals of thymol were added. This gave staining solution of pH 2.5.

**Sections:** Paraffin sections of 5 micron thickness were taken.

6.7.2 **STAINING:**

Alcian blue is a copper phthalocyanin dye containing four isothiouronium groups. Alcian blue stain at pH 2.5 stains both sulphated and non sulphated acid glycosaminoglycans.

**Technique :**

1. Sections were taken to water.

2. Stained in Alcian blue stain for 30 minutes.

3. Rinsed in distilled water.
4. Washed in running water for 5 minutes.

5. Counterstained with fresh light green for 2 minutes.

6. Dehydrated through graded alcohols, cleared in xylene and mounted.

   Positive sites stained – Cytoplasm – Light blue

   Blood vessels stained Light green.
6.8 MODIFIED GOMORI’S METHOD: PLACENTAL ALKALINE PHOSPHATASE (PALP)\textsuperscript{146}

Serum alkaline phosphatase of both normotensive controls and preeclamptic cases was recorded to correlate with PALP.

Whole thickness tissue was cut out from central and peripheral part of placenta. Frozen sections of 10 micron thickness were taken from this fresh unfixed tissue. Sections were then placed on non albuminised slides. These were further stained by modified Gomori’s method.

6.8.1 INCUBATING MIXTURE:

Was prepared as follows:

- 3 \% aqueous Sodium beta glycerophosphate - 20 ml
- 2 \% aqueous Sodium barbitone - 30 ml
- 2 \% aqueous Calcium chloride - 4 ml
- 2 \% aqueous Magnesium sulphate - 2 ml
- Distilled water - 30 ml

6.8.2 STAINING:

Placental alkaline phosphatase activity was demonstrated by using modified Gomori’s method.

Technique:

1. Sections were washed with distilled water and were immersed in incubating mixture for one hour at 37 degree Celsius.

2. Tissues were taken out from the incubator and were brought to room temperature.

3. Stained by 2 \% Cobalt nitrate solution for 5 minutes.

4. Then they were stained by freshly prepared Ammonium sulfide solution for 2 minutes. (Ammonium sulfide solution was prepared by adding 50 drops of concentrated solution into 20 ml of water)
5. Tissues were washed with distilled water after every step.

At the end deposition of black precipitate was observed at the sites of enzymatic activity.
6.9 SUDAN BLACK : LIPIDS 144.145

Whole thickness tissue was cut out from central and peripheral part of placenta. Frozen sections of 10 micron thickness were taken from this fresh unfixed tissue. Sections were then placed on non albuminised slides.

6.9.1 SUDAN BLACK STAIN

Stain was prepared as follows:

i. 0.7 gram of Sudan Black was dissolved in 100 ml of propylene glycol slowly while stirring.
ii. Heated to 100 degree Celsius for a few minutes stirring constantly.
iii. Filtered through Whatman # 2 filter paper.
iv. Cooled and filtered again through frittered glass filter of medium porosity.
v. Stored at 60 degree Celsius.

6.9.2 STAINING

Technique

1. Slides were fixed in 10% formal saline for 15 minutes.
2. Washed well in tap, rinsed in distilled water, excess water was drained off.
3. Two changes of propylene glycol were given for 5 minutes each.
4. Stained with Sudan Black for 7 minutes and agitated.
5. 85% Propylene glycol for 3 minutes.
6. Rinsed in distilled water.
7. Counterstained with Nuclear fast red for 3 minutes.
8. Washed in tap water and rinsed in distilled water.

Positive sites stained -Fat blue-black
Figure 3

A: Wax bath, B: L molds, C: Semi motorized Microtome, D: Water bath

Figure 4

1. Xylene
2-6: Series of alcohol
7. Haematoxylin
8. Eosin
9. Periodic acid
10. Schiff’s reagent
11. Incubation mixture for PALP
12. Cobalt nitrate
13. Ammonium sulphide
14. Sudan black
15. Alcian blue
Figure 5

A: Computer, B: Trinocular microscope with microphotographic attachment