CHAPTER 3

MATERIALS AND METHODS

The study was carried out in the Department of Anatomy in SRM Medical College, Hospital & Research Centre, Potheri from September 2010 to 2014. A total of 300 placentae were collected immediately after delivery for the study. Out of these 300 placentae, 150 were from normal pregnant women who had no hypertension, proteinuria and 150 were from preeclampsia women who had new onset of hypertension, proteinuria and edema. Placentae from patients with previous hypertension, proteinuria or renal disease were excluded (Appendix 1). Informed consent was taken from the patients (Appendix 2) and HOD of the department of Obstetrics and Gynaecology. The work was approved by ethical committee of SRM University 83/IEC/2010 (Appendix 3).

3.1 GROSS MORPHOLOGY

All the collected placentas were tagged with code numbers before the commencement of the study, for the purpose of identity. The placentas were weighed after trimming the membranes, cutting the umbilical cord at a distance of 1cm from the site of insertion, washing in running tap water and drying with blotting paper (Figure 3.1). The volume of the placenta was determined by water displacement technique [53]. The placental diameter was measured with vernier caliper (cm) (Figure 3.2). The number of cotyledons was counted (Figure 3.3). The length of the umbilical cord was measured using measuring scale (cms) (Appendix 4). The thickness of the placenta was estimated by performing two sections: one in the middle and one cut 2cm from the margin of the placenta. The birth weights of newborn babies, APGAR score, gestational age and maternal age were noted from the patients medical record. The feto-placental ratio (fetal weight/placental weight) was calculated in each case [184].
Figure 3.1 Measuring weight of placenta

Figure 3.2 Measuring diameter of placenta

Figure 3.3 Counting number of cotyledons
3.2 HISTOMORPHOMETRY OF CHORIONIC VILLI

The histomorphometric study of chorionic villi of the placenta was done using H&E staining. The measurement was done with ocular micrometer (Figure 3.4) and reticule (Figure 3.5).

Systematic uniform random sampling (SUR sampling) protocol was adopted to avoid any human bias in selecting the sections. For the quantification of stereological study a 1cm$^2$ grid of square lattice called reticule containing 121 intersections is used in this study. Following parameters were estimated:
The volume of villi and intervillous space were obtained by counting the number of point of intersections that falls on the villi/space. The surface area of villi was measured by counting the number of complete square that falls on the villi. Five fields per sections were analyzed.

**Volume density or volume fraction (Vv):** Volume density of various components was estimated by using the point count method. Volume density (Vv) of a given component per unit volume of organ was obtained by dividing the sum of points falling on the component of interest (point or intersection-Pi) by the total number or points in the test grid (Pt). Five fields per sections were analyzed.

\[ Vv = \frac{Pi}{Pt} \text{ cumm/µm} \]

Where,  
\( Vv \) = Volume density  
\( Pi \) = Point or intersection  
\( Pt \) = Total point in reference space/area

**Analysis of tubular profile (diameter of villi):** The tubular profile and cellular axial ratio was obtained by using ocular micrometry.

In two planes

Axial ratio = \( L+B/2 \)

Where, \( L \) – Length or greatest diameter in any plane  
\( B \) – Breadth or greatest diameter right angle to length

The length value was the value obtained per unit area. A range of 10-15 villi were analyzed per section [185].

Data were analyzed using Microsoft excel and values were expressed as ± standard error or the mean.
3.2.1 **Hematoxylin and Eosin Technique:** (Appendix 5)

- Fixed in 10% formalin solution
- Embedded in paraffin wax. Serial Paraffin sections of tissue taken
- Dewaxed sections and hydrated through graded alcohols to water
- Stained in an alum haematoxylin for 10 minutes
- Washed well in running tap water until sections ‘blue’ for 5 minutes
- Differentiated in 1 per cent acid alcohol (1 percent Hcl in 70% alcohol) for 5-10 seconds
- Washed well in tap water until sections are again ‘blue’ for 5 minutes
- Stained in 1% eosin for 10 minutes
- Washed in running tap water for 1-5 minutes
- Dehydrated through alcohols, cleared and mounted in DPX

3.3 **STUDY OF VILLOUS HISTOPATHOLOGY**

To study the villous histopathological changes the following stains were used.

3.3.1 **Van gieson’s Technique**

The stain used to demonstrate the stromal fibrosis (Appendix 5). The following procedure is used for staining the tissue.

**Procedure**

- dewaxed sections and brought to water
- stained nuclei by the Celestine blue-haemalum sequence
- washed in tap water
- differentiated in acid alcohol
• washed well in tap water
• stained in van gieson solution for 3 minutes and blotted with blotting paper
• dehydrated through graded alcohols
• Cleared in xylene and mounted in DPX.

3.3.2 PAS Technique (Periodic Acid - Schiff Reaction)

The stain used to study the thickening of basement membrane, fibrinoid necrosis and proliferation of cytotrophoblast (Appendix 5).

Procedure:

• dewaxed sections and brought to distilled water
• treated with periodic acid for 5 minutes
• washed well with several changes of distilled water
• covered with schiff’s solution for 15 minutes
• Washed in running tap water for 5-10 minutes
• stained nuclei with Harris’s haematoxylin, differentiated with appropriate acid alcohol and
• blueing was done
• washed in water
• rinsed in absolute alcohol
• Cleared in xylene and mounted in DPX.
3.4 IMMUNOHISTOCHEMISTRY

3.4.1 CD 68

Serial sections of 5 µm thickness were collected on poly-L-lysine coated slides (Sigma, St.Louis, MO, USA) and incubated overnight at 56°C. Tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Sections were then boiled in a microwave oven in 10mM citrate buffer, pH6.0, for 4 times, each for 5min and a total of 20min. Thereafter the slides were left to cool for another 20min. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide in methanol for 20min. Afterwards, sections were incubated in a blocking serum (ultra V block, TP-060-HL; Neo-marker, Fremont, CA, USA) for 7 min in order to block non-specific binding without the washing step, sections were subsequently incubated overnight at 4°C with mouse monoclonal anti-human CD 68 for the identification of placental macrophages. Sections were incubated with the biotinylated secondary antibody (TP-060-HL; Lab Vision, Fremont, CA, USA) and a streptavidin-peroxidase complex (TP-060-HL; Lab vision), respectively, for 15min. The resulting signal was developed with diaminobenzidine (DAB) (K3466; Dako). Each step was followed by three washes in phosphate buffered saline (PBS). Finally sections were counterstained with hematoxylin and mounted.

3.4.2 sFLT-1

Paraffin sections were mounted on glass slides, dewaxed in xylene, and rehydrated in descending ethanol gradient. Antigen retrieval was performed by heating in sodium citrate solution (10 mmol). Endogenous peroxidase was quenched with 3% (vol/vol) hydrogen peroxide in PBS for 30 min. After blocking (5% normal goat serum for 1 h), the slides were incubated overnight with primary antibody (anti-human soluble VEGFR-1, 1:150 dilution). Slides were washed in 1×PBS and exposed to peroxidase-conjugated secondary antibody (1:300, goat anti-rabbit, Vector Laboratories) for 45 min at room temperature. Finally, avidin biotin complex (Vector Laboratories) was applied for 1 h, and staining was detected with the diaminobenzidine chromogen after 5 min. Slides were counterstained with hematoxylin.
3.4.3 VEGF

Paraffin sections were mounted on glass slides, dewaxed in xylene, and rehydrated in descending ethanol gradient. Antigen retrieval was performed by heating in sodium citrate solution (10 mmol). Endogenous peroxidase was quenched with 3% (vol/vol) hydrogen peroxide in PBS for 30 min. After blocking (5% normal goat serum for 1 h), the slides were incubated overnight with primary antibody (VEGF, 1:150 dilution). Slides were washed in 1×PBS and exposed to peroxidase-conjugated secondary antibody (1:300, goat anti-rabbit, Vector Laboratories) for 45 min at room temperature. Finally, avidin biotin complex (Vector Laboratories) was applied for 1 h, and staining was detected with the diamino-benzidine chromogen after 5 min. Slides were counterstained with hematoxylin.

3.4.4 eNOS

Tissues were fixed in formalin, embedded in paraffin, and cut into 5µm thick sections, which were collected on slides coated with poly-L-lysine. After the paraffin was removed, the sections were rehydrated. Immunostaining was performed by the streptavidin-biotin-peroxidase method. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Antigen retrieval was carried out in a microwave oven for 15 min in 10nM citrate buffer (pH 6.0) for eNOS. The sections were incubated at room temperature for one hour with rabbit polyclonal antibodies reactive with eNOS. After washing in phosphate buffered saline with tween -20, the tissues were incubated with a biotin conjugated secondary antibody and then with a biotin streptavidine complex for 30min at room temperature, reactions were visualized with 3-3-diaminobenzidine tetrahydrochloride(DAB). Sections were counter stained with hematoxylin, rinsed and mounted (Appendix 6).

Evaluation of immunohistochemical staining: The intensity of the staining reaction of sFLT1 in Hofbauer cells was evaluated by two investigators blind to the purpose of the study. Immunoreactivity for antibodies was scored using a semi-quantitative scale for intensity of staining: 0 negative, no staining; 1+ weak positive; 2+moderately positive; 3+ strongly positive.
3.5 STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS for windows (version 13.0, Chicago IL, USA). Data were expressed as mean ± standard deviations for gross morphology and histomorphometry of chorionic villi. The differences of groups were analyzed by Student’s test.

The intensity of sFLT1, VEGF and eNOS for each slide, a value designated ‘H score’ was obtained by application of the following algorithm: “H score” = Σ(I×PC), where I and PC represent intensity and percentage of cells that stain at each intensity, respectively and corresponding H-score were calculated separately.

Statistical analysis was carried out using SPSS for windows (version 13.0, Chicago IL, USA). Data were expressed as mean ± standard deviations. The differences of groups were analyzed by Mann-whitney U test. P value < 0.001 was considered statistically significant.