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"MACROSCOPIC PLACENTAL CHANGES ASSOCIATED WITH CLINICAL CONDITIONS IN WOMEN WITH OR WITHOUT HYPERTENSIVE PREGNANCY"

Dr.K Ayesha Siddikhakhatoon[1], Dr.Bikshapathy Macharla[2]

[1]Associate Professor, Department of Anatomy, Maheshwara Medical College And Hospital
[2*]Assistant Professor, Department of General Medicine, Maheshwara Medical College and Hospital

***Corresponding Author:** Dr.Bikshapathy Macharla
*Assistant Professor, Department of General Medicine, Maheshwara Medical College and Hospital

Introduction

Pregnancies with hypertensive disorders are prone to a higher risk of preterm deliveries and low birth weights compared to healthy pregnancies. [1] The risk of hypertensive disorders of pregnancy occurs mostly among mothers affected with severe chronic hypertension as well as those with superimposed preeclampsia on chronic hypertension. [2] Hypertensive disorders are common complications of pregnancy. Most adverse events like maternal and neonatal mortality and morbidity are attributed directly to the preeclampsia syndrome, characterized by new-onset hypertension with proteinuria during pregnancy. [3] Hypertensive disorders during pregnancy are classified into 4 categories, as recommended by the National High Blood Pressure Education Program Working

Hypertension is one of the medical problems that mostly affect pregnant women andit remains an important cause of both maternal and foetal morbidity/ mortality. Studies show that 10–15% of pregnancies will be complicated by high blood pressure. [6] Up to about one- quarter of all antenatal admissions will be hypertensive related cases. Over the last century, maternal mortality rates in high-income countries have steadily declined. [7] Every year about 70,000 women die and there are half a million stillbirths or neonatal deaths owing to hypertensive disorders of pregnancy (HDP)– the vast majority being in the developing world.[8
The identification of the Hypertensive disorder and its effective treatment play a beneficial role in pregnancy outcomes for the mother and the foetus, and hence a reduction in both maternal and perinatal mortality. Hypertensive disorders are associated with low birth weight, fetal growth restriction and prematurity which greatly contribute to perinatal morbidity and mortality. [9] Many pregnancy complications that are associated with high foetal morbidity and mortality have shown gross deviations from the normal placental morphology and anatomy. [10] With the placenta serving as the image for the health status of the mother and foetus, complications like hypertension in pregnancy has reflected in the placenta in a significant way, either microscopically or macroscopically. [11]

The placenta is the growing organ of the human body. The normal placenta parenchyma is divided into 10-40 lobes or lobules separated by grooves or septa. During the first twelve weeks of development, the placenta consists of mesenchymal villi after this period, subsequently stem or anchoring villi are formed. [12] Microscopically the bulk of the villi consist of connective tissue in which blood vessels are found. The outer part of villus is surrounded by the syncytiotrophoblast which resembles like a cuboidal epithelium. Most of the cells in the connective tissue core of the villi are fibroblast. [13]

In the first few weeks of development the whole placenta consists of mesenchymal villi and after approximately 12 weeks, immature intermediate villi are formed. Immature intermediate villi are no

longer present after 24 weeks of pregnancy. The terminal villi can berecognized by the presence of syncytio-vascular membranes. [14] Under normal conditions, terminal villi can be recognized from 30-32 weeks onwards and around term 40% of the placental villi consist of these terminal villi. [15]

Many of the disorders of pregnancy which are associated with high perinatal morbidity and mortality are accompanied by gross pathological changes in placenta. Abnormal maturation can be seen in several different conditions. Accelerated maturation i.e. premature formation of terminal villi can be seen as a reaction or adaptation of the placenta to a decreased materno- placental perfusion. [16] Histologically it can be recognized by a decrease of villous diameter and by accelerated formation of syncytio- vascular membranes. Failure of the second phase of trophoblast invasion of the spiral arteries is generally believed to give rise to several pregnancies induced hypertensive. Disorders of pregnancy e.g. pre- eclampsia delayed maturation can be seen in several different clinical situations it is well known in association with maternal diabetes but it can be seen also in macrosomic placentasin mothers without diabetes. [17]

The attachment of the umbilical cord to the placenta is usually near the centre of fetal surface of this organ, but it may attach at any point. For instance, insertion at the margin the placental margin produces a battledore placenta, and its attachment to the fetal membranes isa velamentous insertion of the cord. The umbilical cord is usually 1 to 2 cm in diameter and 30 to 90 cm in length (average 55cm). Excessively long or short cords are uncommon. There are usually two arteries and one vein in the umbilical cord that are surrounded by mucoid connective tissue (Wharton Jelly). [18] The umbilical arteries are spirally arranged and present thick wall. In the early part of pregnancy, the umbilical veins are two in number. Later the right umbilical vein disappears and the left one persists to convey oxygenated blood from placenta to the fetus. Nerves are not detected in the umbilical cord. [18]

Rationale of the study

How pregnancy initiates or aggravates hypertension remains unsolved despite decades of intensive research. Both umbilical cord and placenta are morphologically adapted to the essential function of bringing fetal and maternal blood streams in close association in normalcy. Among the high-risk pregnancies, hypertensive disorders are the most common medical complication of pregnancy and a major cause of maternal and perinatal morbidity and death. Pregnancy-induced hypertension also complicates the pregnancy and is associated with congenital malformations, intrauterine deaths, perinatal and maternal mortality andmorbidity.

Like other developing countries, India still has alarming maternal and fetal mortality rates. Compensatory changes, therefore, are likely to occur in these organs in disease situations. Changes in the morphology of umbilical cord component is, therefore, considered as area of real importance and interest with implications in both basic and clinical research.

REVIEW OF LITERATURE

Majumdar S et al expressed that, the mothers with hypertensive pregnancy were havingminor and asymmetrical placentae with peripheral attachment of umbilical cord. The highlights of vascular inadequacy like localized necrosis, apoplexy and calcification were more in hypertensive placentae. In the histological examination they noticed, the cytotrophoblastic multiplication, syncytial ties, fibrin plaque development likewise more in hypertension. They assessed that these variations of placenta may be the explanation behind low birth weight of child in pre-eclampsia. (19)

Sharmishtha and Sandhya (Ref.) exhibited that the placentae were little and unpredictable and its volume demonstrated decrease in pregnancy induced hypertension. The seriousness of hypertension unfavourably influences both placental and fetal result. (20)

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Dahlmstrom et al showed the little placentae were related with preeclampsia, and more unequivocally with preterm than term preeclampsia. (21)

Udainia and Jain expressed that the placental weight was diminished fundamentally in pregnancy instigated hypertension. Placental weight under 250gms were discovered uniquely in hypertensive pregnancy. As seriousness of hypertension builds, placental weight diminishes as affirmed by least placental load of 250gms in mild hypertension and 200gms inextreme hypertension. (22)

Muhammed Ashfaq contrasted the diabetic placenta and hypertensive placenta and found a critical expansion in weight, thickness and measurement. He noticed a variety in placental connection of rope in diabetes, though in hypertensive pregnancy, there is nocritical contrast in every one of these boundaries. (23)

Segupta kishwara et al noticed a reduction in diameter, thickness, number of cotyledons and volume in preeclamptic placentae. They stated that the insufficiency of blood supply to the intervillous space of placenta was the reason for these changes in preeclampsia.(24)

MATERIALS AND METHODS

Study design: This is prospective and observational and case-control study.
Study area, Population and period: The study will be performed in the Department of Anatomy, Pathology, Gynaecology and Obstetrics, Tertiary care Teaching Hospital over a period two years.
Sampling Selection of hypertensive and normotensive pregnant mothers: Pregnant women having hypertension (Blood Pressure (BP) ≥ 140/90mm Hg), will be selected from the patients attending the antenatal clinic in the Department of Obstetrics and Gynaecology.

A total of 55 patients will be selected. Out of 55 selected hypertensive pregnancy, 40 patients fulfill the inclusion and exclusion criteria at the time of delivery and thereafter in the postnatal period of more than 12 weeks. Normotensive of pregnant women will be selected randomly from antenatal clinic after applying inclusion and exclusion criteria for selection. Total 50 normotensive pregnant mothers will be selected. Out of 50 selected normotensive patients, 40 will be traced at the time of delivery and thereafter fulfilling the criteria for inclusion and exclusion and the placentae will be collected.

Inclusion criteria: The pregnant mothers in the age group 20 years to 40 years attended for antenatal checkup. Patients having detailed history, clinical data, consent & cooperation of patients, blood and urine report and specimen of placenta available for examination.

Pregnant women having hypertension (blood pressure (BP) ≥ 140/90mm Hg). Normotensive (blood pressure of less than 140/90mmHg) pregnant mothers without any illness. Specimen of placenta collected from the full-term delivery cases (i.e, cases completed37 weeks of gestation)

Exclusion criteria: Patients with age below 20 years and above 40 years. Other Medical or Surgical illness. Blood and urine report and consent not available and patients lost or missing in the follow-up period.

Study Techniques

1.Case history and clinical examination2.Collection of the specimen 3.Examination of the specimen
4. Follow-up
5. Statistical Analysis.

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Techniques in detail:
It includes clinical study of the cases and examination of placenta after delivery as described below:

1. Detail Case history including LMP recorded and Clinical examination done
2. Collection of the specimen.

Fresh placentae will be collected at labor room /Operation theatre, labeled and sent to Pathology Department.

Examination of the specimen: Examinations of placenta will be conducted according to proforma adopted by Benirschke and later modified by Woodly et al [13].

Gross examination: Following parameters of the placenta will be determined: - dimensions, surface area, weight, volume, shape, examination of the fetal surface, insertion of the umbilical cord and amniotic membranes. Examination of the maternal surface, blood clots, infarcts, number of cotyledon and examination of cut sections.

HISTOLOGY
Slide PreparationUmbilical Cord
1cm length of the cord will be taken from three sites, one at foetal end (that is 2cm away from cut end after clamping), one from maternal end (4cm away from placental attachment) and from middle of the cord for histological and immunohistochemical study. The segments will be fixed in 10% formalin for 24 hours and followed the routine steps for tissue processing with automatic tissue processing machine (figure 3.5) and slide preparation. 5 micron thick sections will be taken and stained with Haematoxylin and Eosin and Vangieson stains.

Placenta
2 cm sized placental pieces will be taken from the central and peripheral part of the placenta and fixed in 10% formalin. After taking the 5micron thick sections, the routine steps for slide preparation will be followed. The sections will be stained with Haematoxylin & Eosin, Vangieson and Periodic acid schiff stains.

Haematoxylin and Eosin Staining Procedure

- The tissues will be fixed in 10% formalin
- Embedded in paraffin wax and prepared paraffin blocks.
- Serial sections of the tissue will be taken
- The sections will be dewaxed with xylene and hydrated through graded alcohols towater
- The slides will be 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 5-10seconds
- The slides will be washed well in tap water for 5minutes until sections are again‘blue’
- Then the section will be stained in 1% eosin for 10 minutes
- Washed in running tap water for 1-5 minutes
- Dehydrated through alcohols, cleared and mounted in DPX

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Van Gieson Technique

- The sections will be dewaxed and brought to water
- Stained nuclei by the Celestine blue-haem alum sequence
- Sections will be washed in tap water
- It was differentiated in acid alcohol
- Washed well in tap water
- This stained in van gieson solution for 3minutes and blotted with blotting paper
- It will be dehydrated through graded alcohols
- It cleared in xylene and mounted in DPX.

PAS Technique (Periodic Acid-Schiff Reaction)

The stain used to study the thickening of basement membrane, fibrinoid necrosis andproliferation of cytotrophoblast.

- Dewaxed the sections and brought to distilled water
- It will be treated with periodic acid for 5minutes
- Washed well with several changes of distilled water
- It will be 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 will be done
- Washed in water
- Rinsed in absolute alcohol
- Cleared in xylene and mounted in DPX.

HistomorphometryUmbilical Cord

The thickness of the wall and lumen of the artery and vein will be measured by using ocular micrometer and surface area with a reticule. All the above dimensions will be measured in12, 3, 6 and 9' O Clock positions of each cord.

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Area of vessels- The diameter of artery and vein will be measured with micrometer. Diameter of vessel (d) = (L of vessels+B of vessel) /2
Where, L = largest diameterB = shortest diameter Radius of vessel (r) =d/2 Area of vessel = πr^2

Area of Wharton's jelly

The diameter of umbilical cord measured with micrometer.Diameter (D) = (L of cord+B of cord) /2
Where, L = largest diameterB= shortest diameter
Radius of cord (R) =D of cord/2Area of cord= πR^2
Area of Wharton's jelly = Area of cord - Area of vessels

The thickness of the amniotic epithelium will be also measured with ocular micrometer. The type of amniotic epithelium will be evaluated under high power.

The wall lumen ratio will be calculated by dividing the wall thickness with lumen. Thickness of individual layers of vessels, intima and media, and endothelium thickness of vessels will be also measured by using ocular micrometer. From the total thickness, the % of intima and media will be

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also calculated. The type of endothelium will be evaluated under high power.

Placenta
Randomly selected 5 fields of every slide will be used for the study. Volume and surface area of the villi will be measured by using reticule and diameter and thickness by ocular micrometer.

Diameter of villi = (L+B)/2
Where L = largest diameter of villiB= shortest diameter of villi

Volume of villi will be measured by dividing the sum of intersections falling on the corresponding tissue with the total number of intersections in a grid

Volume tissue V = Pi/Pt
Where, Pi = point of intersections on the tissue component Pt = total number of intersections in a grid

Surface area is the total number of squares of the reticule occupied by the particular tissue component.

IMMUNOHISTOCHEMISTRY
Slide Preparation
Vasculoendothelial Growth Factor (VEGF)
4 micron thick sections on poly lysine coated slides will be used for immune histochemical procedures. Before starting the IHC protocol, sections will be dewaxed in xylene and rehydrated with descending ethanol gradient. Antigen retrieval will be performed with microwave oven for 15 minutes. Then the slides will be treated with 3% hydrogen peroxide block to remove endogenous peroxidase activity. After washing with PBS, added sufficient drops of VEGF primary antibody (Thermo scientific, Rabbit polyclonal antibody) and incubate for 1hour.Then added secondary antibody. Reactions will be visualized with DAB. The slides will be counter stained with haematoxylin.

Endothelial Nitric Oxide Synthase (eNOS)
4 micron thick sections on poly lysine coated slides will be used for immune histochemical procedures. Before starting the IHC protocol, sections will be dewaxed in xylene and rehydrated with descending ethanol gradient. Antigen retrieval will be performed with microwave oven for 15 minutes. Then the slides will be treated with 3% hydrogen peroxide block to remove endogenous peroxidase activity. After washing with PBS, added enough drops of primary antebody for eNOS (Thermo scientific, Rabbit polyclonal antibody) and incubate for 1hour.Then added secondary antibody. Reactions will be visualized with DAB. The slides will be counter stained with haematoxylin

Scoring of IHC Slides
Semi quantitative scoring method will be used to evaluate the expression of VEGF and eNOS in tissues. Based on the intensity of the staining, the antibodies present in the cells will be scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), 3(strong staining). Expression of antibodies will be scored separately in amniotic epithelium, Whartons jelly, endothelium and smooth muscle cells of artery and vein. All immunostained sections will be reviewed by two histologists, who will be blind to the purpose of study. They calculated the H score by multiplying the intensity of staining with percentage of cells stained.
H score = (% at 0)*0 + (% at 1+)*1 + (% at 2+)*2 + (% at 3+)*3.

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Calculated H score values will be between 0- 300. Based on these values again the staining intensity scored into 0 to 3+. H score value up to 100 is 1+, between 101 to 200 is 2+and from 201 to 300 are 3+. Randomly selected fields will be used for scoring.

Statistical Analysis: Statistical Analysis will be done by using SPSS software Version 25th. Statistical Tests (Pearson Chi-square Test, Independent Samples Test) will be applied whenever it will be necessary. For significance p-value.

RESULTS

In table 1, Birth Weight, Gestational age, Appearance, Pulse, Grimace, Activity, and Respiration (APGAR) score was decreased in hypertensive cases than normal. In the progress of hypertensive condition from gestational hypertension (GH) the birth weight of baby and gestational age was reduced significantly, but the reduction was not significant between GH and control group. Two Intra uterine death cases were reportedin severe preeclampsia group. The details are given in table 1.

TABLE 1: CLINICAL DETAILS

Parameters	Normal Pregnancy(Control)	Hypertensive Pregnancy(Case)
Baby weight in grams	2651.21 ± 313.51	2282 ± 212.33 *
Gestationalage in Wk	41.55 ± 5.55	38.53 ± 5.73 *
Apgar scoreat 1 minute	9.73 ± 1.66	8.93 ± 2.77 *
Apgar score at 5 minutes	10.79 ± 1.31	9.54 ± 1.13 *

* = P<0.05 (Significant), **= P<0.001 (highly significant).

TABLE 2: GROSS MORPHOMETRY – UMBILICAL CORD

Parameters	Normal Pregnancy (Control)	Hypertensive Pregnancy (Case)
Cord length in cm	33.54 ± 5.23	29.54 ± 5.46
Coiling index	0.23 ± 0.02	0.20 ± 0.03
Diameter – maternal end (cm)	1.49 ± 0.31	1.31 ± 0.19*
Diameter – middle (cm)	1.48 ± 0.14	1.02 ± 0.05*
Diameter – Foetal end (cm)	1.65 ± 0.07	1.08 ± 0.07*
Hyrtle anastomosis	Transverse- 26 (65%) Oblique-7 (45%) Fused- 5(12.5%) Absent-2 (5%)	Transverse-24 (60%)Oblique-9(22.5%) Fused-4(10%) Absent-3(7.5%)

There were no significant differences in cord length and coiling index between the groups. Gradual decrease in diameter was observed from normal to mild preeclampsia,so lowest values in diameter of cord were observed in mild PE. The severe group showed an increase in its diameter, which might be due to the edema. Three types of Hyrtl's anastomosis have been observed in all groups. No significant differencefound in Hyrtl's anastomosis among the groups. Details in table 2.

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TABLE 3: GROSS MORPHOMETRY – PLACENTA

Parameters	Normal Pregnancy (Control)	Hypertensive Pregnancy (Case)
Placental weight (grm)	453.62 ± 54.13	352.8 ± 40.21*
Fetoplacental(FP) Ratio	8.52 ± 1.63	9.12 ± 1.73*
Placental volume (cm3)	411.23 ± 44.73	293.83 ± 65.21*
Number of Cotyledon	16.3 ±3.37	12.41 ± 3.73*
Placental thickness (cm)	4.35 ± 2.42	4.13 ± 2.53
Placental diameter (cm)	21.89 ± 4.32	17.83 ± 4.43*
Site of insertion ofcord	Central-13 (32%) Medial-16 (34%) Lateral-10 (30%) Marginal-1 (4%)	Central-16(36%)Medial-12 (30%) Lateral-11 (30%) Marginal-1 (4%)

Weight, volume and thickness were significantly reduced in hypertensive placenta. Among the hypertensive group, very small placentae were observed in severe preeclampsia groups. Number of cotyledon and diameter of placenta were highly decreased in preeclampsia severe cases. Feto-placental Ratio were increased in hypertensive group, highest ratio was found in severe pre-eclamptic group. There was not much difference in the site of insertion of the umbilical cord. Totally all the parameters of placenta were decreased highly in severe case. Values are given in table3.

Summary

The present study showed extensive changes in the structure of placenta and umbilical cord in hypertensive pregnancy. These structural alterations affect the foetal outcome adversely. The abnormalities in the angiogenic factors are responsible for this alteration. In conclusion, the data demonstrated that placental and umbilical cord tissue from pregnancies with hypertensive disorders showed a different expression of angiogenic factors according to different degrees of clinical severity.

Prospects of future study related to the result of present study

- Study on the quantification of VEGF and eNOS both in the serum and tissues of placenta and cord simultaneously in hypertensive mothers with different severity
- Evaluate the difference in the content of VEGF and eNOS in chronic hypertension and gestational hypertension with different severity
- To have an extensive study on comparison of changes of placenta and cord alteration along with its content of VEGF and eNOS in foetus with intrauterinedeath due to preeclampsia.
- The studies can be conducted on a animal model by introducing VEGF and eNOS separately in hypertension induced rats. By this study the changes of vasculature and pregnancy outcome can be evaluated.
- Based on the results of the animal study VEGF and eNOS can be used therapeutically for hypertensive pregnancy

The present study was conducted on the structural changes of Hyrtl's anastomosis in hypertension. Though the study did not find any change in the anastomosis, this suggests that an extensive study in the field of direction of blood flow in the anastomosis and its correlation with hypertension need

to be done.

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