DISSERTATION ON

GROSS MORPHOLOGICAL AND PATHOLOGICAL CHANGES IN PLACENTA OF DIABETIC PATIENTS AND ITS ASSOCIATION WITH FETAL OUTCOME

Dissertation submitted to

TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY
CHENNAI

for

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APRIL 2016

Under the guidance o f

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CERTIFICATE

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I solemnly declare that this dissertation titled "**GROSS MORPHOLOGICAL AND PATHOLOGICAL CHANGES IN PLACENTA OF DIABETIC PATIENTS AND ITS ASSOCIATION WITH FETAL OUTCOME"** is the original and bonafide work done by me under the guidance of Dr.A.JAMILA, M.D., Professor, Department of Pathology at the Government Stanley Medical College & Hospital, Chennai – 600 001, during the tenure of my course in M.D. Pathology from AUGUST -2013 to April-2016 held under the regulation of the Tamil Nadu Dr. M.G.R. Medical University, Guindy, Chennai– 600 032.

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ABBREVIATIONS

ACOG	-	American Congress of Obstetricians and Gynaecologists
ADA	-	American Diabetes Association
APAAP	-	Alkaline Phosphatase-antialkaline phosphatase
CDC	-	Centre for Disease Control and prevention
Cms	-	Centimetres
DAB	-	Diaminobenzidine
DM	-	Diabetes mellitus
DPX	-	DibutylPhathalate Xylene
FBS	-	Fasting Blood Sugar
FGF-2	-	Fibroblastic Growth Factor-2
FGF-2R	-	Fibroblastic Growth Factor-2 Receptor
GDM	-	Gestational DiabetesMellitus
Gms	-	grams
HCl	-	Hydrochloric acid
H&E	-	Hematoxylin & Eosin
IADPSG	-	International Associationof Diabetes and PregnancyStudy Groups
IGT	-	Impaired GlucoseTolerance
IHC	-	Immunohistochemistry
Kg	-	Kilograms

mg/dL	-	milligram/deciLitre
mmol/L	-	millimoles/Litre
NBF	-	Neutral Buffered Formalin
NIH	-	National Institute of Health
OGTT	-	Oral GlucoseTolerance Test
PAP	-	Peroxidase-anti-peroxidase
PAS	-	Periodic Acid Schiff
PP2BS	-	Post Prandial Blood Sugar at 2 hrs
Sq. cm	-	Square Centimetres
TBS buffer	-	Tris buffered saline
VEGF	-	Vascular Endothelial Growth Factor
VEGFR-1	-	Vascular Endothelial Growth Factor Receptor-1
VEGFR-2	-	Vascular Endothelial Growth Factor Receptor-2
VPF	-	Vascular Permeability Factor

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INSTITUTIONAL ETHICAL COMMITTEE,

STANLEY MEDICAL COLLEGE, CHENNAI

Title of the work: Gross morphological and pathological changes in placenta of diabetic patients and its association with fetal outcome

Principal investigator: Dr. Nidhi SantoshKumar Mishra

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INTRODUCTION

Placenta is a latin word which literally means a flat plate or cake. Placenta is a very complex organ which has a very short life span of 9 months and serves as a channel between the foetus and the mother for the selective fetomaternal transport of gases, nutrients and excreta transport of metabolic waste products. Placenta separates the fetal and maternal circulation via endothelium and syncytiotrophoblast respectively.

The development of placental villous vessels continues throughout the pregnancy and comprises of two stages, vasculogenesis and angiogenesis. The stage of vasculogenesis occurs mainly during the period of first and second trimester, in which the mesenchymal cells of the villous core differentiate into the cords of vascular cells and by the process of dehiscence, it forms the vascular lumina. The cells required for the elongation and widening of vessels and the pericapillary cells – pericytes are also derived from the mesenchymal cells. In stem villi, arteries and veins are differentiated from the vessels. The surrounding supporting structures of the walls of the vessels like smooth muscle cells, myofibroblasts and fibroblasts are also recruited from the villous stroma. On the other hand, the stage of angiogenesis takes place during the third trimester. In this process, the already existing stem villous vessels sprouts and give rise to new capillaries, and thus by this way vascularizing the emerging mature intermediate and terminal villi.

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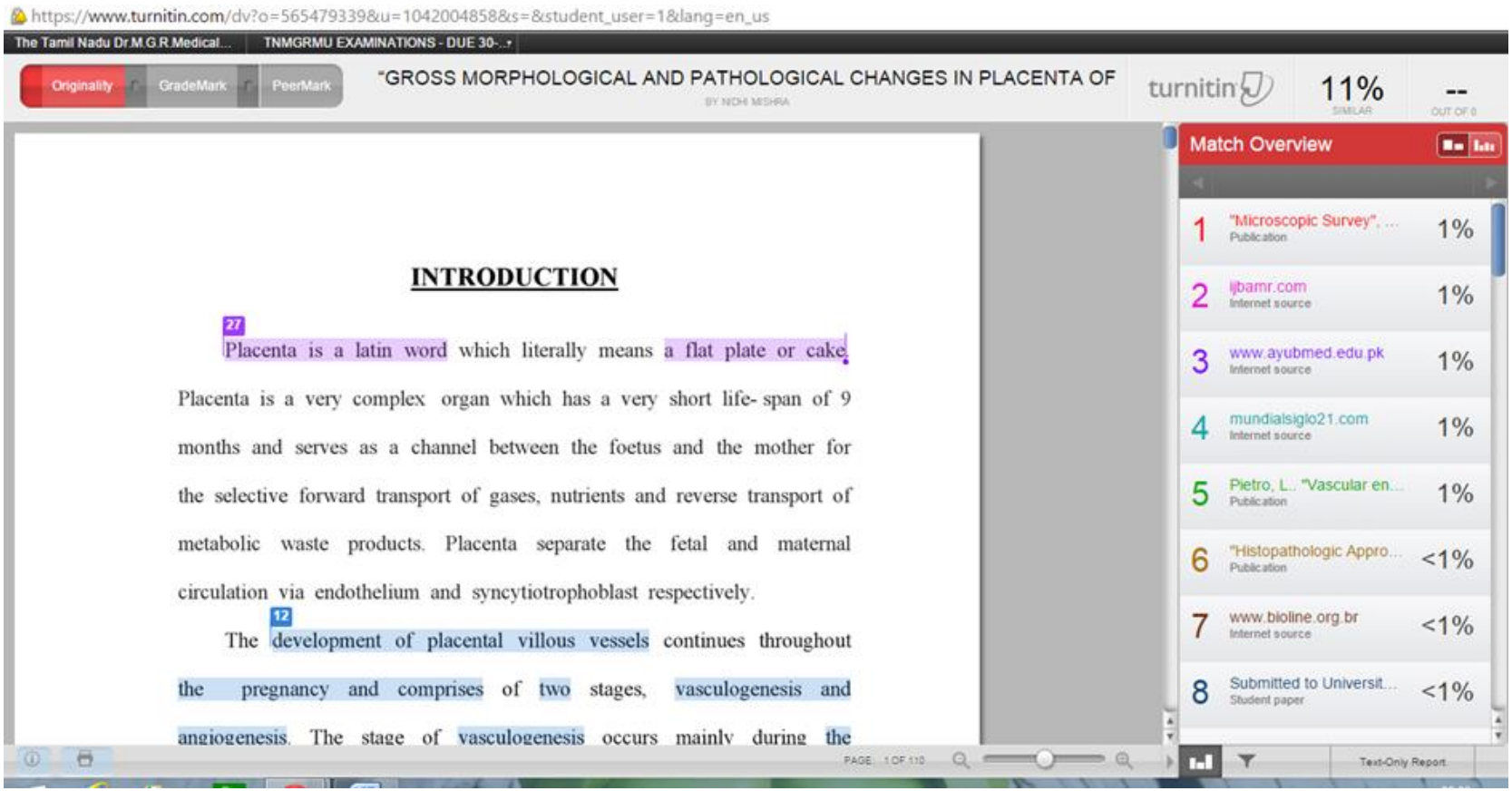
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INTRODUCTION

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Metabolic disease associated with pregnancy such as diabetes mellitus and hypertension can affect the components of placenta for e.g. connective tissue component in chorionic villi and the basement membrane lining the chorionic villi.[1]

The Centres for Disease Control and Prevention (CDC) has shown that the crude incidence of the cases diagnosed with diabetes mellitus has increased, from 3.3 per 1000 to 7.4 per 1000, i.e. 124%,from the year 1980 to 2005 and hence, diabetes mellitus is now considered to be one of the major health problem in our society. Various studies suggested that the increased prevalence of diabetes mellitus (DM) amongst the women of childbearing age is due to increase in sedentary lifestyles, changes in dietary habits and the virtual epidemic of childhood and adolescent obesity.[2]

GDM or Gestational Diabetes Mellitus is defined as variable degree of intolerance to glucose with either onset or first recognition during pregnancy. Maternal glucose intolerance occurs in 3-10% of pregnancies.[3]

Pregnancy complications like gestational diabetes are reflected grossly and microscopically in the placenta. Placental examination can yield information about the existence and effects of maternal, placental or fetal disease, the cause of stillbirth, and potential risks in future pregnancies.

The various pathological changes occurring in the placenta of diabetic mothers are considered to be the important risk factors contributing to fetal anoxia and fetal compromise in pregnancy [4]. Previous studies on functional morphology of placentas from diabetic mothers have produced inconsistent results and conclusions.

AIMS AND OBJECTIVES

- To observe & study the various gross morphological changes in the placentas of diabetic mothers and its comparison with normal term placentas.

- To observe & study the various histopathological changes that occurs in placentas of diabetic mothers and its comparison with normal term placentas and,

- To study the correlation of maternal diabetes with the foetal outcome.

REVIEW OF LITERATURE

Normal anatomy of placenta

The human placenta is described by the terms like "hemochorial" and "hemochorioendothelial". Hemo in general refers to blood and in case of placenta it refers to maternal blood, which bathes the syncytiotrophoblast; The word chorio is for chorion-placenta, which in turn is separated by the endothelial wall of the foetal capillaries that traverses the villous core from the fetal blood.[5]

The placenta is a flattened discoidal mass with a maternal surface which attaches to the decidua of the uterus and a fetal surface facing the amniotic cavity. The maternal surface is finely granular and divided into 15-30 lobes, also termed as cotyledons, by a series of fissures and grooves which are incomplete placental septa. The placental septa are complex structures which comprises of the cytotrophoblastic shell and residual syncytium along with maternally derived material including decidual cells, occasional blood vessels and glandular remnants, collagenous and fibrinoid extracellular matrix and in later months of pregnancy, foci of degeneration**[Figure1]**[6].The glossy appearance of the fetal surface is because of intact epithelial surface of the amnion. The umbilical cord is usually attached to the centre of the fetal surface. The chorionic vessels are seen branching, over the fetal surface, centrifugally from the cord insertion in a star like pattern.

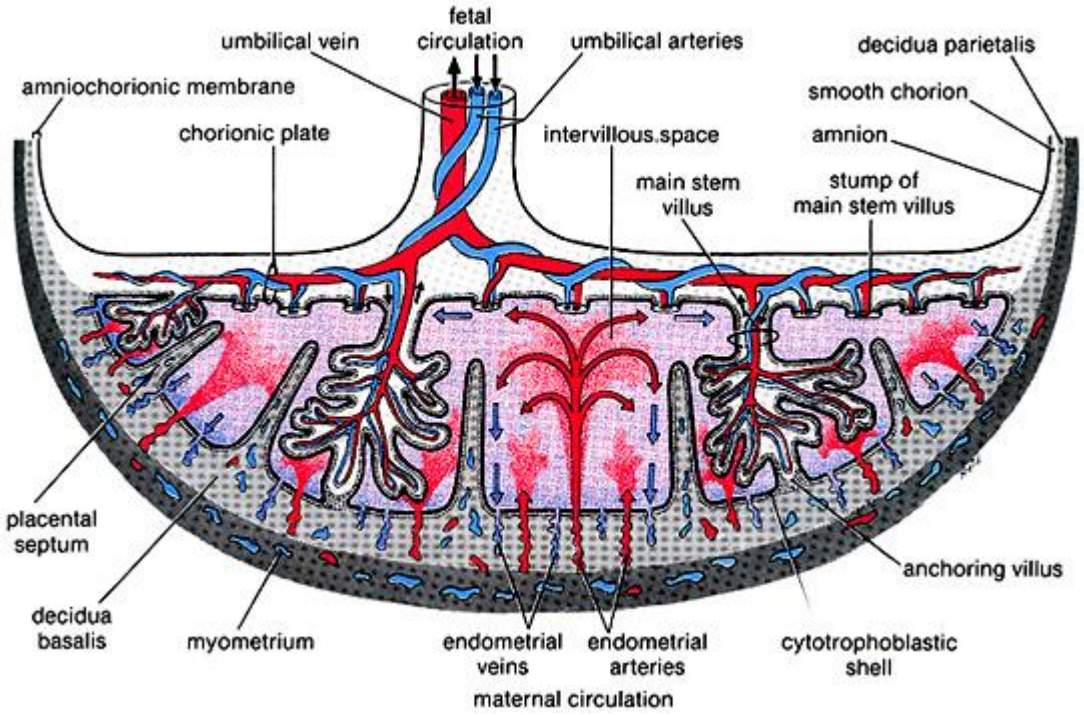


Figure 1 - Anatomy of placenta showing fetal and maternal surface as well as fetal and maternal circulation.[6]

<u>Histology of 3rd trimester placenta</u>[7]Routine histological examination of placenta requires a sections that covers all the placental structure from the chorionic plate via intervillous space down to the basal plate. Structures that are seen from maternal surface to fetal surface are:

1) Basal plate including the anchoring villi which is connected to the septa via cell columns
2) Septa
3) Cell islands
4) Fibrinoid deposits
5) Intervillous space
6) Chorionic plate including chorionic villi of various type.

Decidua[8]: It refers to the gravid endometrium. According to its relation to the site of implantation three regions are described –

A) Decidua Basalis:- It is a part of the decidua which lies deep to conceptus and forms the maternal part of placenta.

B) Decidua Capsularis:- This part of the decidua overlies the conceptus and considered to be the superficial part of the decidua.

C) Decidua Parietalis:- All the remaining part of the decidua.

The decidual cells are enlarged pale staining connective tissue cells of the decidua which usually formed in response to increasing progesterone levels in the maternal blood. This enlargement of the decidual cells is mainly because of cytoplasmic accumulation of glycogen and lipid. As the blastocyst implants, these cellular changes occurring in the endometrium constitutes the decidual reaction.[9]

Basal plate of term placenta shows, from maternal side to fetal side, a compact decidual layer, a layer of Nitabuch fibrinoid followed by extra villous cytotrophoblast and superficial stria of Rohr fibrinoid. The uteroplacental vein is embedded in the decidual layer.

Placental septas are rudimentary pillar shaped structure, which are formed as a result of folding of the basal plate and are supported by tension of anchoring villi. These placental septas are insufficient to subdivide the intervillous spaces into separate chambers.

Fibrinoid is an intensely staining, eosinophilic acellular material which is mostly related to the intervillous space.

The anchoring villi and basal plate are connected by the cell columns. The development of cell column starts when the extra embryonic mesenchyme invades the primary villi. It is composed of an outer incomplete sleeve of syncytiotrophoblasts with a core of multi-layered cytotrophoblast. This outer syncytiotrophoblastic cover of the cell column is replaced by fibrin type of fibrinoid, as soon as the cell columns are buried in the basal plate.

As the extravillous trophoblastic cells proliferate, the cell island starts increasing in size. As a result of degeneration of trophoblastic cells and subsequent liquefaction, large cell islands may contain cysts or central cavities.

The main difference between the cell column and cell island is the topographic relation. **Cell islands** are nothing but freely floating cell columns. Immunological difference between the two is that the extravillous trophoblastic cell columns invades the decidual tissues whereas cell islands only migrate within its own extracellular matrix and finally degenerates leading to formation of cyst or cavities.

The villous tree[Figure 2] measures around 1 to 4cms in diameter. The stem villi forms the central branches of villous tree, which are large calibre villi that ranges in size from eighty to several thousand micrometres in diameter. The highest concentration and the largest

calibre stem villous are found near the chorionic plate. Histologically these stem villous are characterized by one or several arteries and veins or arterioles and venules, with arteries/arterioles showing muscular walls, and are surrounded by fibrous stroma containing few paravascular capillaries.

Immature intermediate villi lies immediately before the stem villi**.** In early pregnancy, these are the dominating villi, but in later stages persist only in small group in the centre of villous trees and are absent in hyper mature placenta.

Mature intermediate villi are slender, multiple curved branches of stem villi, that measures 60 to 100 micrometres in diameter. They lack both the fetal stem vessels and stromal fibrosis seen in the stem villi. The stroma is composed of loose connective tissue in which slender fetal capillaries are embedded. This stromal connective tissue of mature intermediate villi is poor in fibres but rich in cells.

Terminal villi are the terminal side branches of mature intermediate villi, having diameter of 40 to 80 microns. The main structures within the loose stroma of terminal villi are the fetal capillaries, which are highly coiled and sinusoidally dilated. In the third trimester, these terminal villi along with the mature intermediate villi represents the main site of exchange of materials between the maternal and fetal side.

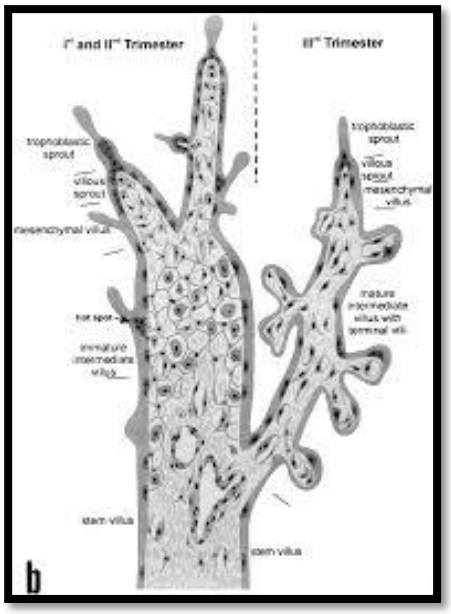


Figure 2 – Types of villi[10]

Syncytial knot is a typical feature of terminal villi (Tenny – Parker changes). It is a polypoidal trophoblastic outgrowth at the villous surface.

Chorionic plate is a multi-layered structure and consist of a spongy layer with numerous clefts, followed by a compact layer of chorionic mesoderm, which is separated from the Langhans fibrinoid stria by a rudimentary basement membrane.

The amnion is made up of a single layer of cuboidal to low columnar cells. It covers the chorionic plate towards amniotic cavity and participates in the turnover of amniotic fluid. There may be foci of squamous metaplasia in some cases that may become upto 15 cell layer thick.

Formation of chorionic villi[6]

The functional element of the placenta are villi which are very small finger like processes and are surrounded by maternal blood. In the substance of villi; there are capillaries through which fetal blood circulates. Exchange between the maternal and fetal circulation takes place through the tissue forming the walls of the villi.

The villi are formed as offshoots from the surface of the trophoblast. As the trophoblast, along with the underlying extraembryonic mesoderm, constitutes the chorion, the villi, arising from it are called chorionic villi.

The chorionic villi are formed all over the trophoblast and grows into the surrounding decidua. Those villi which are related to the decidua capsularis are transitory, and hence when they degenerate, this part of the chorion becomes smooth and is called the chorion leave. The villi that grow into the decidua basalis undergo considerable development later in the stage of pregnancy and along with the tissue of decidua basalis they form the disc shaped mass called placenta. The part of chorion that forms placenta is called the chorionic frondosum.

The trophoblast is single cell layer thick, which then undergo multiplication and forms two distinct layers. The cells which are closest to the decidua, lose their cell borders and forms one continuous sheet of cytoplasm containing many nuclei, which is referred as syncytiotrophoblast or plasmodiotrophoblast.

Deep to the syncytiotrophoblast, the cells of trophoblast retain their cell borders and forms the second layer called the cytotrophoblast, also known as Langhans layer. There are 3 stages involved in the formation of chorionic villi **[Figure 3]:**

a) **Primary villi:-** It consist of a central core of cytotrophoblast surrounded by a layer of syncytiotrophoblast.

b) **Secondary villi:-** It shows three layers, an inner layer of extraembryonic mesoderm, outer syncytiotrophoblast and an intermediate layer of cytotrophoblast.

c) **Tertiary villi:-** These are like secondary villi except that there are blood capillaries in the mesoderm.[11]

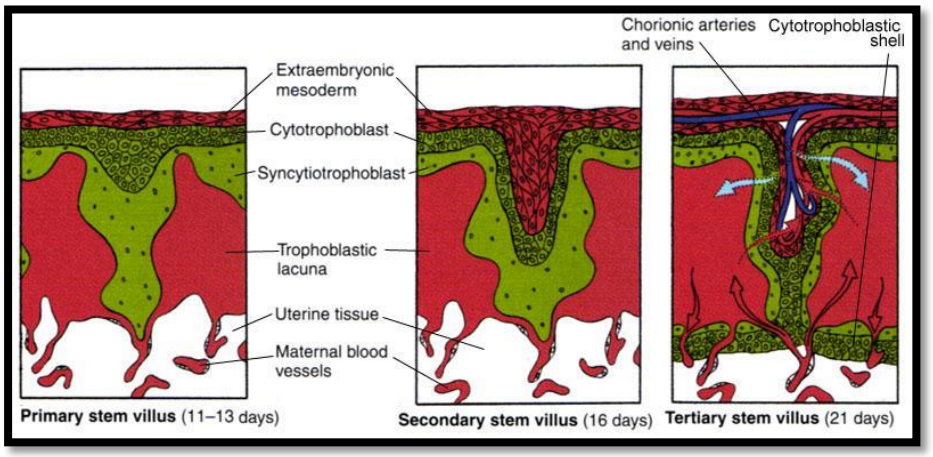


Figure 3 – Formation of chorionic villi[11]

Placental membrane

In Placenta, the maternal blood circulates through the intervillous space while the fetal blood circulates through the blood vessels within

the villi. The fetal and the maternal blood do not mix with each other. They are separated by layers of the villous, forming placental barrier or membrane. These layers from the fetal to maternal surface are:

a) The endothelium of foetal blood vessels within the villi, and its basement membrane.
b) Surrounding mesoderm (connective tissue)
c) Cytotrophoblast and its basement membrane.
d) Syncytiotrophoblast.

The total area of this membrane varies from 4 to 14 m^2. In the later part of pregnancy the efficiency of membrane is increased by considerable thinning of the connective tissue and by disappearance of the cytotrophoblastic layer from most villi. But towards the end of pregnancy, a fibrinoid deposit appear on the membrane, and this reduces its efficiency.[6]

Variations of placenta[7]

1) **ANOMALIES OF SHAPE OF PLACENTA:-** normal placenta is disc shaped.Following variation occur.

a) Bidiscoidal placenta:- It consists of two discs.

b) Lobed placenta:- Exhibit two or more lobes

c) Placenta membranous:- A thin diffuse placenta is formed when chorionic villi persist all-round the blastocyst cavity.

d) **Placenta Circumvallate:-** Placenta is covered, at the peripheral edge, by a circular fold of decidua.

e) **Placenta succenturiate:-** Placenta with an accessory lobe having vascular connection with the main placenta.

f) **Placenta fenestrated:-** Is when there is a hole in placental disc.

2) **ACCORDING TO THE ATTACHMENT OF UMBILICAL CORD**

a) **Battle dore placenta**:- Umbilical cord is inserted to the margin of the placenta and gives it a club like appearance.

b) **Vilamentous placenta**:- The umbilical cord is attached to the chorion and amnion instead of placenta and the vessels branch between the membrane before they extend over the placenta.

c) **Placenta furcate**:- Blood vessels divides before reaching the placenta.

3) **ACCORDING TO THE DISTRIBUTION OF UMBILICAL ARTERIES**

a) **Disperse type:-** In this umbilical arteries divide in dichotomatous manner and undergo successive reduction in calibre.

b) **Magisterial type:-** In this, the arteries maintain almost a uniform calibre upto periphery of the placenta and give off number of smaller side branches.

Functions of placenta

Placenta exhibits great structural and functional variability amongst species. The only structural component that is common in all placental types are the two separate circulatory system: the foetal and the maternal circulatory systems.

The following are the functions done by placenta during pregnancy…

a) Gas transfer.
b) Catabolic and resorptive functions.
c) Numerous metabolic and secretory functions of liver.
d) Synthetic and secretory functions of most endocrine gland.
e) Haematopoiesis.
f) Heat transfer of the skin.
g) Excretory function, water balance, pH regulation.
h) Immunological functions.

Gestational diabetes

GDM(Gestational Diabetes Mellitus) is a type of hyperglycemia or glucose intolerance disorder that is of transient type and occurs or is diagnosed during pregnancy [12]. In many pregnant females with GDM, the glucose level will return to the normal after delivery of the baby. If in a woman, even after delivery, the glucose levels does not returns to normal she will no longer be considered to have GDM but will be re-diagnosed with type 2 diabetes mellitus [13]. Type 2 diabetes mellitus

represents the most common metabolic complication of pregnancy and is associated with increased maternal and foetal morbiditiy[14]. The factors which have been postulated to influence the risk of GDM in mothers include advancing maternal age, treatment for infertility, positive family history of diabetes, diabetes in previous pregnancy, obesity, prematurity, preeclampsia, macrosomic infant and unexplained neonatal death.[15]

Pathophysiology of GDM

Like all the other forms of hyperglycaemia, GDM is characterized by levels of insulin which are insufficient to meet the demand of the body. The cause of insulin insufficiency in GDM is not fully known, but thought to be caused by 1) B cell dysfunction which is associated with chronic insulin resistance, 2) highly penetrant genetic abnormalities or 3) autoimmune B cell dysfunction[16]. During pregnancy, the insulin resistance is because of change in many factors, like alteration in cortisol (insulin antagonist) and growth hormone secretion, secretion of human placental lactogen (promotes lipolysis) and insulinase (facilitates metabolism of insulin), which is produced by the placenta along with oestrogen and progesterone which causes glucose intolerance [17].As the pregnancy progresses, the placenta grows, thus increasing the level of these hormone production and so the level of insulin resistance also increases. This process of increased hormone production usually starts between 20 and 24 weeks of pregnancy. Once the placenta is delivered the production of these hormones stops and hence strongly suggesting that these hormones are responsible for the development of GDM.[18]

Apart from all these hormones, adipose tissue also has an important role in the development of GDM. Numerous factors, known as adipocytokines are produced by the adipose tissue, most of which acts as hormones. These adipocytokines have been involved in the regulation of insulin resistance and maternal metabolism [19].Adipocytokines like leptin and TNF-alpha could impair insulin signalling and thus causes insulin resistance.[20,21]

Complications of GDM during pregnancy

Women having GDM are twice more prone for urinary tract infection than normal women. This increased incidence of infection is due to glycosuria. There is also an increased risk of pre-eclampsia, pyelonephritis and asymptomatic bacteriuria. There is 10% risk of polyhydramnios in GDM mothers which in turn may increase the risk of abruptio-placenta and pre-term labor. There is also 10% risk of developing type 2 diabetes mellitus per year after the pregnancy has been complicated with GDM.[22]

Effects of GDM on the mother

- Women with GDM have increased chance of birth trauma , because of heavy weight baby.[23]
- They are more prone to develop type 2 diabetes.[24]
- Are more likely to get high blood pressure.[25]

Effects of GDM on the fetus

- Can lead to heavy birth weight babies.[26]
- Newborn of a mother with GDM has three times higher risk of shoulder dystocia as compared to newborn of a mother without GDM, which can lead to temporary or permanent nerve damage in the shoulder.[27]
- Newborn of a mother with GDM is at increased risk for hypoglycaemia after birth, hyperinsulinemia, hypocalcemia, polycythemia, and jaundice.
- Infants born to mothers with GDM are at a higher risk of becoming obese and having long-term glucose intolerance or developing early onset type 2 diabetes.

Diagnosis of GDM[28, 29]

Criterias for the diagnosis of GDM were developed by IADPSG (International Association of Diabetes and Pregnancy Study Groups), and were endorsed by the ADA(American Diabetes Association). Women having high risk factors for the development of GDM should under go a glucose tolerance test, if possible at the first antenatal visitor soon thereafter.

At 24 to 28 weeks gestation, all the women who were not known to have diabetes (including the high-risk women in which the initial testing was normal) should undergo screening with glucose tolerance test. Either two-step or one-step screening tests may be used. The IADPSG (International

Association of Diabetes and Pregnancy Study Groups) recommends a one-step test, while a two-step test is recommended by the National Institute of Health (NIH) and the American Congress of Obstetricians and Gynaecologists (ACOG). The ADA has the datas to support both the approaches.

One-step method: In this method a 75-gram OGTT(Oral Glucose Tolerance Test) is performed in women not previously diagnosed with overt diabetes, at 24 to 28 weeks of gestation, with measurement of plasma glucose during fasting and at 1 and 2 hours, .

- The OGTT should be performed after an overnight fasting of at least 8 hours, in the morning.
- The diagnosis of GDM is made when any one of the following plasma glucose values are exceeded:
    - Fasting ≥ 92 mg/dL (≥5.1 mmol/L)
    - 1 hour ≥ 180 mg/dL (≥10.0 mmol/L)
    - 2 hours ≥ 153 mg/dL (≥8.5 mmol/L).

Two-step method: This test is performed during the fasting state. In this method 50 gms of oral glucose tolerance test is performed. The diagnosis is established when two or more plasma glucose levels are at or above the following thresholds:

- **Fasting**: 95 mg/dL or 105 mg/dL (5.3/5.8mmol/L)
- **1 hr:** 180 mg/dL or 190 mg/dL (10.0/10.6mmol/L)

- **2 hr:** 155 mg/dL or 165 mg/dL (8.6/9.2mmol/L)
- **3 hr:** 140 mg/dL or 145 mg/dL (7.8/8.0mmol/l)

A review has been made on the important contribution to the gross and histopathological changes in placenta of diabetic mothers and its effect on neonatal weight.

In the year 1951, Hamilton showed that at term human placenta is a flattened mass with circular and oval outline and is determined by the form of villi finally left on chronic sac.[30]

Cardwell in the year 1953, Taylor in 1962 and Zacks in 1963 concluded from their respective studies that the placentas from diabetic women did not show any unusual features.[31]

Burstein et al in the year 1957, observed that the placentas of diabetic mothers had shown villous lesion on light microscopic examination, such as increased synctial knots formation. It was also suggested that reduced amount of maternal blood flow to the intervillous space leads to low pO_2 and hence development of endarteritis, which may reduce the utero-placental blood flow, resulting from endothelial cell damage followed by proliferation which may lead to narrowing of the lumen of maternal blood vessels.[32]

In the year 1958 Thomsen K showed histological villous lesions in diabetic placentas such as synctial degeneration and excessive synctial knot formation.[33]

Hughes in the year 1961 and Horky in 1964 showed that villous immaturity was seen more commonly among diabetic placenta.[34, 35]

In the year 1963, Pav J and Jezkova Z noted that antiinsulin antibodies were found in all pregnant diabetics cases but not in normal patients. The antibodies were also found in the infants of diabetic mothers.[36]

In the year 1965, Holzner and Thalhammer studied that placenta of diabetic women showed increased thickness of placental vasculo-synctial membrane and villous fibrinoid necrosis.[37]

Driscoll SG in the year 1965, showed that, at the same gestational age, the placentas from the diabetic pregnant women tends to be heavier than that of general population.[38]

In the year 1967, Aljadem observed that in more than half of the diabetic cases, the placentas were thicker, heavier and larger than the control group of the same gestational age, although there were many diabetic placentas which were normal.[39]

Kjeldsen and Pederson in the year 1967 reported that placentas of poorly controlled diabetic mothers were plethoric, thick and enlarged which was generally thought to be the manifestation of maternal hyperglycemia and fetal hypervolemia.[40]

In the year 1967, Nelson et. al noticed in their study that, the acidic components are present at lower density on the inter-microvillous surface membrane than on the surface of membrane microvilli in normal pregnancies. It was also noticed that the thickening of the basement membrane in diabetic placentas was the result of deposition of mucopolysacchrides.[41]

Salvatore, in the year 1968, observed that there was continued branching of terminal villi in cases of prolonged ischemia. He also observed that in case of pre-eclampsia, atherosclerosis affects the uterine vessels, causing narrowing of their lumen which in turn leads to decreased blood flow in the intervillous space.[42]

Fox in the year 1969 studied 48 diabetic placentas and reported principal histological abnormalities like villous fibrinoid necrosis, thickening of the trophoblastic basement membrane and obliterative endarteritis of fetal stem arteries, all resulting from immunological damage. Increased syncytial knot formation and villous fibrosis were also observed.[43]

Laga EM in the year 1973, had shown that attachment of umbilical cord was normally at the centre of the fetal side of placenta. They also suggested that growth of placenta occurs in two phases, hyperplasia followed by hypertrophy.[44]

In the year 1977, Jone and Fox reported increased number of syncytiotrophoblast nuclei in the placentas of gestational diabetes

mellitus. The nuclei showed chromatin clumping and were usually seen arranged in clusters forming syncytial knots.[45]

Fletcher, in the year 1981, observed in his study that the incidence of intrauterine growth retardation, congenital malformations and fetal macrosomia was high in pregnant mothers with poorly controlled diabetes mellitus.[46]

Geppert in 1982 suggested that the degree of placental dysmaturity in diabetes was mainly influenced by the variability of the blood sugar level during pregnancy.[47]

Singer in the year 1984 showed that the placentas of gestational diabetic mothers were larger than normal and had various structural abnormalities, leading to disturbance in fetal growth and development.[48]

Sala et al. in their study mentioned that, fibrinoid degeneration in human term placenta showed regional variation in the frequency of distribution. They also suggested that the fibrinoid degeneration of villi can be stimulated by hypoxia or relative stasis at increased sugar levels.[49]

In the year 1986, Gewolb et al. showed that the apparent functioning of placenta in diabetic pregnancy and the discrepancy between the fetal size and placental size can be explained by study of microscopic structure of the placenta.[50]

In the year 1981, Teasdale observed in his study that the weight of the foetuses as well as the placental weight of diabetic mothers were significantly higher than the control group of mothers of same gestational age.[51]

In the year 1988 Frank Stoz conducted histometric study on diabetic placentas and found that there was statistically significant increase in the size of terminal villi in diabetic placentas. They also found that number of villous vessels was decreased in diabetic placentas.[52]

Brudenell and Droddridge in the year 1989 observed that villous edema was common in diabetic placentas[53]. Cheung et al in the year 1990 reported that glucose intolerance disorder, such as gestational diabetes mellitus caused an increased incidence of fetal wastage such as prematurity, abortion, macrosomia and congenital anomalies.[54]

A study conducted by Yang in the year 1993 showed placental changes like increased syncytial knots, proliferation of small fetal vessels and villous immaturity, in placentas of diabetic patients.[55]

In the year 1994, Al-okail and Al-Atlas studied the human placenta from poorly controlled gestational diabetes and overt diabetics, using direct light microscopy and specific staining techniques. They reported changes like villous edema, fibrin thrombi, thickening and hyperplasia of basal membrane of trophoblast in placentas of the mothers with gestational diabetes mellitus. This study indicated that structural

changes in the placental cells may result from poorly controlled diabetes during gestation, shown by high HbA1c levels, which leads to accumulation of fat droplets and carbohydrate compounds in the basement membrane of the placentas.[56]

Fox in the year 1997 observed from his study that villous structure of placenta in gestational diabetes mellitus may be relatively immature; with an increased incidence of trophoblastic proliferation showed by increased number of villous trophoblast cells.

Lao TT in the same year i.e. 1997, conducted a retrospective case control study, in which he compared 478 singleton IGT/GDM pregnancies with a control group with normal OGTT, performed on the same day as each index case. As compared with the control group, the placental ratio and the placental weight alone, was found to be significantly increased in the GDM and IGT groups respectively .[57]

King H in the year 1998 reported in his study that the prevalence of impaired glucose tolerance was usually more than diabetes in women of child bearing age. Obesity, maternal age and parity all predispose to gestational diabetes mellitus and, there was low incidence of gestational diabetes in the absence of risk factors.[58]

Radaelli et al in the year 2003 and Segregur et al in 2009 observed in their studies that large babies were usually delivered in mothers with gestational diabetes mellitus. Lao et al in the year 1997 and Taricco et al

in 2003 observed in their studies that placentas of diabetic mothers were of larger size.

Emmanuel Odar in the year 2004 studied that pregnancy is a diabetogenic state manifested by insulin resistance and hyperglycemia. He recruited 90 mothers with gestational ages between 24-32 weeks from April to September 2001 and followed them upto the time of delivery. In this study the age group at risk of getting gestational diabetes mellitus was between 20-39 years in 96.8% of cases.[59]

In the year 2010, Fahima Akhter carried out a morphologic study on preterm placentas of gestational diabetes mellitus. This study was carried out on 44 placentas out of which 22 samples belonged to mothers with GDM and 22 samples belonged to mothers with normal pregnancy. The placenta were examined for weight, volume, thickness, diameter and cotyledons number. According to this study the GDM group showed significantly higher values for weight, diameter and volume.[60]

Ranjana Verma in the year 2010 studied the cellular differences that might contribute to the larger size of the placenta. Light microscopic analysis was done for 20 placentas of mothers with gestational diabetes mellitus and 5 control group. According to the study gross abnormalities were uncommon in the study group, but microscopic examination revealed changes like villous fibrosis, villous edema, fibrinoid necrosis, increased syncytial knots and capillary proliferation.[61]

In the year 2011, Vineeta Tewari et al did a study on total 60 cases, with 30 diabetic cases and 30 normal cases and concluded that histological changes like, thickening of trophoblastic basement membrane, increased synctial knots, villous stromal fibrosis, fibrinoid necrosis, crowding of villi, villous edema, fibrin deposition and vessel wall thickening were common features in diabetic placenta.[62]

Lavinia Gheormanet al. in the year 2012 studied the histopathological changes in placenta of 19 diabetic patients and concluded that villous edema, villous immaturity, chorangiosis, intra- and extravillous fibrinoid, presence of basement membrane thickening, and deposit of glycogen were suggestive and specific for pregnancy with diabetes but were not pathognomonic for this association.[63]

Lal Baksh Khaskhelli et al in the year 2013 studied 80 cases, 40 from diabetic patients and 40 from normal patients and observed that diabetes mellitus produces gross as well histopathological changes in the placenta which may result in large for date babies because of fetal compromise.[64]

In the year 2014,Ambedkar Raj Kulandaivelu et al studied that the morphometric parameters of the placenta for eg. diameter, number of cotyledons, weight of the placenta and fetal birth weight were increased in case of diabetic mothers.[65]

PERIODIC ACID SCHIFF STAINING:

The PAS technique was first used for the demonstration of mucin by McManus in the year 1946[66]. Subsequently the utility of the PAS technique for demonstration of other carbohydrate-containing molecules, such as glycogen and certain glycoproteins has been demonstrated by Lillie & McManus.[67, 68]

In the year 1987 Hennigar described that the PAS technique is an important means of assessing the basement membrane thickness as it reacts with the glycoproteins of the basement membrane.[69]

Vineeta Tewari et al in the year 2011 did a study on 60 cases, with 30 diabetic cases and 30 normal and concluded that on histochemical study diabetic placentas showed stronger reactivity to PAS and Sudan black as compared to normal placentas.[62]

IMMUNOHISTOCHEMISTRY:

Immunohistochemistry is the technique which is used to demonstrate the expression or absence of a particular antigen on a particular site with the help of specific antibodies which are labelled either directly or by using a secondary labelling method. The principle behind the IHC is antigen antibody reaction.

In 1941 Albert H. Coons was the first to describe a new way of visualizing tissue constituents using an antibody labelled with a fluorescent

dye. Fluorescence microscope was used for the visualization of these labelled complex.[70]

In the year 1958 Riggs et al observed from his study that the antibody conjugates much easily to fluorescein isothiocyanate molecule than to fluorescein isocyanate, and the results obtained with it were more stable. Hence fluorescein isocyanate, which was the first fluorescent dye to be attached to an antibody was replaced by fluorescein isothiocyanate.[71]

Many limitations seen previously were overcome with the introduction of enzymes as labels. In the year 1966, Nakane and Pierce mentioned in their study that, simultaneous evaluation of morphological details and immunohistochemistry is possible, if the cells are labelled with an enzyme such as horseradish peroxidase, conjugated to an antibody, and visualized with an appropriate chromogen such as diaminobenzidine (DAB) and then the nucleus is counterstained with hematoxylin.[72]

In the year 1970,Sternberger et al. first described the peroxidase-anti-peroxidase (PAP) technique. Engvall and Perlman, in the year 1971,mentioned in their study the use of alkaline phosphatase labelling, and Cordell et al. in the year 1984described the alkaline phosphatase-antialkaline phosphatase (APAAP) technique.[73,74,75]

In the year 1977, Heggeness and Ash proposed the use of avidin-biotin for immunofluorescence, which was later modified by Guesden et al. in the year 1979 and Hsu et al. in 1981,who used a horseradish

peroxidase label. Later on the streptavidin-biotin labelling superseded the avidin-biotin labelling, and was one of the most popular techniques used in diagnostic laboratories. However, now labelled polymer detection system is the most popular choice for most diagnostic laboratories.[76, 77, 78]

METHODS OF IHC [79]

- ***Direct labelling method***

Antibody is attached with a label by chemical means and directly applied to tissue sections. It is rapid and easy procedure and carries the disadvantage of multiple antigens which requires separate incubation with respective antibodies.

- ***Indirect labelling technique***

Enzymes are labelled with the secondary antibody, which is produced against primary antibody .This method is more sensitive and easy to handle. The advantages also include increased versatility, higher working dilution of primary antibody, secondary antibodies against primary antibodies of different species and easy to prepare.

- ***Avidin biotin techniques***

High affinity binding between biotin and avidin is used in this procedure. Biotin is chemically linked to primary antibody thus localizing the peroxidase moiety at the site of antigen. Disadvantage of this technique is that the endogenous biotin, produces nonspecific background staining.

- ***Avidin biotin conjugate procedure***

In this technique first primary antibody is added which is then followed by biotinylated secondary antibody and next by preformed complex of avidin and biotin horse radish peroxidase conjugate. This is amore sensitive method.

- ***Biotin streptavidin system***

Streptavidin is used in place of avidin. Streptavidin is more stable than avidin.

- ***Immunogold silver staining technique***

This is used in ultrastructural immunolocalisation. Gold particles are enhanced by the addition of several layers of metallic silver. The fine silver deposits in the background creates confusion when small amount of antigen are identified.

Vascular endothelial growth factor (VEGF), an angiogenic growth factor, is a homodimeric glycoprotein with approximate size of 45kDa

and has widespread tissue distribution. It is responsible for the development of both physiological and pathological angiogenesis, was demonstrated in the study done by Ferrara and Henzel in the year 1989 and Ferrara in 1993.[80,81]

VEGF was first described by Senger et al. in the year 1983 in guinea pig and called it as Vascular Permeability Factor (VPF), as it was found to induce vascular leakage in the skin. The purification of the amino acid sequence by Senger et al,. however did not occur until 1990.[82]

In the year 1989, Connolly et al., Leung et al., and Keck et al., did an extensive research on VEGF and found through cDNA cloning that both VEGF and VPF were the same molecule. It is more commonly recognized as VEGF because it acts primarily on vascular endothelial cells[83, 84, 85].Later on it was found that VEGF belonged to a family of secreted glycoproteins, including VEGF-B, -C, -D, and placenta growth factor (PlGF).

Sharkey et al in the year 1993 showed in his study, that mRNA which encodes VEGF was expressed by the villous trophoblast at an increased concentration towards term as compared to low concentrations seen in first trimester.[86]

J. C. Cooper et al in 1995 investigated the expression of VEGF and flt-like immunoreactivity in first trimester and in term placentae. In the first trimester, VEGF immunoreactivity was localized to the decidua, placental macrophages (Hofbauer cells), maternal macrophages and

glandular epithelium. In the term placenta, VEGF immunoreactivity was present in the extracellular material and in extravillous trophoblast.[87]

Satu Helske et al, in their study done in 2001, demonstrated that immunoreactivity for VEGF was strongly located in the capillary endothelial cells of the villi and larger vessels. They also mentioned that there was no significant difference in the localization or intensity of the staining between the pathological and normal tissues examined.[88]

Jan Janota et al, in the year 2003, observed in their study that the regulation of angiopoietic genes VEGF, Angiopoietin-1, Angiopoietin-2, and their receptors VEGFR-1 (Vascular Endothelial Growth Factor Receptor-1), VEGFR-2 (Vascular Endothelial Growth Factor Receptor-2), and Tie-2, as well as FGF-2 (Fibroblastic Growth Factor-2) and FGF-2R (Fibroblastic Growth Factor-2 Receptor) were same in normal term placentas and placentas of well-controlled type 1 diabetes mellitus.[89]

L. Pietro et al in the year 2010 studied 12 cases, 3 normoglycemic cases, 3 cases of mild hyperglycemic, 3 cases of gestational diabetes and 3 cases of overt diabetes and found out that VEGF was generally detected in muscle cells and vascular endothelial cells of the intermediate villi,in the cytoplasm of the syncytiotrophoblast, in the mesenchymal cells and in the capillary endothelial cells and cytotrophoblastic cells in the basal decidua proximity to the maternal vessels. The women with gestational diabetes showed somewhat different pattern of staining of VEGF. In women with clinical diabetes, VEGF was detected in vascular

smooth muscle cells, but not in endothelial cells. In contrast to the other groups of women, pregnant women with gestational diabetes showed no staining for VEGF in the vascular endothelial and smooth muscle cells of the villi. Staining was observed in the cytoplasm of the syncytiotrophoblast and mesenchymal cells, but in the extravillous cytotrophoblast, it was rather weak.[90]

MATERIAL AND METHODS

The study was conducted for the period of 1 year from September 2014 to August 2015, after clearance from the Institutional Ethical Committee.

Type of study: Prospective, Cross sectional study.

The total number of specimens studied in the present study was 80, 40 placentas were from the mothers with uncomplicated/normal pregnancy which were taken as control group and 40 placentas from the mother with either gestational or overt diabetes which were taken as study group. The specimens were collected from the RSRM Govt. Stanley Maternity Hospital for a period of 1 year and the study was conducted in the Department Of Pathology, Stanley Medical College, Chennai.

Inclusion criteria:

1) Placentas of women who are diagnosed as diabetic either during the period of gestation (gestational diabetes) or before gestation (overt diabetes).

2) Placentas of women with normal pregnancy with no associated diabetes, as control.

Exclusion criteria:

Placentas of women with other associated condition like pregnancy induced hypertension /pre eclampsia, hypothyroidism.

The gestational history of the mother and weight of the newborn was taken from the case sheets and blood sample were collected from the diabetic mothers for the values of FBS and PP2BS.

Collection and gross examination of placenta

Placenta with attached membranes and umbilical cord was collected immediately after delivery, either by normal vaginal delivery or by caesarean section, washed in running tap water to remove all the blood and kept in 10% Neutral Buffered Formalin(NBF) for fixation. After 12 hours the amniotic and chorionic membrane is trimmed off from the placenta in all cases and umbilical cord is cut at a distance of 6cm from its insertion to maintain a uniform length in all the cases.

The placenta was washed again in running tap water to clear off the blood, photographs were taken with appropriate labels and following parameters were recorded:

- ***Weight:*** by spring balance weighing machine calibrated in grams.
- ***Diameter :*** measured twice with a measuring tape up to the nearest 0.5 cm. The mean of maximum (d1) and minimum (d2) diameters was taken as the diameter (d) of the placenta.

- ***Thickness:*** it was taken at the centre, at the margin and midway between the centre and margin. The average of the three readings was taken as the thickness of the placenta.

- ***Area(A):*** the placental area was computed from the formula:

  $A = (\pi/4) \times d1 \times d2$[91]

- ***Circumference:*** the circumference P was estimated from $P = \pi \times d$

- ***Cord insertion site*:** the minimum distance of the site of cord insertion to the margin of the placenta was measured and denoted as '*x*'. Assuming the placenta to be a perfect circle the mean radius 'r' was obtained from the formula $r = d/2$ and then the centricity (C) was computed from $100 \times (x/r)$. The placentas were divided into 4 groups:

  Central cord insertion (C = 76 – 100);
  Moderately eccentric cord insertion (C = 51 – 75);
  Highly eccentric cord insertion (C = 26 – 50);
  Marginal cord insertion (C = 0 – 25).

- **Fetal placental ratio:** it is the ratio of fetal weight (taken from the case sheet of the mother) and the placental weight.

Maternal surface was then examined for its completeness, any areas of calcification and infarction.

After gross examination parallel cuts were made at a distance of 1cm each, perpendicular to the long axis of placenta and again kept in

fresh 10%NBF for 24hrs for adequate fixation. After fixation 2 random bits were taken from each placenta at a distance of 2cm from the peripheral margin and submitted in 2 cassettes for processing.

TISSUE PROCESSING:[79]

Tissue processing is a technique which is used for the removal of all extractable water from the tissue and replacing it with a support medium which provides sufficient rigidity to the tissue to enable its sectioning without parenchymal damage or distortion.

After proper fixation, the placental bits were processed routinely by dehydrating in ascending grades of isopropyl alcohol, clearing in 2 changes of xylene followed by impregnation and embedding in the paraffin wax, thus forming a paraffin block which helps to cut thin sections using rotatory microtome.

With each block 5-7 micro-meter sections were taken with microtome on 3 different slides, of which 2 were egg albumin coated, 1 for Hematoxylin and eosin (H&E) stain, 1 for Periodic acid Schiff (PAS) stain, and one slide was positively charged with chrom-alum for Immunohistochemistry(IHC). The slides for H&E and PAS staining were placed in incubator at 70^0c for 1 hour.

HEMATOXYLIN AND EOSIN STAIN[79]

Hematoxylin & Eosin stain, is the most commonly used stain in histopathology for routine microscopy and reporting.

Hematoxylin is a naturally available basic dye and is extracted from the core or heartwood of the tree Haematoxylon Campechianum and stains the nucleus of the cells, while eosin is an acid xanthene or phthalein dye which is a counterstain and gives a pleasant contrast to the nuclear stain.

The stains used for the staining of slides were Harris's hematoxylin and eosin Y. The staining procedure was as follows:

1) After taking out the slides from the incubator, the sections were immersed in first xylene bath for 5 minutes and then in 2nd and 3rd xylene bath for 3 minutes each. Xylene dissolves the paraffin wax and remove it from the sections.

2) The xylene in turn was removed by immersing in 2 baths of absolute alcohol, each for 2 minutes and then the sections were rehydrated by immersing in descending grades of alcohol i.e. 90%, 80% 70% and then placed in running tap water or distilled water for 2 minutes.

3) Sections were stained in Harris's hematoxylin for 5-7 minutes.

4) Rinsed quickly in water and then differentiated in 1% acid alcohol (1%HCl in 70%alcohol) by dipping the sections in it for about 5-10 seconds (1 to 2 times).

5) Washed in water until the sections were blue (10-15 minutes, known as blueing).

6) Sections were then stained with acidified 1% aqueous eosin Y for 1-2 minutes, and washed again with tap water for 2 minutes.

7) Dehydrated through increasing grades of alcohol, cleared with xylene 2 changes, 5 minutes each and mounted with DPX.

Once the H&E slides were ready, all the cases were viewed/screened under 100x and 400x view using binocular light microscope. The observer had been blinded for the case data. For each case, 100 terminal villis were counted and the presence of synctial knot, villous edema, villous fibrosis, fibrinoid necrosis was recorded in percentage and presence or absence of chorangiosis was noted.

PERIODIC ACID SCHIFF (PAS) STAINING[79]

PAS staining was used to demonstrate the thickness of the basement membrane. The procedure used to prepare the Schiff's reagent was "Lillie's cold Schiff" procedure:-

In this 100ml of 0.15N HCl was taken and 1gm of basic fuchsin and 1.9gms of sodium metabisulphite was dissolved in it. The solution was mixed properly by shaking frequently at intervals for 2 hours. Then

0.5gms of activated charcoal was added and after shaking and mixing properly was kept overnight. The solution was then filtered next day with Whatmann's filter paper no. 1 in brown stock bottle and kept at 0-4^{0}c in refrigerator. Following procedure was done for PAS stain:

1) The sections were first deparaffinised by immersing in first xylene bath for5minutes and then in 2nd and 3rd xylene bath for 3 minutes each.

2) The sections were then rehydrated by immersing in 2 baths of absolute alcohol, each for 2 minutes and then by immersing in descending grades of alcohol i.e.90%, 80% 70% and then placed in running tap water or distilled water for 2 minutes, to remove the xylene and hydrate the tissue.

3) Sections were then oxidized with 0.5% aqueous periodic solution for 5 minutes.

4) Washed in tap water for 1-2 mins.

5) Sections were placed in Schiff's reagent for around 15-20 minutes.

6) Sections are then washed in tap water for 5-10 minutes.

7) The nucleus is counterstained with Harris's hematoxylin for 30 seconds and differentiated in tap water for 1-2 minutes or till the sections became blue.

8) The sections were then dehydrated through ascending grades of alcohol, cleared with xylene, 2 changes, 5 minute each and mounted with DPX.

Note: Section of appendix was taken as control for PAS, the mucous cells/goblet cells of the appendix which is a neutral mucin will takeup the PAS positivity.

The PAS stained slides/sections were then reported under the 100x and 400x magnification for basement membrane thickening which was seen as the thickness between the syncytiotrophoblast and vessel endothelium. Here also the observer had been blinded for case data. The following grading system had been used for the evaluation of PAS staining[62]….

- HAZY - ±
- TRACES - +1
- MILD - +2
- MODERATE - +3
- SEVERE - +4

IMMUNOHISTOCHEMISTRY (IHC)[79] -

10% neutral buffered formalin was used for specimen fixation. The tissues were processed in automated histokinette through various grades of alcohol and xylene. Paraffin blocks were prepared. Sections were cut using semi-automated microtome with disposable blade and suitable sections were chosen for IHC. Slides were coated with chrome alum and sections were transferred onto these slides. Sections were subjected to antigen retrieval using pressure cooker technique with TRIS–EDTA buffer solution.

PROCEDURE FOR IHC:

1) Sections were deparaffinised using xylene – 2 changes –each for 15 minutes
2) Rehydrated with absolute alcohol – 2 changes 5 minutes each followed by 90% alcohol for 3 minutes and 70 % alcohol for 3 minutes
3) The Sections were then rinsed in distilled water – 1 minute
4) Rinsed in TBS buffer – 5 minutes
5) Antigen retrieval was done by pressure cooker method in citrate buffer – 2 whistles
6) The sections were then cooled to room temperature for 5 -10 minutes
7) Rinsed in TBS buffer – 2 changes – each 5 minutes
8) The sections were kept under Peroxidase block -15 minutes
9) Rinsed in TBS buffer – 2 changes – each 5 minutes
10) Slides were drained and sections were covered with primary antibody for 45 minutes
11) Rinsed in TBS buffer – 2 changes – each 5 minutes
12) Covered the sections with Super enhancer for 15minutes
13) Rinse the sections in TBS buffer – 2 changes – each 5 minutes
14) Secondary antibody labelled with horse raddish peroxidase was kept on the sections for 15 minutes
15) Rinsed in TBS buffer – 2 changes – each 5 minutes
16) Sections were covered with DAB and substrate solution for 3-5 minutes

17) Rinsed in TBS buffer – 5 minutes then in distilled water for 5 minutes

18) Counterstained with hematoxylin for 30 seconds

19) The sections were then dehydrated with increasing grades of alcohol, cleared with xylene and mounted with DPX.

EVALUATION OF IMMUNOSTAINING:

VEGF i.e. vascular endothelial growth factor is a dimeric glycoprotein which has the property to start angiogenesis. For this study, mouse monoclonal antibody, which shows cytoplasmic/membranous staining in IHC.

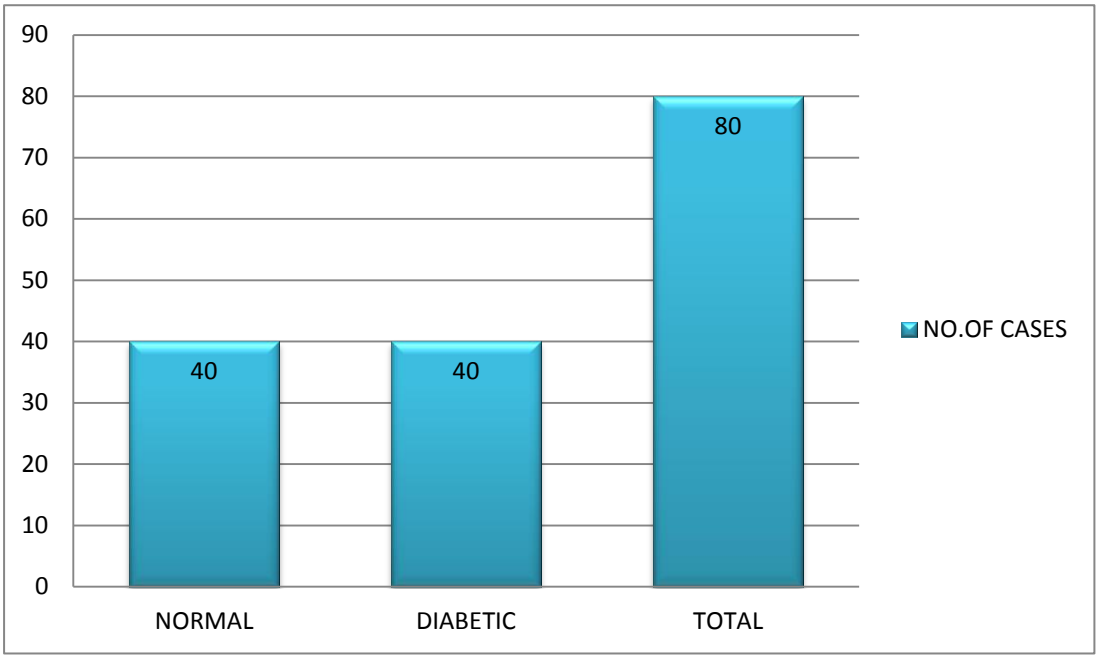
Control: Moderately differentiated Colonic adenocarcinoma and normal colonic mucosa has been taken as positive and negative control for VEGF.

The staining of VEGF antibody was observed in the trophoblastic cells and endothelial cells of the villous capillaries and the following grading system had been used for the staining intensity for both the locations.[90]

- NEGATIVE 0
- WEAK 0.5
- MODERATE 1.0
- STRONG 2.0

OBSERVATION & RESULTS

In this present study we included total 80 placentas out of which 40 placentas were of diabetic mothers and 40 placentas were of normal mothers, fulfilling the inclusion and exclusion criteria **[Graph no.1]**

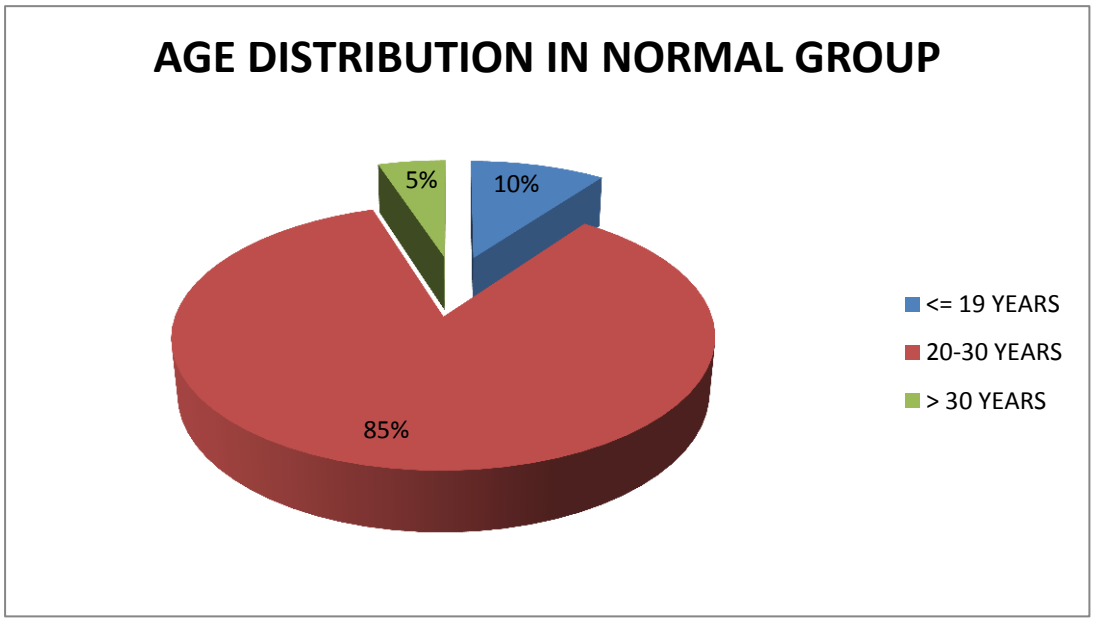


Graph no. 1 - Total no.of cases

Majority of cases in both the normal and diabetic group were between 20 to 30 years of age, about 34 cases in both the groups. 5 cases were more than 30 years of age in diabetic group and 2 cases in normal group. The eldest patient in diabetic group was 39 years, while in the normal group it was 38 years. While only 1 case in diabetic group and 4 cases in normal group were less than or equal to 19 years**[Table no. 1]**.

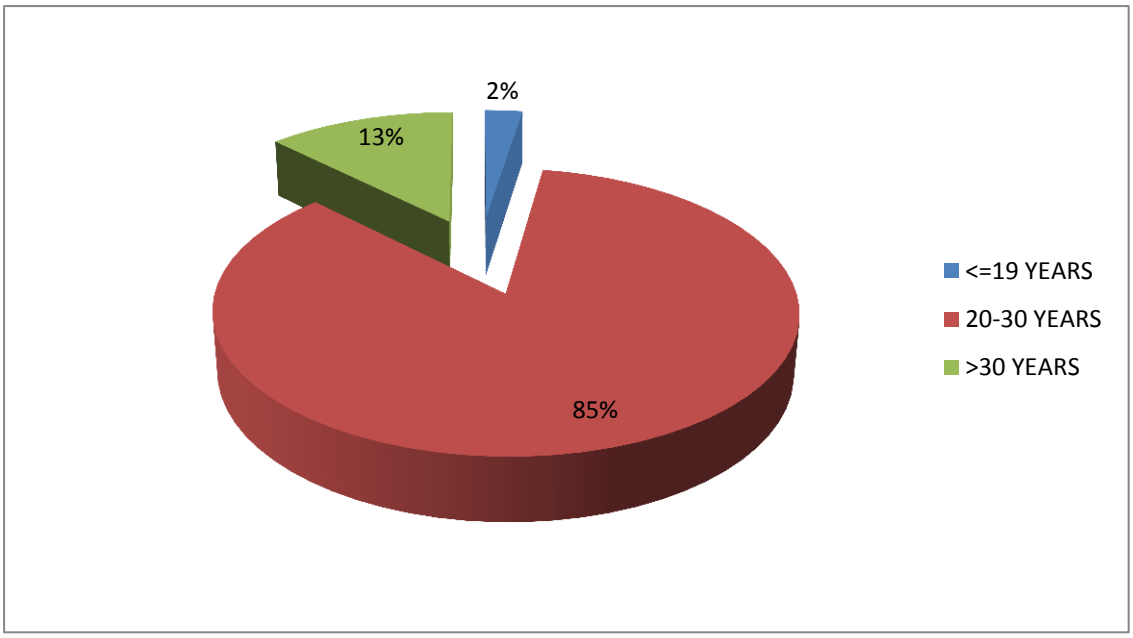
Table no. 1 – Age distribution in both groups

			GROUP		
			DIABETES	NORMAL	Total
AGE GROUP	< = 19 YEARS	Count	1	4	5
		% within GROUP	2.5%	10.0%	6.3%
	20 - 30 YEARS	Count	34	34	68
		% within GROUP	85.0%	85.0%	85.0%
	> 30 YEARS	Count	5	2	7
		% within GROUP	12.5%	5.0%	8.8%
Total		Count	40	40	80
		% within GROUP	100.0%	100.0%	100.0%



Graph no. 2 – Age distribution in normal group

The above pie chart **[Graph no.2]** shows the age distribution of cases in normal group with majority of the cases i.e. 85% between the age of 20-30 years, 10% cases were > 30 years of age while only 5% cases were <=19 years of age.



Graph no.3 – Age distribution in diabetic group

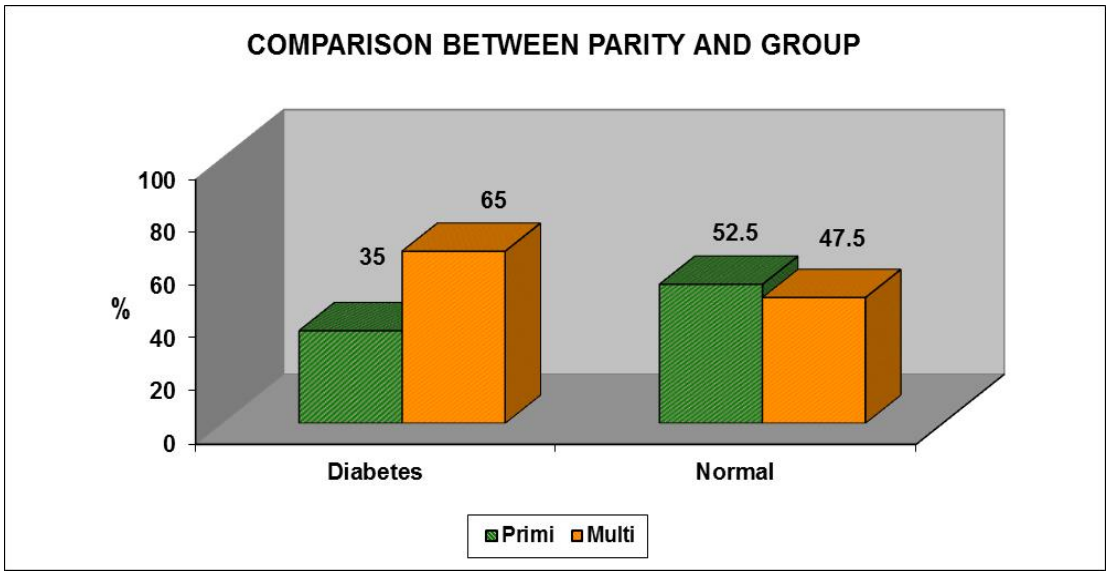
The above pie chart **[Graph no. 3]** shows the age distribution of cases in diabetic group with majority of the cases i.e. 85% between the age of 20-30 years, 12.5% cases were > 30 years of age while only 2.5% cases were <=19 years of age.

Table no.2 - Parity in both groups

			GROUP		
			DIABETES	NORMAL	Total
PARITY	PRIMI	Count	14	21	35
		% within GROUP	35.0%	52.5%	43.8%
	MULTI	Count	26	19	45
		% within GROUP	65.0%	47.5%	56.3%
Total		Count	40	40	80
		% within GROUP	100.0%	100.0%	100.0%

Here as shown in above table[**Table no.2**], in our study, 21 cases were primigravida while 19 cases were multigravida (>=2) in normal

group, while only 14 cases were primigravida and 26cases were multigravida in the diabetic group.



Graph no. 4 – Parity in both groups

The above graph**[Graph no.4]** shows that 52.5% cases of normal group were primi as compared to 35% cases in diabetic group, while only 47.5% cases were multigavida in normal group as compared to 65% cases in diabetic group.

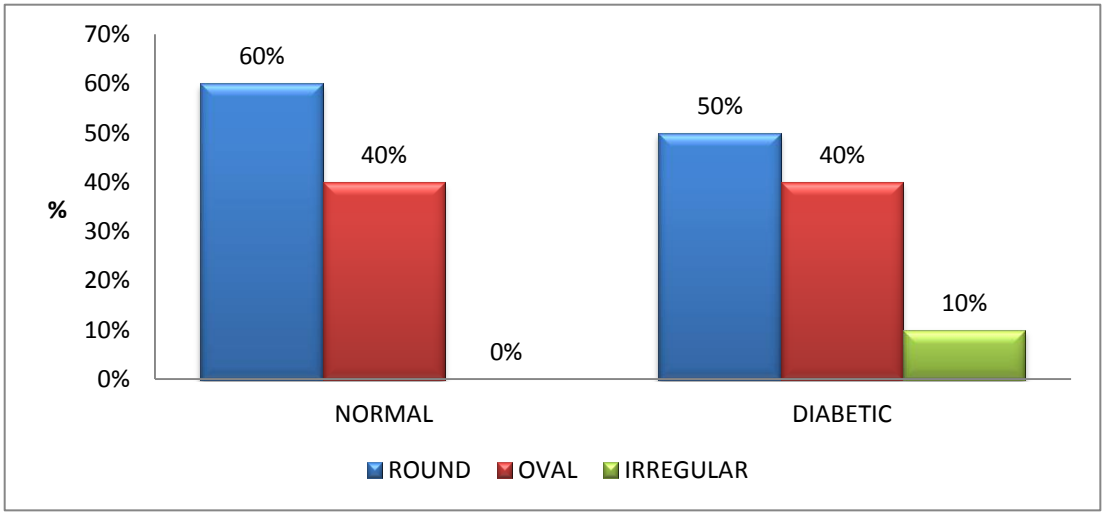
Placenta can have various shapes of which the most common are round, oval and irregular shape. Placenta succenturiate is a variation in the shape of placenta in which an acessory lobe is present having vascular connection with the main placenta. We have got 3 cases of placenta succenturiate which are included in irregular shape category.

As shown in the tablebelow [**Table no.3**] the most common shape encountered in both the normal group and diabetic group was round**[Figure 4]** with 24 cases in normal group and 20 cases in diabetic

group. 16 placentas were oval**[Figure 5]** in shape in both the groups while the diabetic group showed4 cases with irregular shape**[Figure 6]** of placenta, including the placenta with suceenturiate lobe[**Figure 7**].

Table no.3 – Placental shape distribution in both groups

			GROUP		Total
			DIABETES	NORMAL	
SHAPE	ROUND	Count	20	24	44
		% within GROUP	50.0%	60.0%	55.0%
	OVAL	Count	16	16	32
		% within GROUP	40.0%	40.0%	40.0%
	IRREGULAR	Count	4	0	4
		% within GROUP	10.0%	.0%	5.0%
Total		Count	40	40	80
		% within GROUP	100.0%	100.0%	100.0%



Graph no.5 - Shape of placentas in both groups

The above graph **[Graph no. 5]** shows, in normal group 60% cases had round placenta, 40% cases had oval shape placenta while none of the cases showed irregular shape of the placenta. In comparison the diabetic group showed 50% cases with round shape, 40% cases with oval shape and 10% cases with irregular shape of placenta.

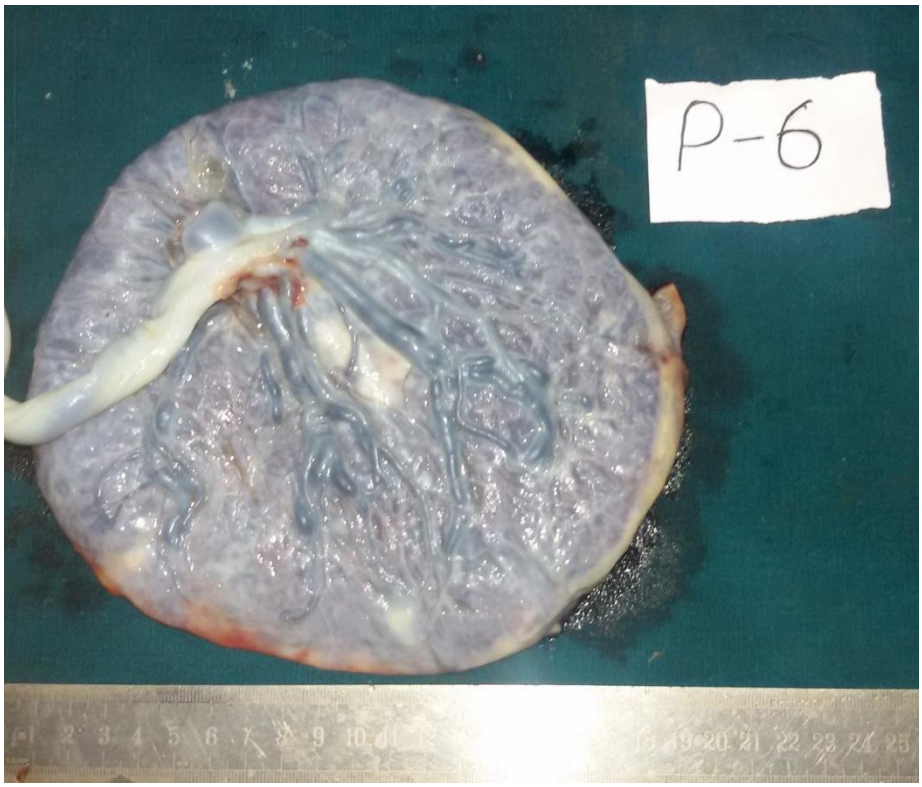


Figure 4 – Round shape of placenta

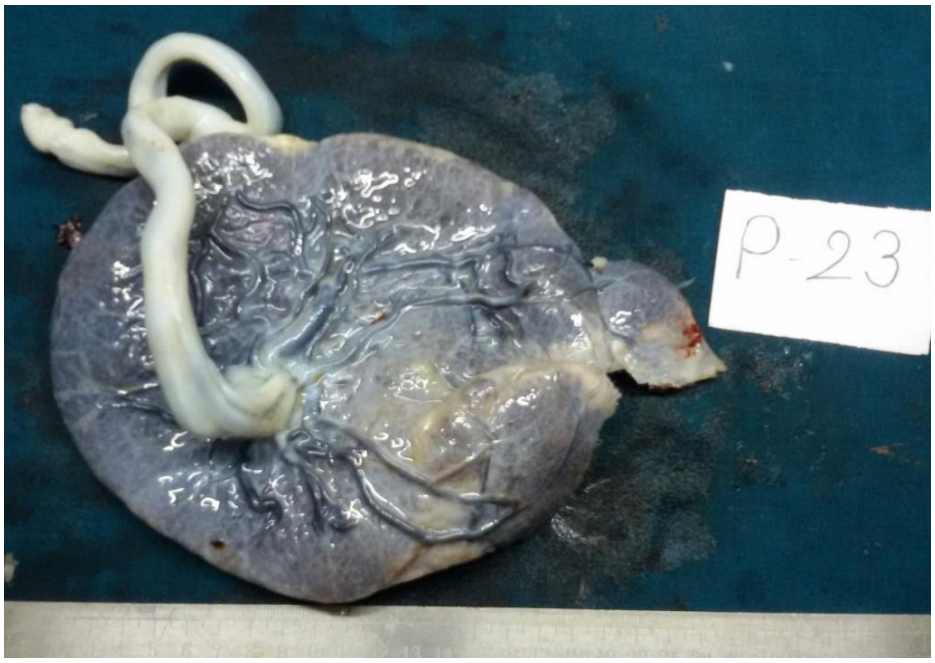


Figure 5 – Oval shape of the placenta

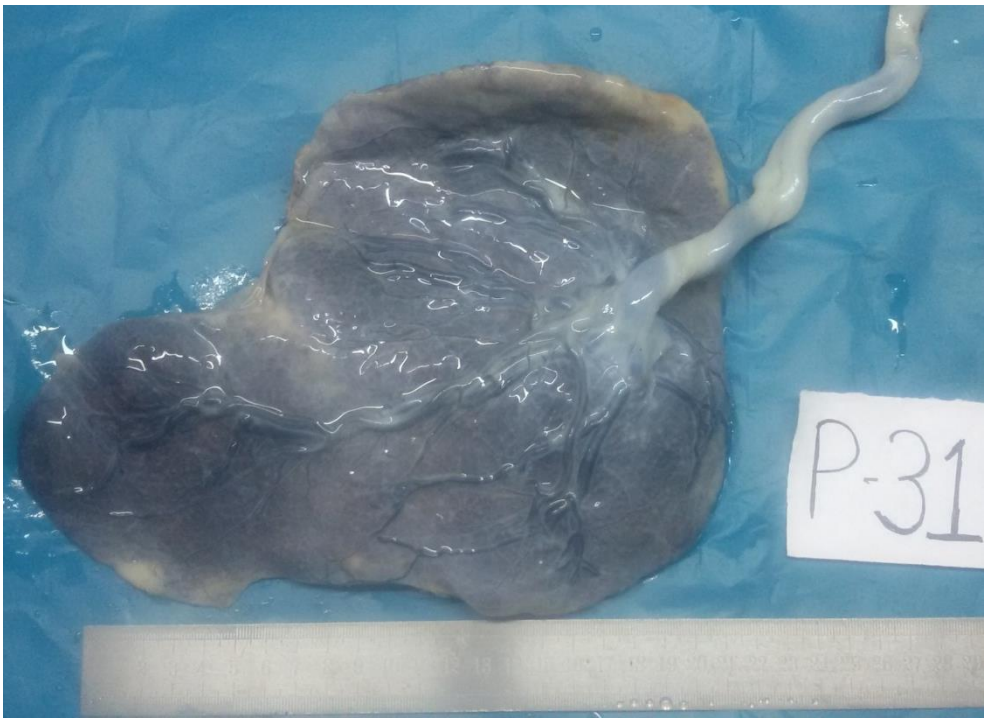


Figure 6 – Irregular shape of the placenta

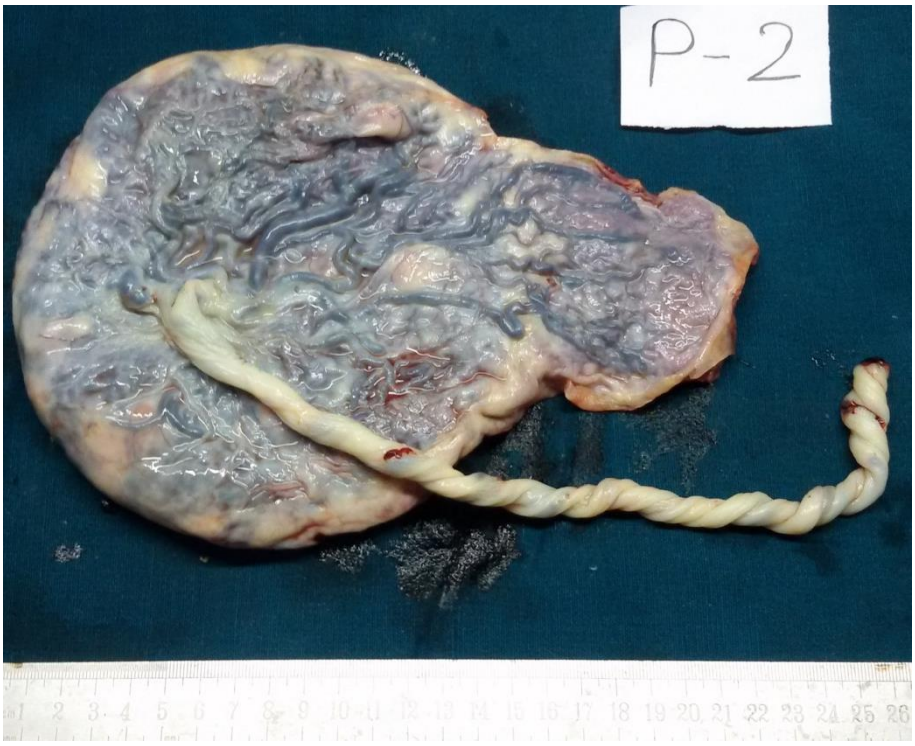
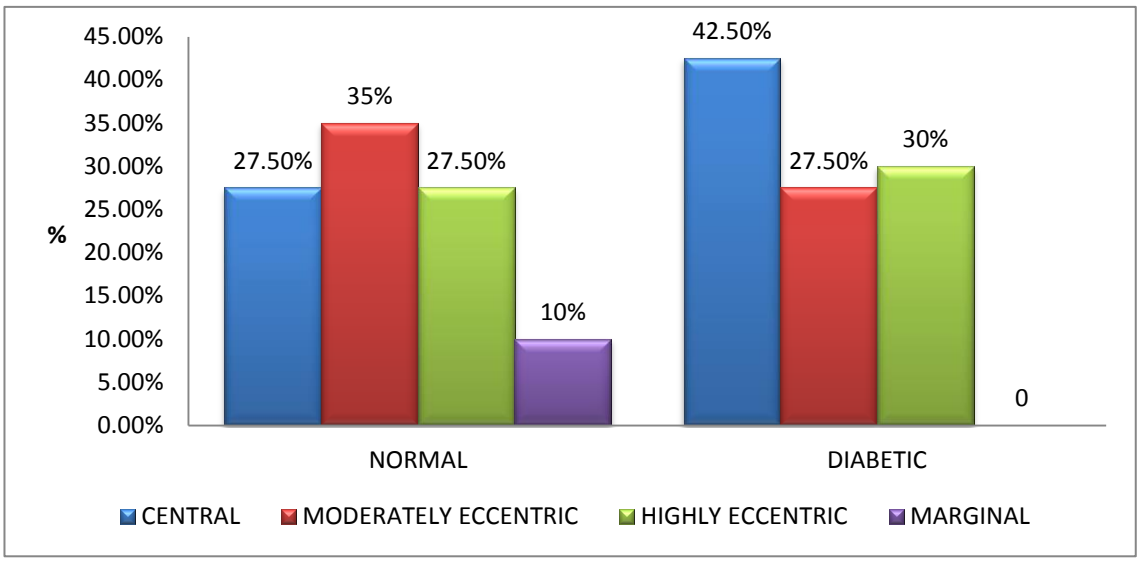


Figure 7 – Succenturiate lobe of placenta

Table no. 4 – Site of cord insertion

			GROUP		
			DIABETES	NORMAL	Total
CORD INSERTION	CENTRAL	Count	17	11	28
		% within GROUP	42.5%	27.5%	35.0%
	MODERATELY ECCENTRIC	Count	11	14	25
		% within GROUP	27.5%	35.0%	31.3%
	HIGHLY ECCENTRIC	Count	12	11	23
		% within GROUP	30.0%	27.5%	28.8%
	MARGINAL	Count	0	4	4
		% within GROUP	.0%	10.0%	5.0%
Total		Count	40	40	80
		% within GROUP	100.0%	100.0%	100.0%

As shown in above table[**Table no.4**] shows that overall the most common site of insertion of umbilical cord was central[**Figure 8**] accounting for about 28 cases out of total 80 cases, and the least common was marginal[**Figure 9**] shown only in 4 cases out of 80.



Graph no. 6 – Site of cord insertion in both groups

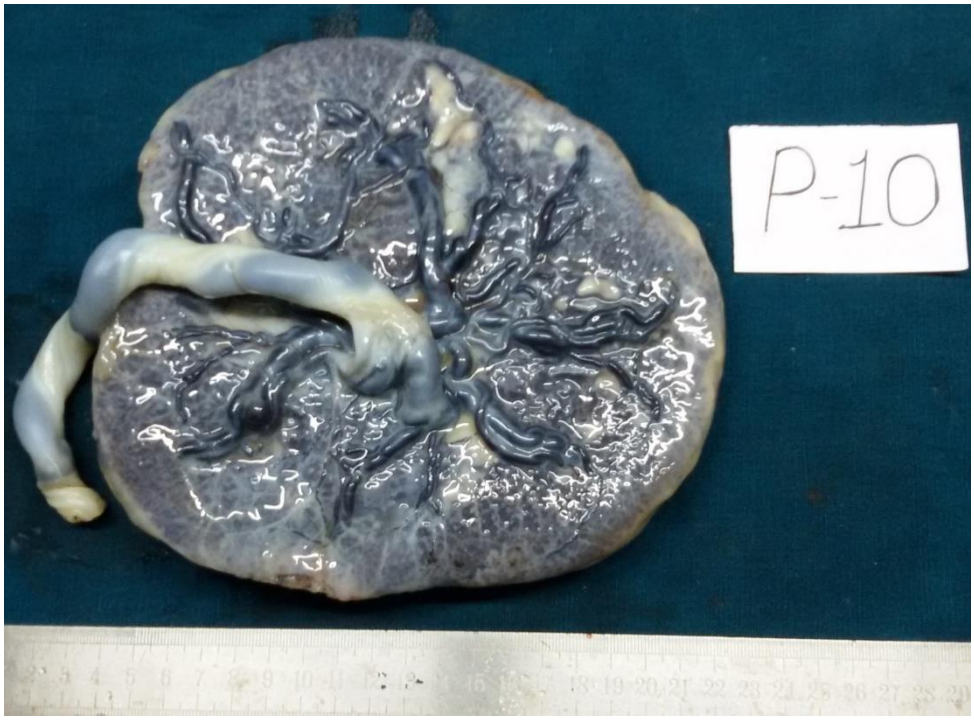


Figure 8 – Central attachment of Umbilical cord

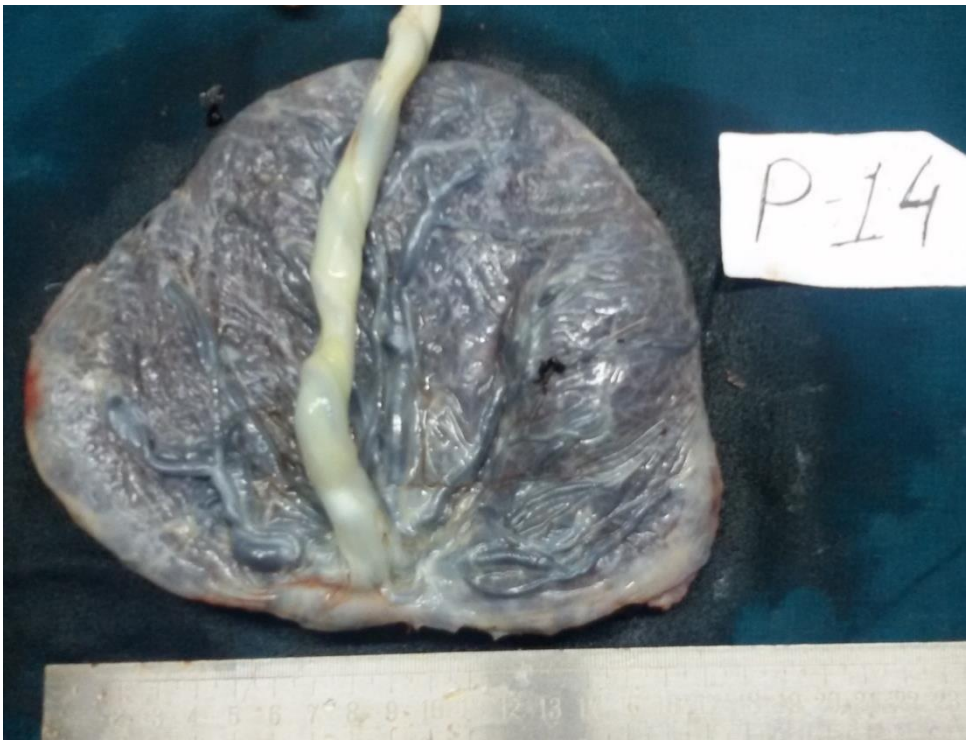


Figure 9 – Marginal attachment of Umbilical cord

The above graph[**Graph no.6**] shows that in normal cases the most common site of insertion was moderately eccentric[**Figure 10**]that was seen in 35% of cases while in diabetic group it wascentral with 42.5% of cases. 10% of cases in normal group showed marginal attachement of the umbilical cord but none of the cases in diabetic group showed marginal attachment of the placenta. Highly eccentric attachement of umbilical cord **[Figure 11]** was seen in 27.5% of normal cases and 30% of diabetic cases.

STATISTICS FOR QUANTITATIVE DATA FOR GROSS MORPHOLOGICAL PARAMETERS

Descriptive statistical analysis was done for all the quantitative gross morphological data and paired t test was applied with a confidence interval of 95%. Statistical significance was taken when $p < 0.05$. The data was analysed using Microsoft Excel 2010 and SPSS (Statistical Package for Social Science).

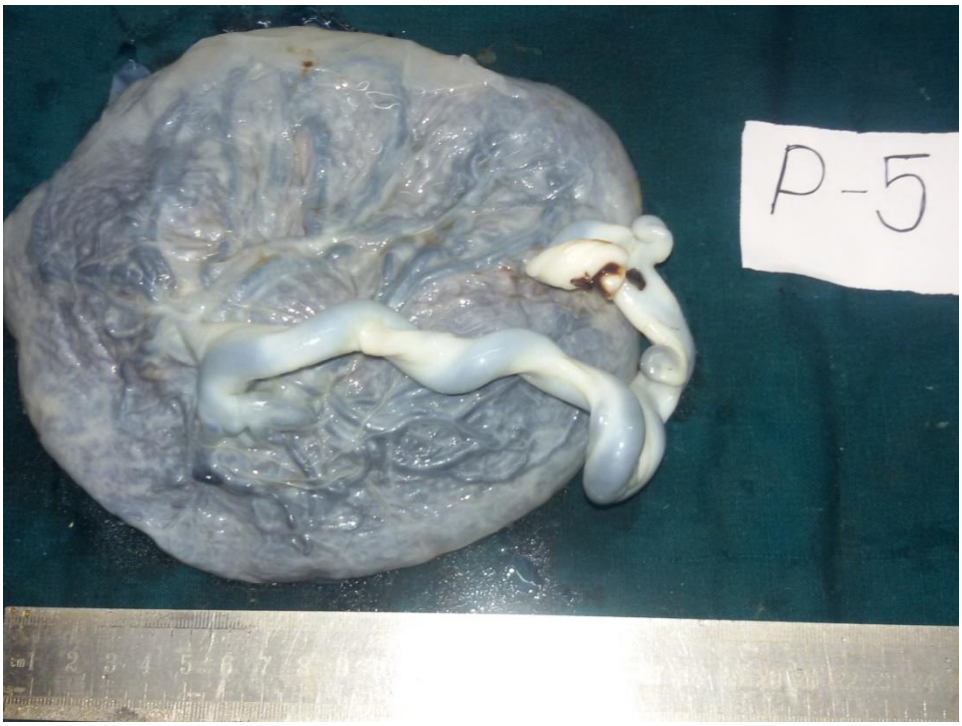


Figure 10 – Moderately eccentric attachment of umbilical cord

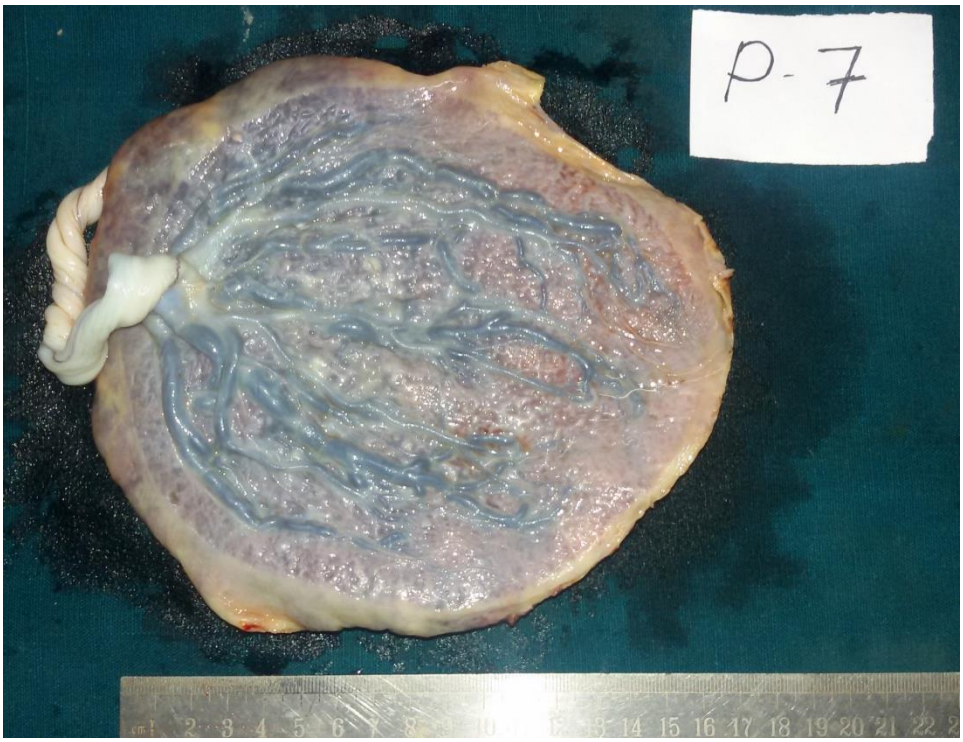
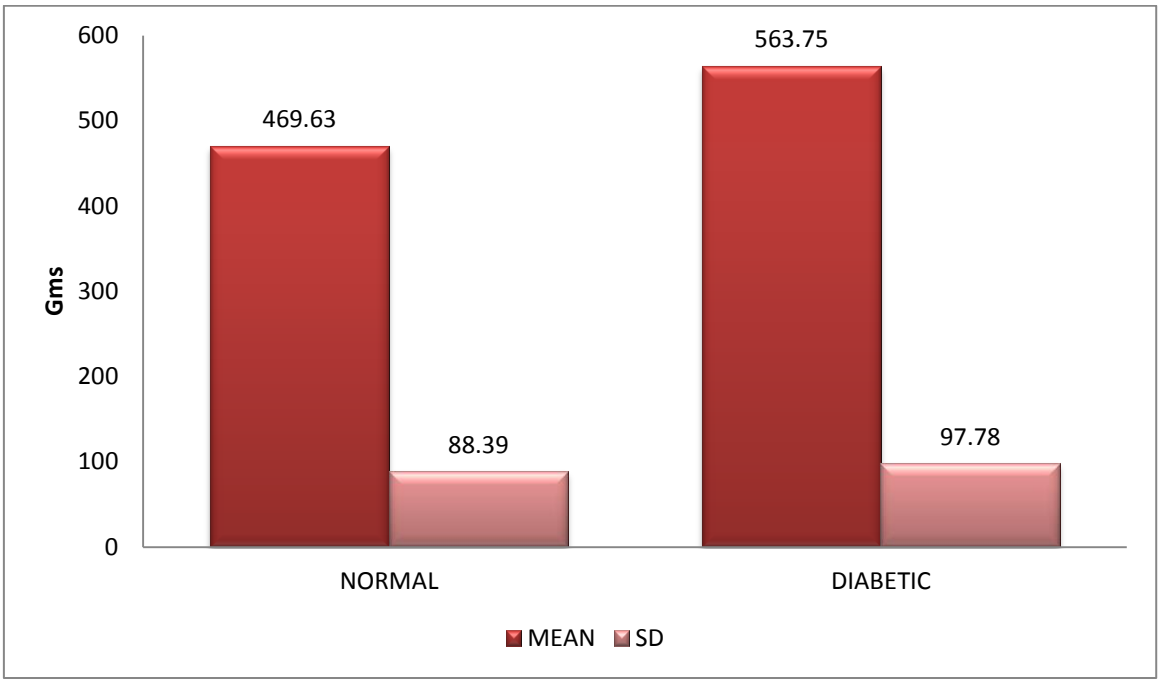


Figure 11 –Highly eccentric attachment of umbilical cord

TABLE NO. 5 – Comparison of gross morphological parameters of diabetic placenta with reference to normal placenta

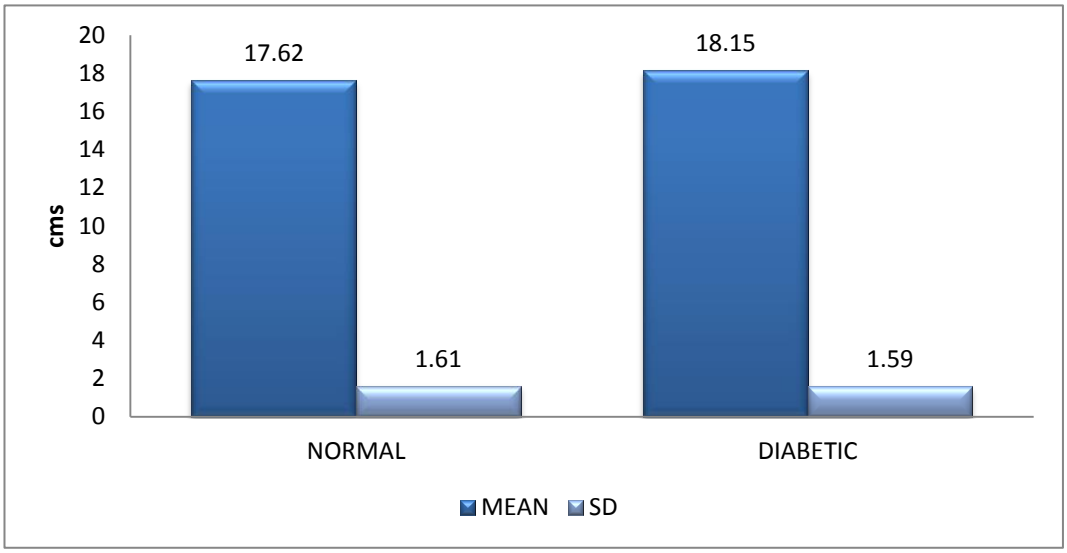
Parameters	Normal Mean±SD	Diabetic Mean±SD	P value
Placental weight(gms)	469.63±88.39	563.75±96.78	.000
Diameter(cm)	17.62±1.61	18.15±1.59	.145
Circumference(cm)	55.39±5.05	56.98±5.02	.161
Area(sq.cm)	243.43±45.93	258.87±44.76	.132
Central thickness(cm)	1.79±0.37	2.51±0.57	.000
Baby weight(Kg)	2.82±0.36	3.14±0.35	.000
Fetal/placental ratio	6.10±0.60	5.68±0.75	.008



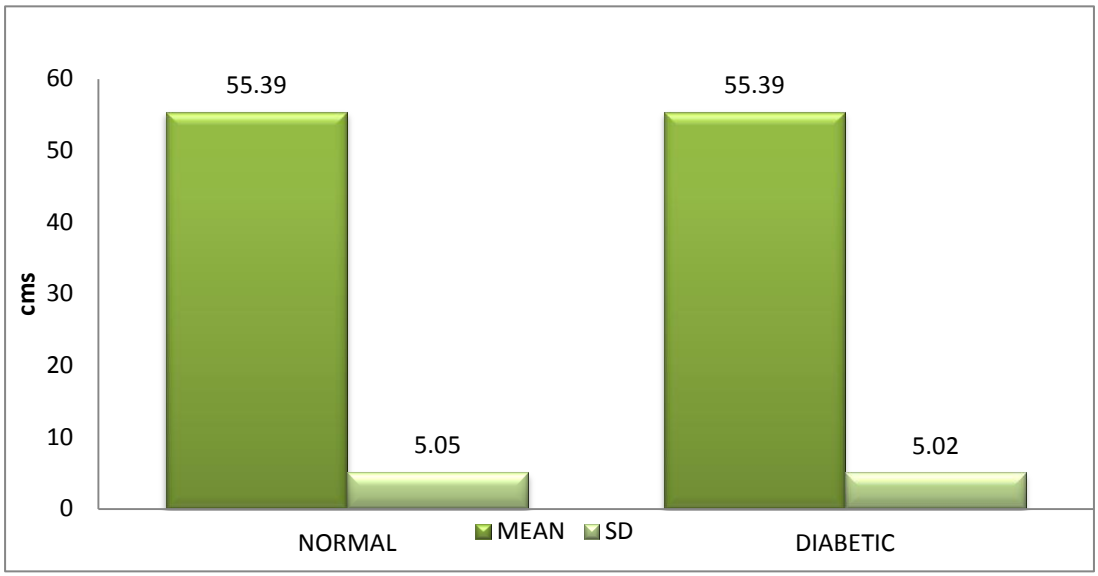
Graph no. 7 – Mean weight of placenta in gms

The above graph [**Graph no.7]** shows that there was a significant difference between the weight of the placenta amongst the normal and diabetic group. The mean weight of the placenta in normal group was 469.63 gms while in diabetic group the mean weight was 563.75gms and the difference between 2 groups was statistically significant. (<0.05) [**Table no.5**].

The **graph no.8** shows there was no significant difference between the diameter of the placenta amongst the two groups. The mean diameter of the placenta in normal patient was 17.62cm while in case of diabetic patient it was 18.15cm, and the p value is .145(>0.05) and hence the difference between the 2 groups was not statistically significant in our study[**Table no.5**].

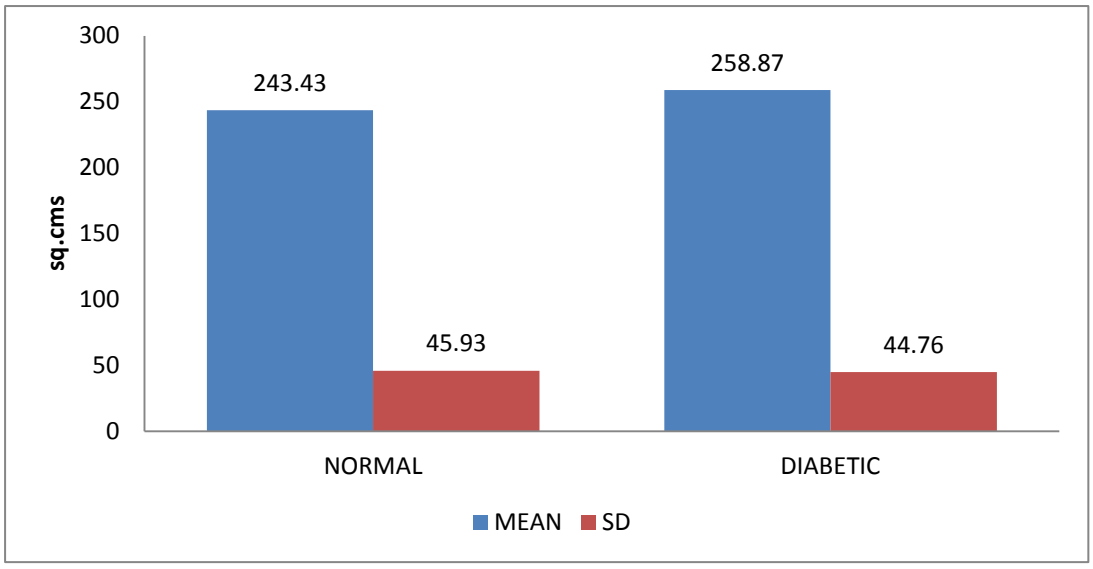


Graph no.8 – Mean diameter of placenta in cms.



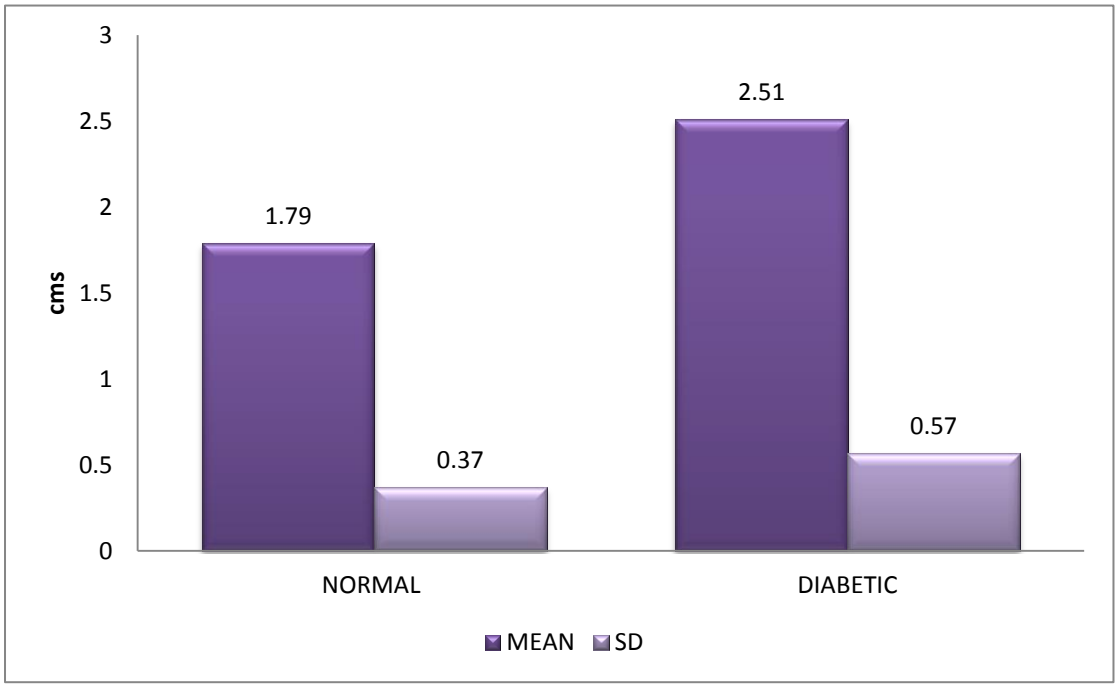
Graph no. 9 – Mean circumference of placenta in cms

The **graph no.9** shows that there was no significant difference between the circumference of the placenta amongst the 2 groups. The mean circumference of the placenta in normal patient was 55.39cm while it was 56.98cm in case of diabetic patient, and the p value is 0.161 (> 0.05) and hence the difference between the 2 groups was not statistically significant[**Table no.5**].



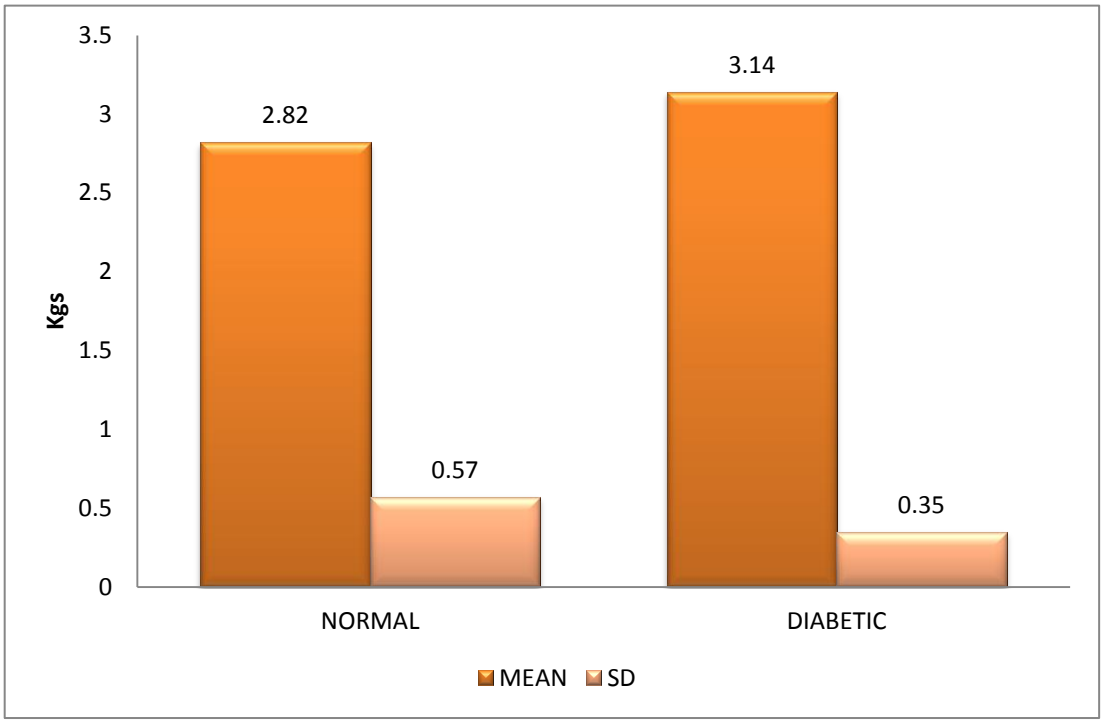
Graph no.10 – Mean areas of the placentas in both the groups in sq.cms

The **graph no. 10** shows there was no significant difference between the areas of the placenta amongst the two groups. The mean area of the placenta in normal patient was 243.43sq.cm while in case of diabetic patient it was 258.87sq.cm, and the p value is 0.132 (> 0.05) and hence the difference between the 2 groups was not statistically significant [**Table no.5**].



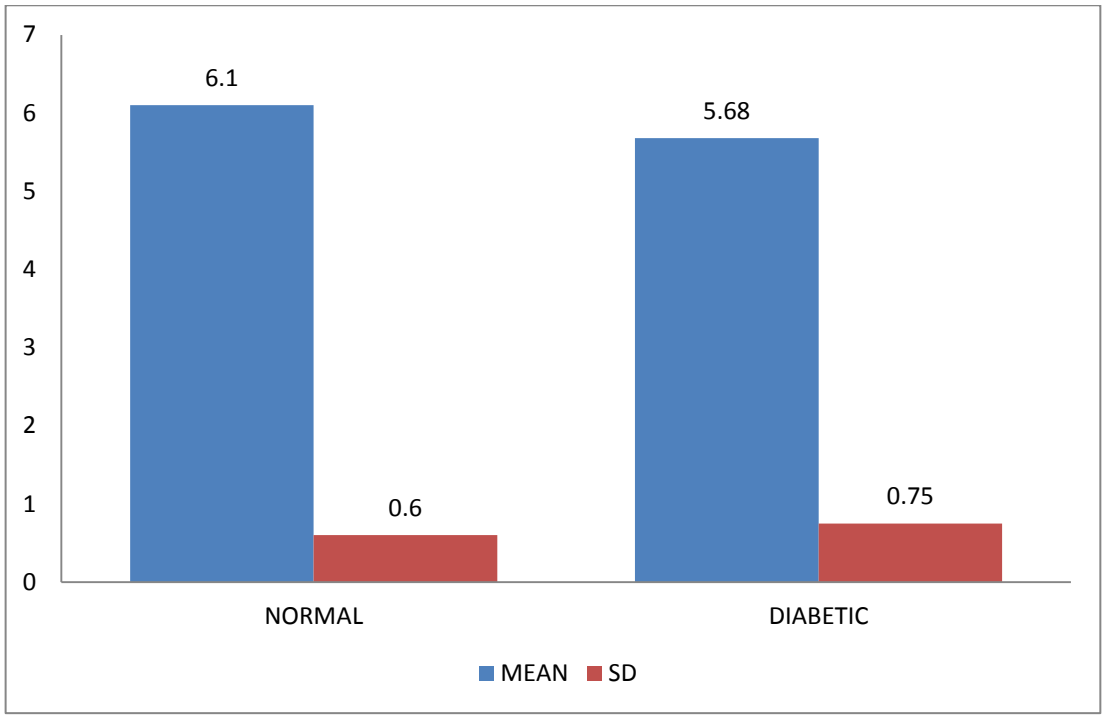
Graph no.11 – Mean central thickness of placentas in both the groups in cms

The **graph no. 11** shows that there was a significant difference between the central thickness of the placenta amongst the two study groups. The mean placental thickness in normal patient was 1.79cm while it was 2.51cm in case of diabetic patient , and the p value is 0.000 (< 0.05) and hence the difference between the 2 groups was statistically significant [**Table no.5**].



Graph no. 12 – Mean birth weight of the baby in both groups in kgs

The **graph no. 12** shows that there was a significant difference between the birth weight of the baby amongst the two study groups. The mean birth weight of the baby in normal patient was 2.82Kg while in case of diabetic patient it was 3.14Kg, and the p value is 0.000 (< 0.05) and hence the difference between the 2 groups was statistically significant[**Table no.5**].



Graph no.13 – Mean fetal/placental ratio in both the groups

The **graph no. 13** shows that there was a significant difference between the fetal/placental ratio amongst the two study groups. The mean fetal/placental ratio in normal patient was 6.10 while it was 5.68 in case of diabetic patient , and the p value is 0.008(<0.05) and hence the difference between the 2 groups was statistically significant [**Table no.5**].

Next we tried to find out the degree of correlation between the birth weight of the newborn and various placental parameters like placental weight, diameter, circumference, area, central thickness of the placenta and with fetal/placental ratio in both the groups by using Pearson's correlation coefficient 'r'. Interpretation of correlation coefficient: 'r' > 0.7 = strong correlation, 0.7 ≥ 'r' > 0.5 = good correlation; 0.5 ≥ 'r'> 0.3 = fair

correlation; 0.3 ≥ ‘r’ = poor correlation; r > 0.0 = negative correlation. we got the following result:

Table no.6 – Pearson’s r correlation between weight of the baby and various gross parameters of placenta in both groups

		Placental weight	Diameter	Circumference	Area	Central thickness	Fetal/placental ratio
Diabetic	Baby birth weight	0.682	0.154	0.195	0.141	0.515	-0.048
normal	Baby birth weight	0.839	0.451	0.448	0.428	0.371	-0.25

As shown in above table[**Table no.6],** in the normal group there was a strong correlation between the weight of the baby and placental weight. Correlation between the birth weight of the baby and the diameter, circumference, area and central thickness of placenta was fair, while the fetal/placental ratio shows a negative correlation.

On the other hand in diabetic group, there was fair correlation between the birth weight of the baby and the placental weight as well as central thickness. There was a poor correlation between the birth weight of the baby and the diameter, circumference and area of the placenta in diabetic group.

STATISTICS FOR QUANTITATIVE DATA FOR HISTOPATHOLOGICAL PARAMETERS

Descriptive statistical analysis was done for all the quantitative histopathological data and Mann-Whitney U test was applied. Statistical significance was taken as $p < 0.05$. The data was analysed using Microsoft excel 2010 and SPSS.

Table no. 7 – Comparison of histopathological parameters of diabetic placenta with reference to normal placenta

Parameters	Normal Mean of rank	Diabetic Mean of rank	P value
Villous edema (figure 12)	25.44	55.56	0.000
Villous fibrosis (figure 13)	28.58	52.43	0.000
Synctial knots (figure 14)	24.15	56.85	0.000
Fibrinoid necrosis (figure 15)	22.53	58.48	0.000

Table no. 7 shows that the p value for all the 4 parameters i.e. villous edema, villous fibrosis, syncytial knots and fibrinoid necrosis seen on histopathological examination, was <0.05 and hence there was a significant difference between these findings in both the groups.

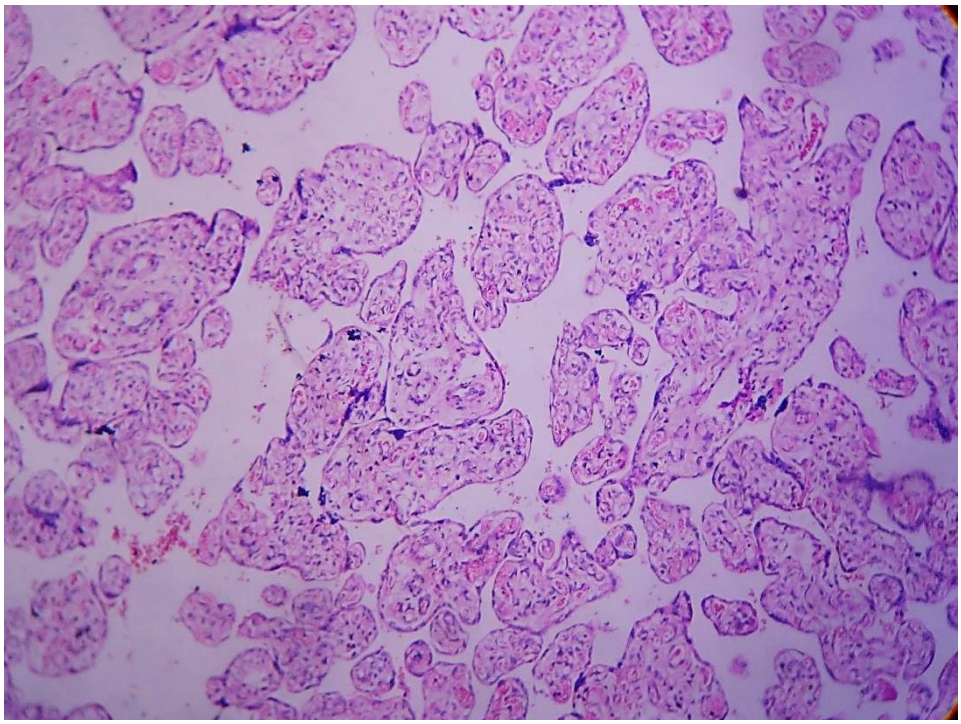


Figure 12 – Sections showing villous edema – 10x view

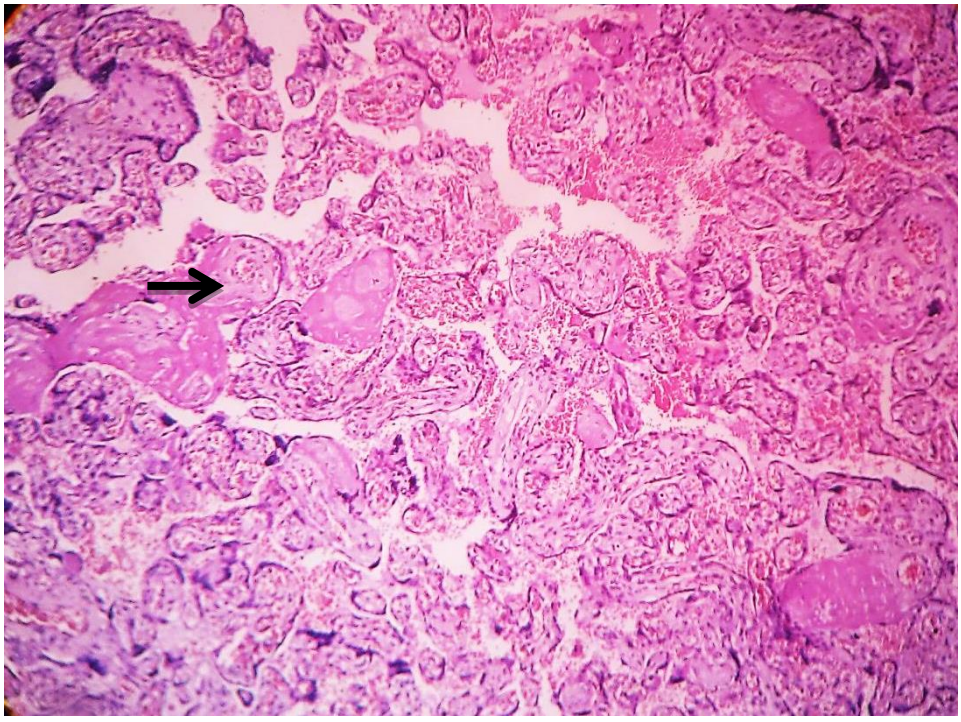


Figure 13 – Section showing villous fibrosis – 10x view

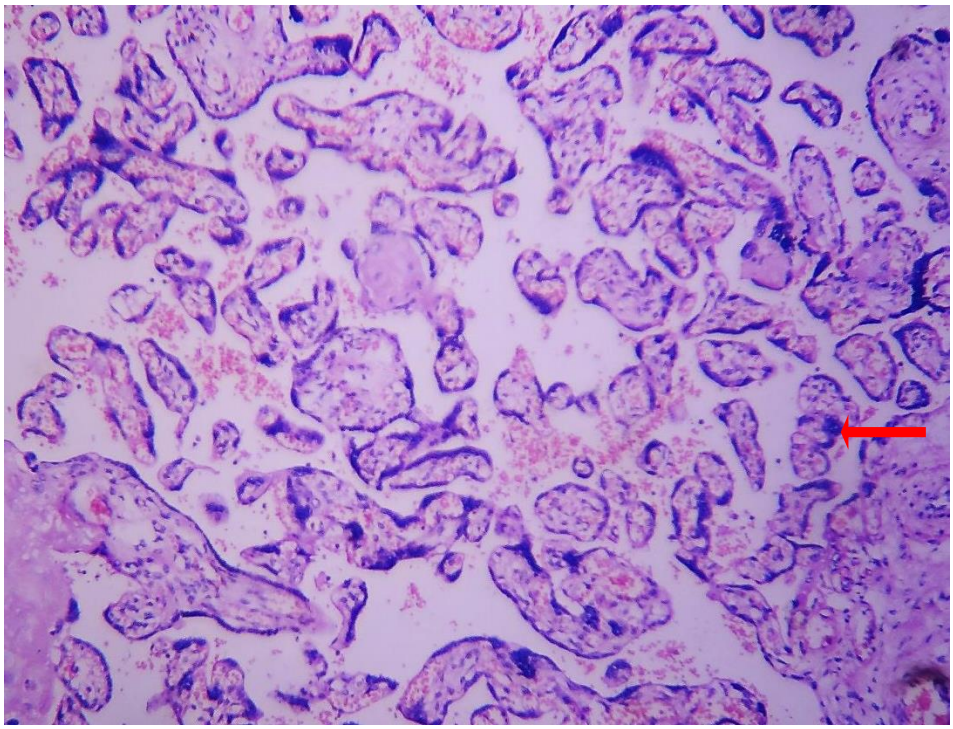


Figure 14 – Section showing increased synctial knot – 10x view

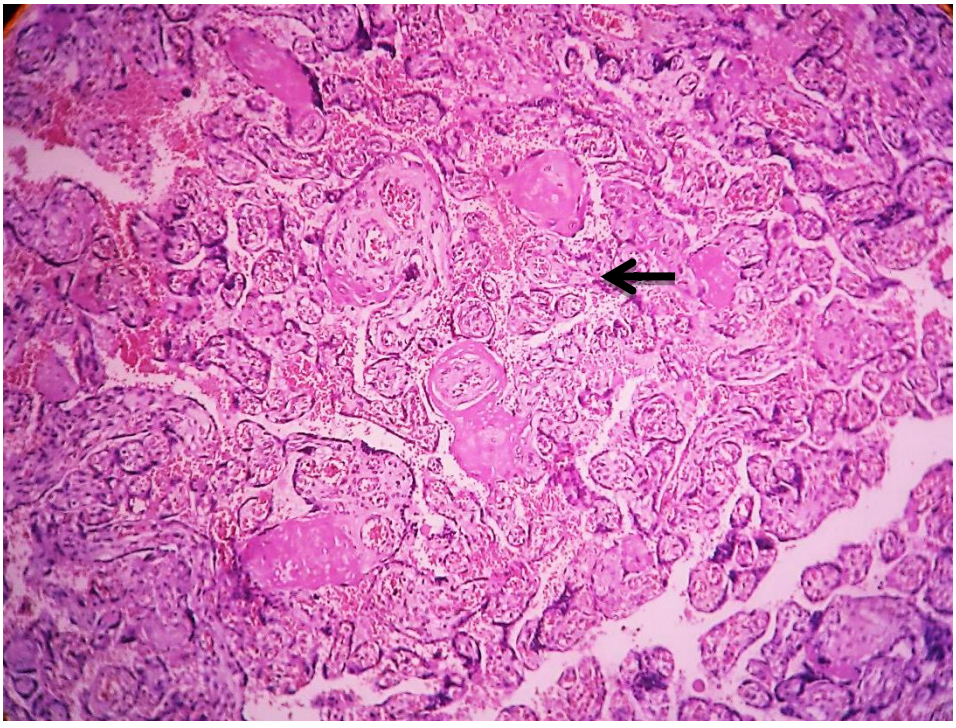
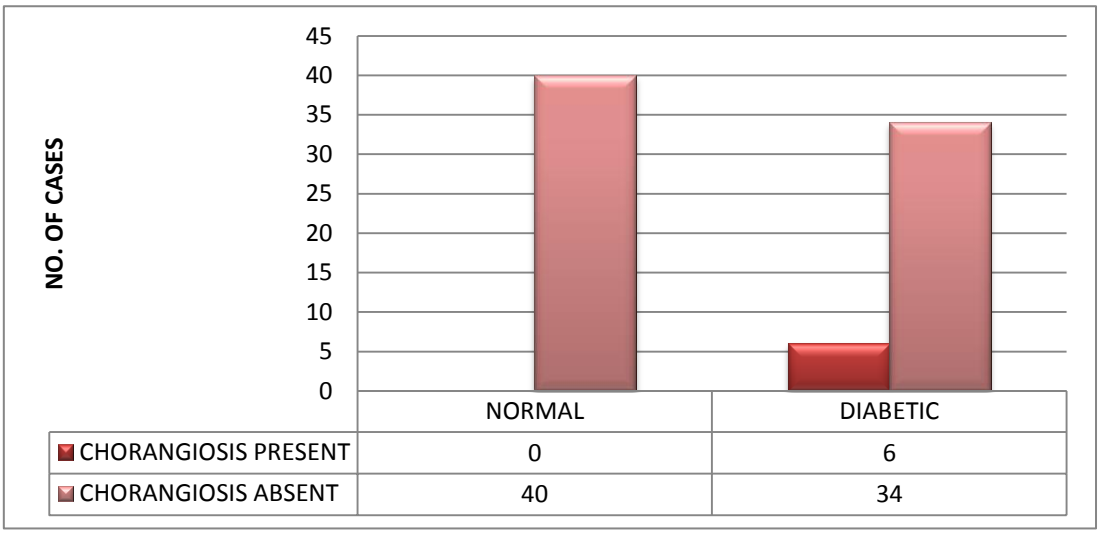


Figure 15 - Section showing fibrinoid deposition – 10x view

Chorangiosis is one another histopathological finding that we saw in our study**(Figure 16).** The following table[**Table no. 8]** shows only 15% of diabetic cases had features of chorangiosis while no such finding was seen in normal cases.

Table no.8 - Distribution of chorangiosis in 2 groups

			GROUP		
			DIABETES	NORMAL	Total
CHORIOANGIOSIS	PRESENT	Count	6	0	6
		% within GROUP	15.0%	.0%	7.5%
	ABSENT	Count	34	40	74
		% within GROUP	85.0%	100.0%	92.5%
Total		Count	40	40	80
		% within GROUP	100.0%	100.0%	100.0%



Graph no. 14 - Distribution of chorangiosis in 2 groups

The above graph[**Graph no.14]** shows chorangiosis is seen in 6 cases from diabetic cases but none of the normal case had this feature on histopathological examination.

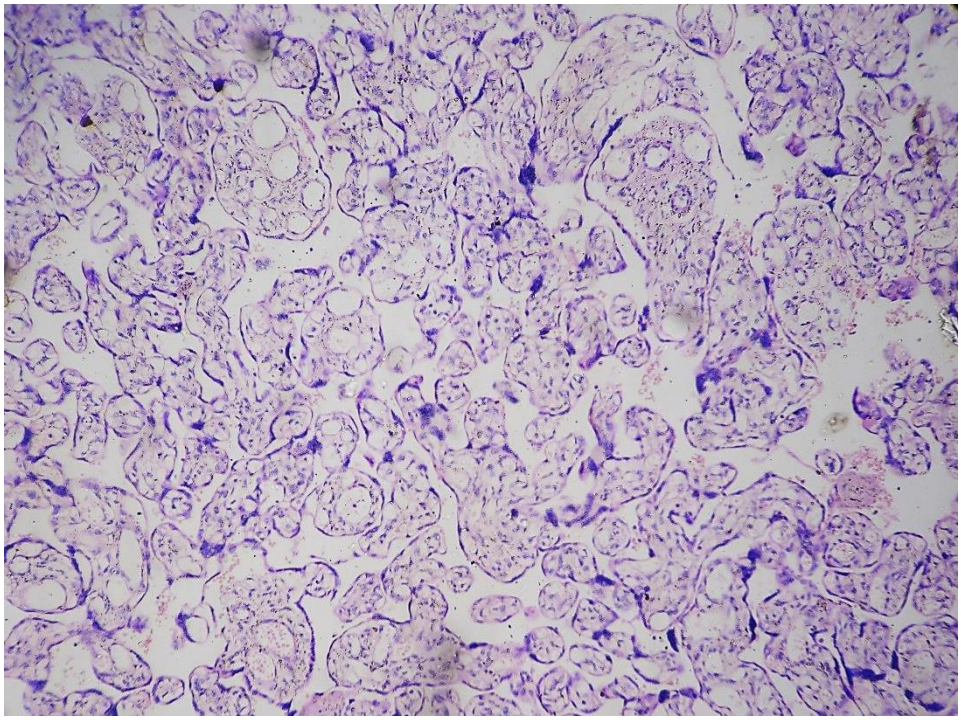
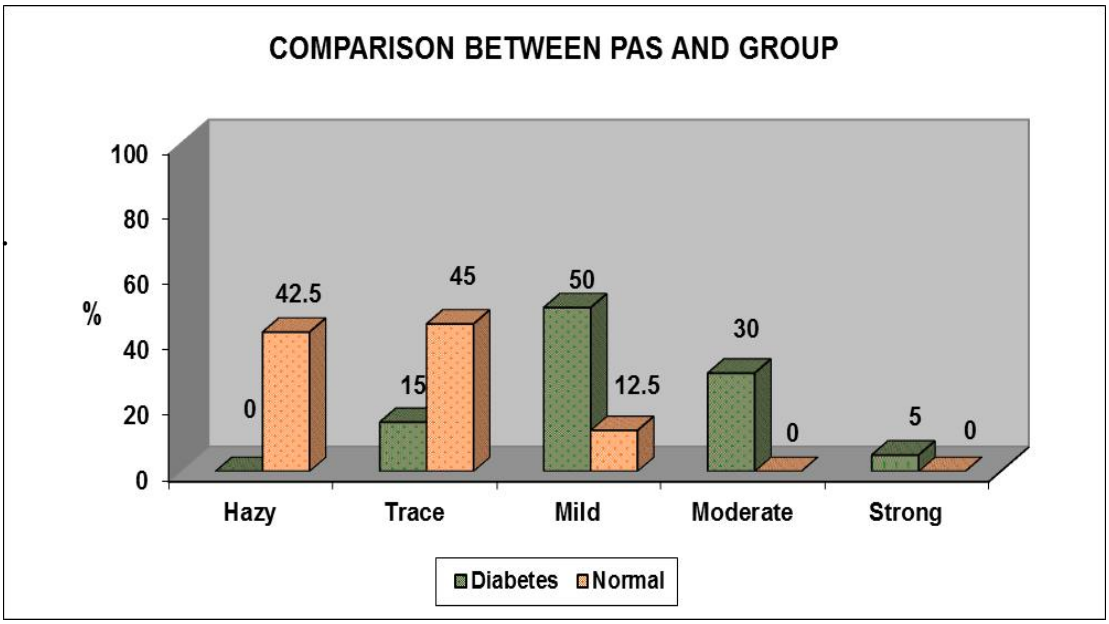


Figure 16 – Section showing chorangiosis – 10x view

After reporting the histopathological slides, all the cases had been screened for PAS staining and the following table shows the result of PAS amongst the 2 groups.

Table no.9 – Distribution of PAS grading in 2 groups

			GROUP		
			DIABETES	NORMAL	Total
PAS	HAZY	Count	0	17	17
		% within GROUP	.0%	42.5%	21.3%
	TRACE	Count	6	18	24
		% within GROUP	15.0%	45.0%	30.0%
	MILD	Count	20	5	25
		% within GROUP	50.0%	12.5%	31.3%
	MODERATE	Count	12	0	12
		% within GROUP	30.0%	.0%	15.0%
	STRONG	Count	2	0	2
		% within GROUP	5.0%	.0%	2.5%
Total		Count	40	40	80
		% within GROUP	100.0%	100.0%	100.0%



Graph no.15 – Distribution of cases in 2 groups depending upon PAS reactivity

As shown in **table no.9 and graph no.15**, PAS grading was divided into 5 grading depending upon the thickness of the membrane. In normal group maximum cases i.e. 45% showed a staining reactivity of trace**(Figure 17)** while 42.5% cases had hazy**(Figure 18)** and 12.5% cases showed mild reactivity**(Figure 19)** of PAS staining with none of the cases showing moderate or strong reactivity. In diabetic group, 50% of cases showed PAS staining with mild reactivity, 30% with moderate**(Figure 20)**, 15% with trace and 5% with strong reactivity**(Figure 21)**.

As findings of PAS reactivity is a qualitative data, chi square test had been applied which gave a value of $p = 0.000$ which is <0.05 and hence the difference in the distribution of PAS reactivity between the 2 group was statistically significant.

Localization of VEGF:

In various studies, VEGF-positive cells were observed in different cellular components of the maternal and fetal placenta. In chorionic villi, VEGF was detected in the cytoplasm of the syncytiotrophoblast, endothelial cells of fetal capillaries and vessels, vessel smooth muscle cells and mesenchymal cells.[79]

In our study, monoclonal anti VEGF antibody had been used, the cytoplasmic staining pattern was observed in trophoblasts and fetal endothelial cells and the intensity of staining has been graded into 4 category.

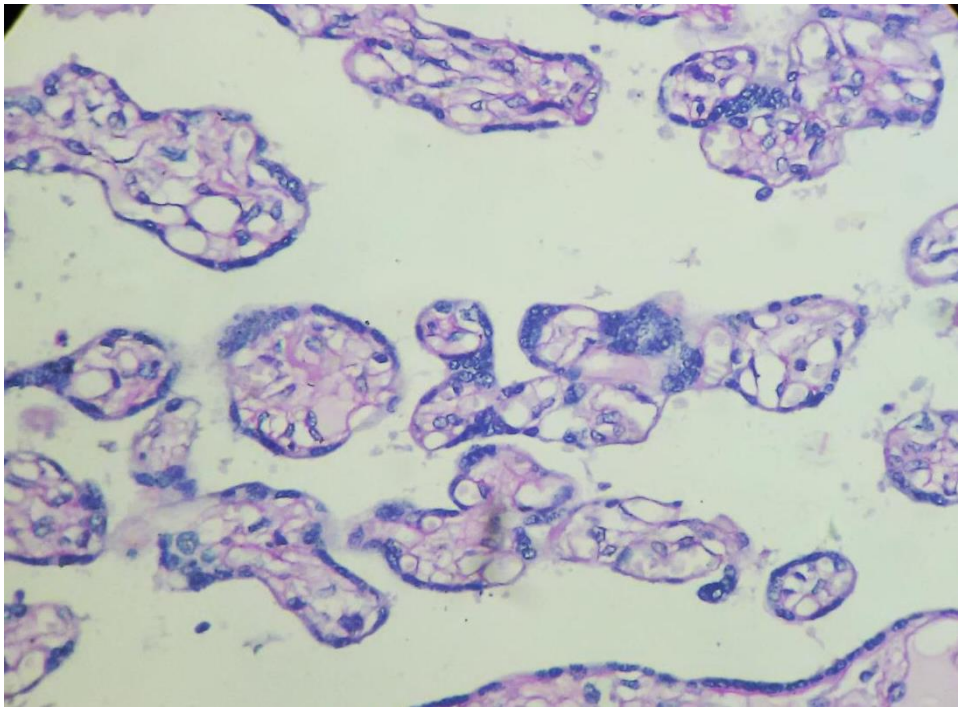


Figure 17 – Trace staining of PAS in the basement membrane – 40x view

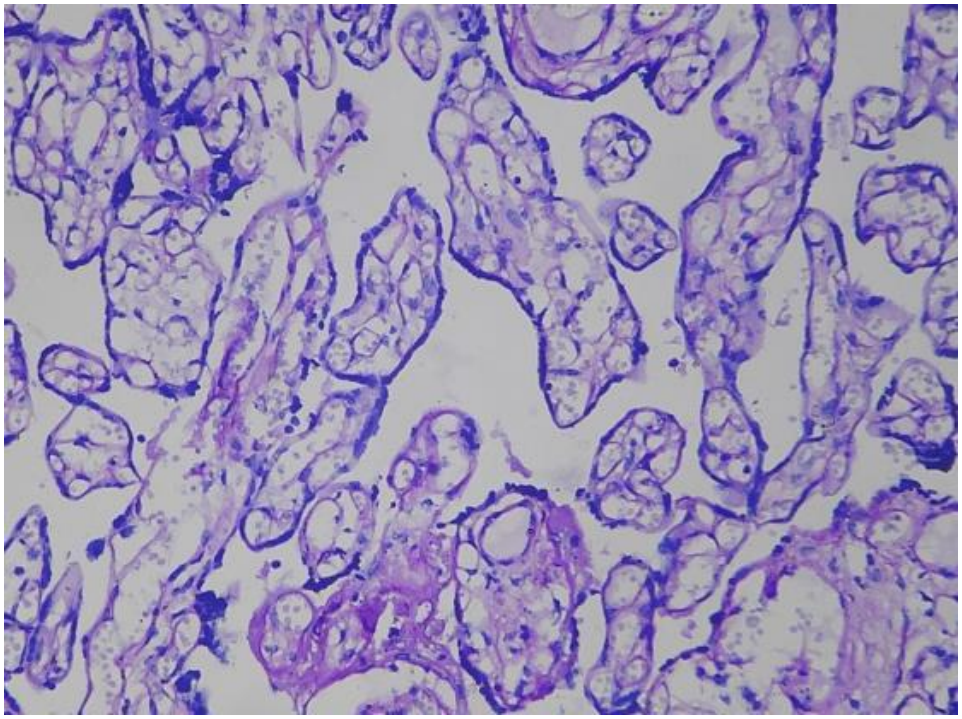


Figure 18 – Hazy staining by PAS in the basement membrane – 40x view

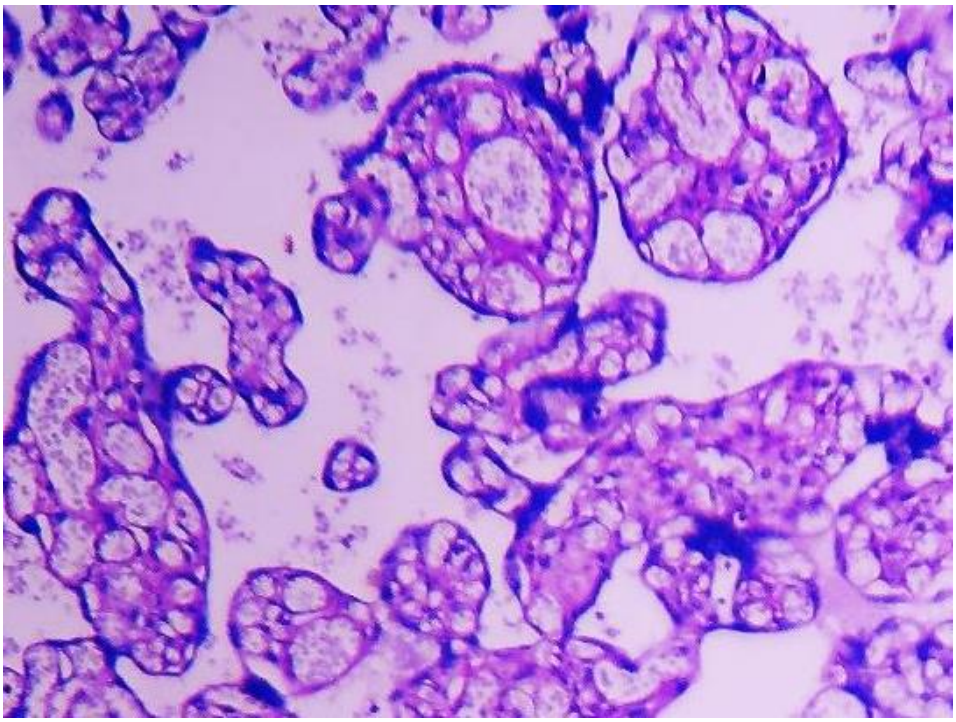


Figure 19 – Mild staining of basement membrane by PAS – 40x view

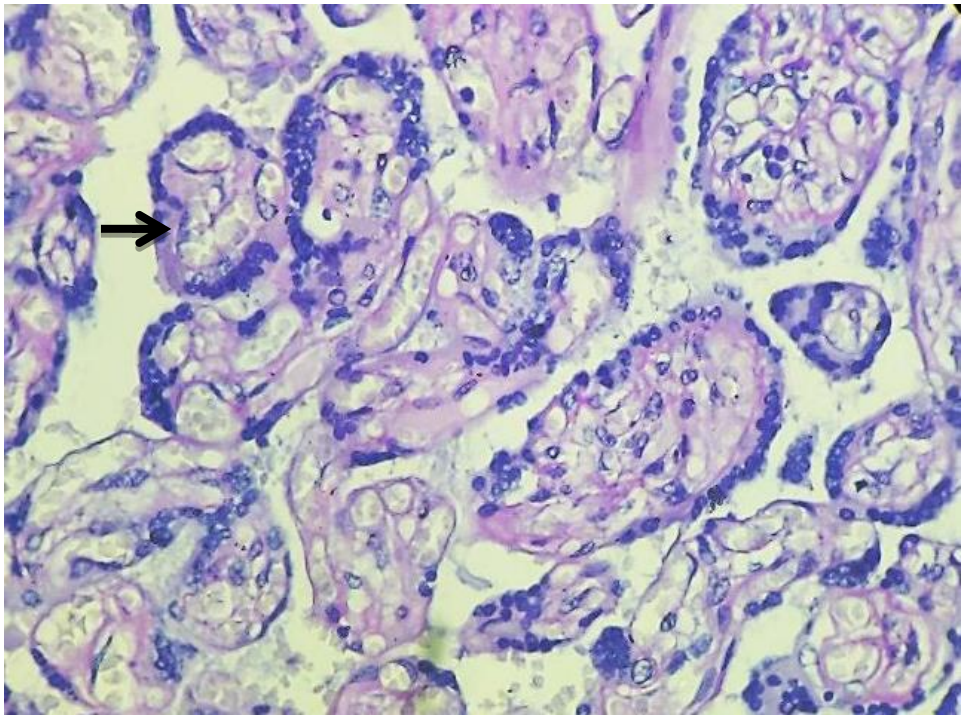


Figure 20 – Moderate staining of basement membrane by PAS – 40x view

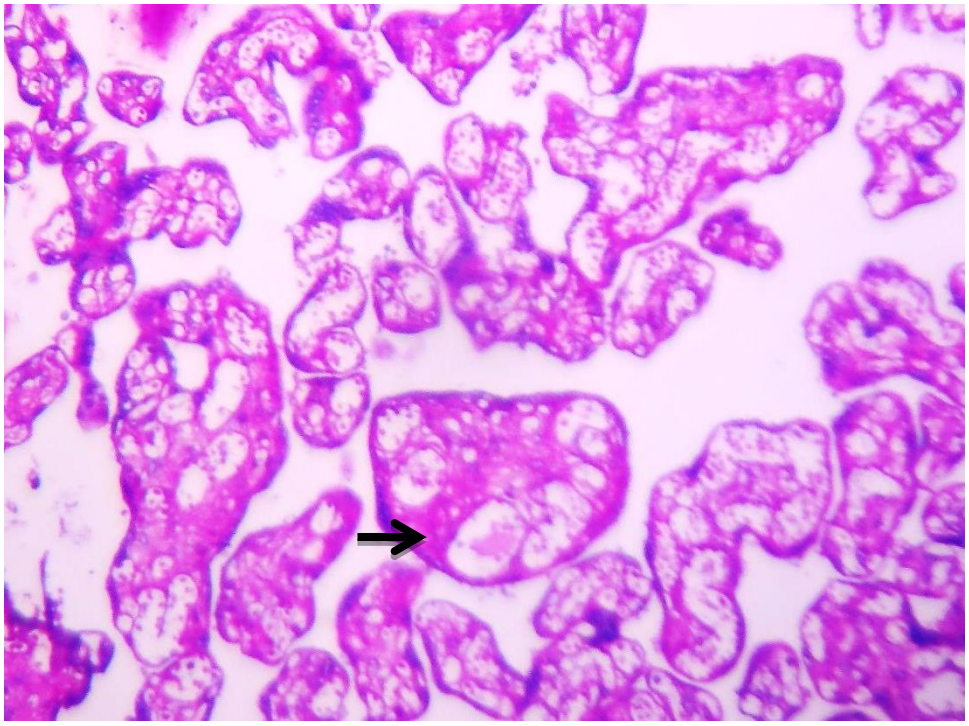


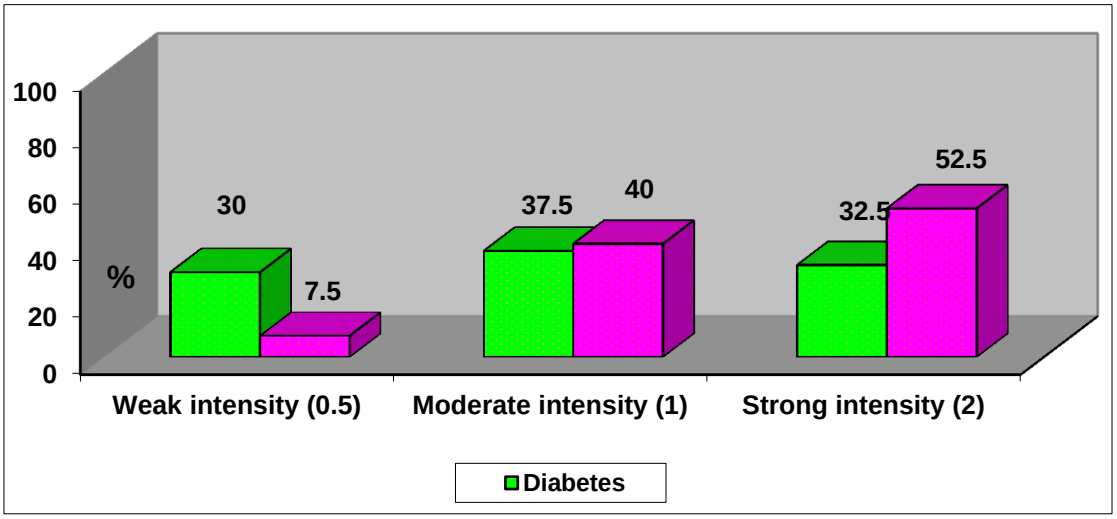
Figure 21 – Strong staining of basement membrane by PAS – 40x view

The following table[**Table no.10]** shows the distribution of cases amongst 2 groups with varying grades of intensity in trophoblastic cells.

Table no.10 – Distribution of VEGF in trophoblastic cells amongst 2 groups

			GROUP		
			DIABETES	NORMAL	Total
VEGF IN TROPHOBLAST	WEEK INTENSITY(0.5)	Count	12	3	15
		% within GROUP	30.0%	7.5%	18.8%
	MODERATE INTENSITY(1)	Count	15	16	31
		% within GROUP	37.5%	40.0%	38.8%
	STRONG INTENSITY(2)	Count	13	21	34
		% within GROUP	32.5%	52.5%	42.5%
Total		Count	40	40	80
		% within GROUP	100.0%	100.0%	100.0%

As shown in above table[**Table no.10],** in normal group maximum number of cases i.e. 21 cases showed strong positivity for VEGF as compared to 13 cases in diabetic group. None of the cases in both groups showed negative staining for VEGF.



Graph no. 16 – Distribution of cases in 2 study groups according to VEGF reactivity in trophoblast

As shown in above graph[**Graph no. 16],** maximum number of cases i.e. 52.5% cases in normal group showed strong positivity for VEGF in trophoblastic cells**(Figure 22)** while in diabetic group, maximum no. of cases i.e. 37.5% cases showed moderate positivity in trophoblastic cells**(Figure 23)**, weak positivity in the trophoblastic cells **(Figure 24)** were shown in 7.5% of normal cases and in 30% of diabetic cases, with no cases in both group showing negative staining for VEGF.

Next in this study, we studied VEGF expression in the fetal endothelial cells amongst the cases in 2 groups, the distribution of which is given in next table[**Table no. 11].**

Table no. 11- Distribution of cases amongst 2 study groups based on VEGF reactivity in fetal endothelial cells.

			GROUP		
			DIABETES	NORMAL	Total
VEGF IN ENDOTHELIAL CELLS	NEGATIVE (0)	Count	27	1	28
		% within GROUP	67.5%	2.5%	35.0%
	WEEK INTENSITY(0.5)	Count	8	1	9
		% within GROUP	20.0%	2.5%	11.3%
	MODERATE INTENSITY(1)	Count	1	16	17
		% within GROUP	2.5%	40.0%	21.3%
	STRONG INTENSITY(2)	Count	4	22	26
		% within GROUP	10.0%	55.0%	32.5%
Total		Count	40	40	80
		% within GROUP	100.0%	100.0%	100.0%

As Shown in above table**[Tableno.11]** 22 cases from the normal group showed strong staining for VEGF in the endothelial cells as compares to 4 cases in diabetic group. Maximum number of cases in diabetic group showed negative staining for VEGF in the endothelial cells.

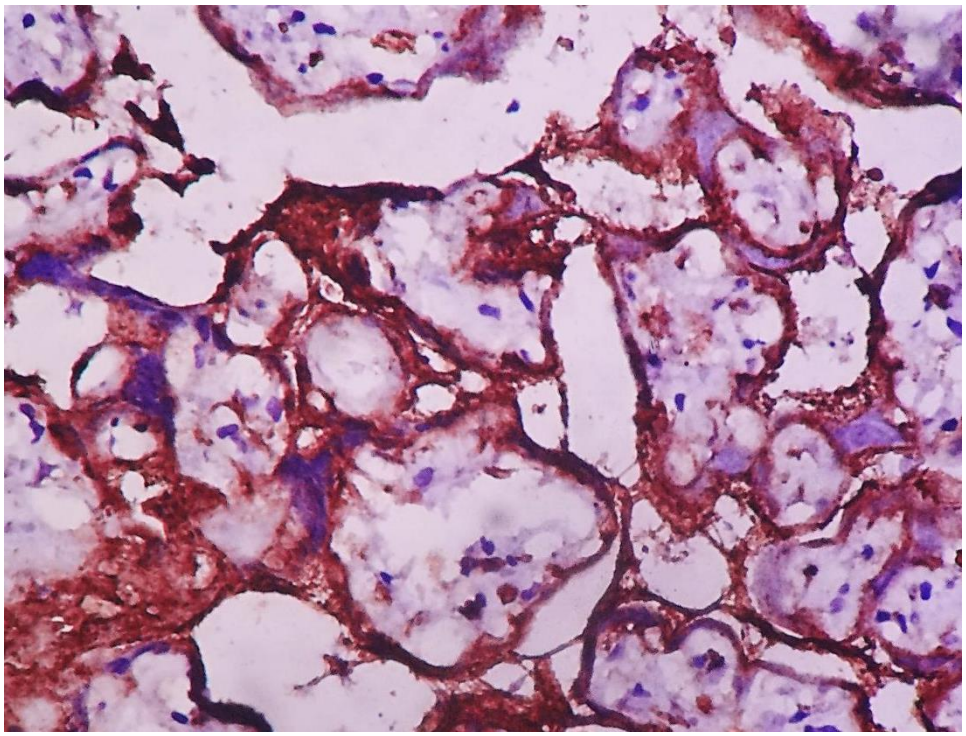


Figure 22 – Strong staining of VEGF in trophoblastic cells – 40x view

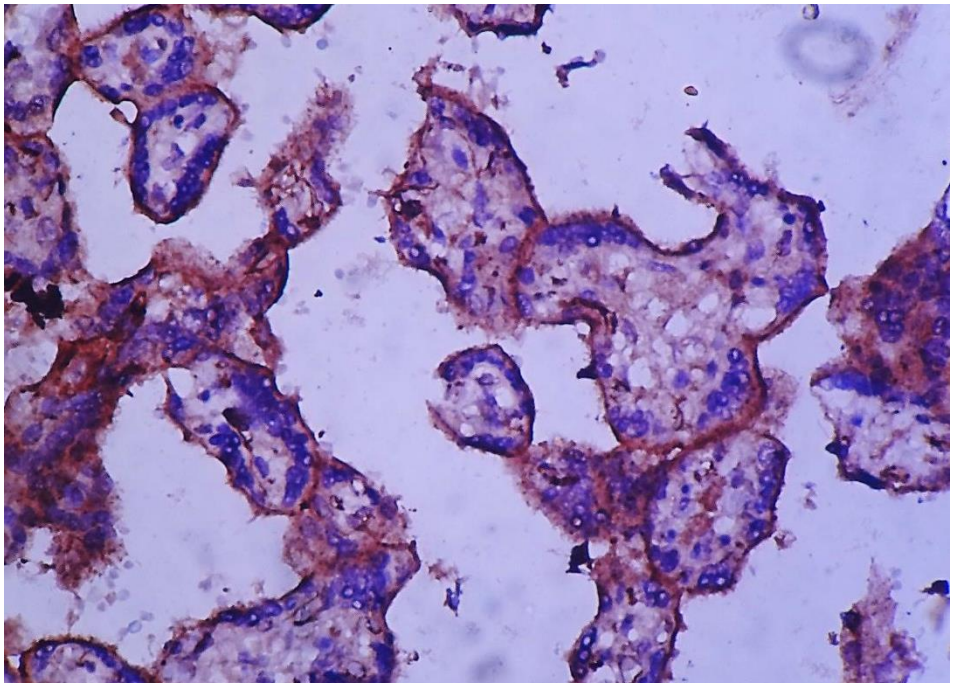


Figure 23 – Moderate staining OF VEGF in trophoblastic cells – 40x view

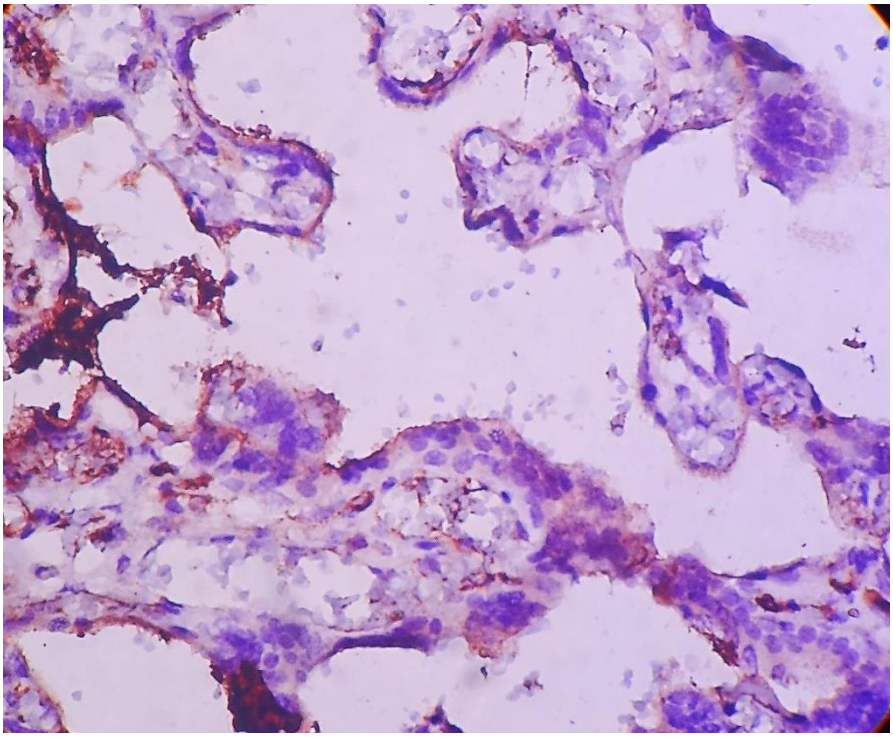
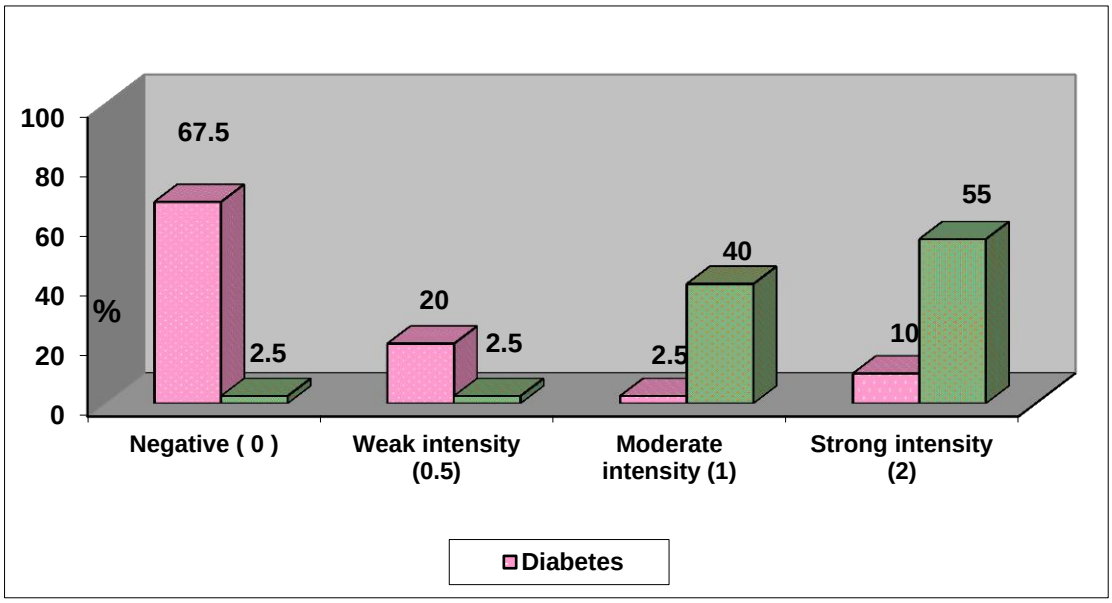


Figure 24 – Weak staining of VEGF in trophoblastic cells – 40x view



Graph no. 17 – Distribution of VEGF in fetal endothelial cells amongst the 2 study groups

As shown in above graph[**Graph no.17],** 55% cases in the normal group showed strong intensity for VEGF in the fetal endothelial cells**(Figure 25)**, with 40% cases showed moderate positivity**(Figure 26)**, 2.5% case showed weak positivity and 2.5% case showing negative staining for VEGF in fetal endothelial cells. As compared to normal group, in diabetic group only 10% cases showed strong positivity for VEGF in fetal endothelial cells with maximum number of cases i.e. 67.5%, showing negative staining, thus suggesting that the expression of VEGF is reduced in the endothelial cells in terminal villi of diabetic placentas.

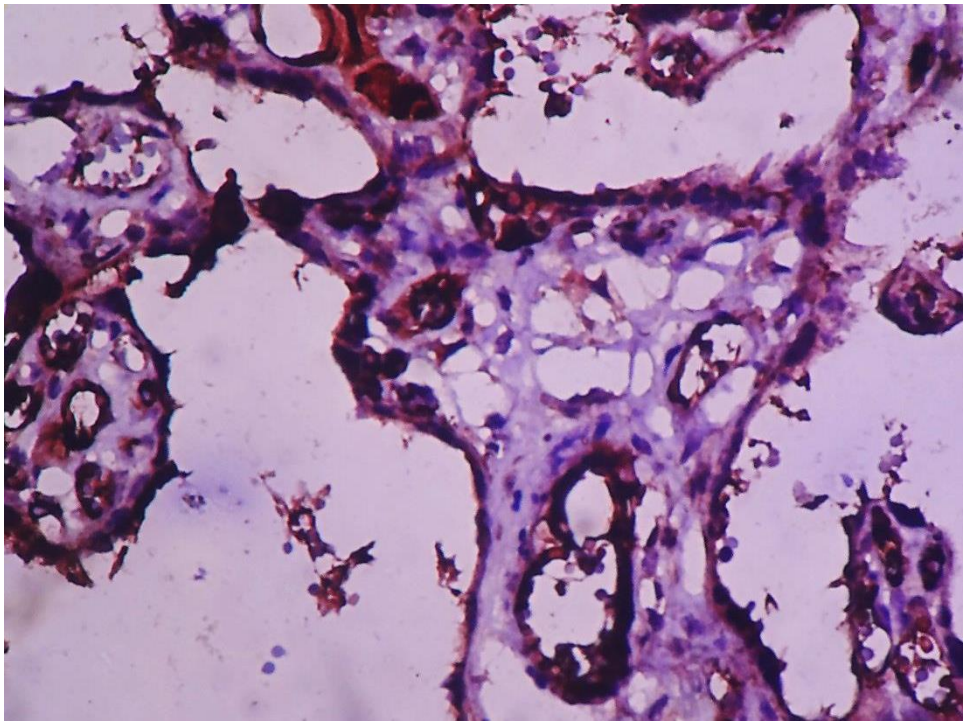


Figure 25 – Strong expression of VEGF in endothelial cells – 40x view

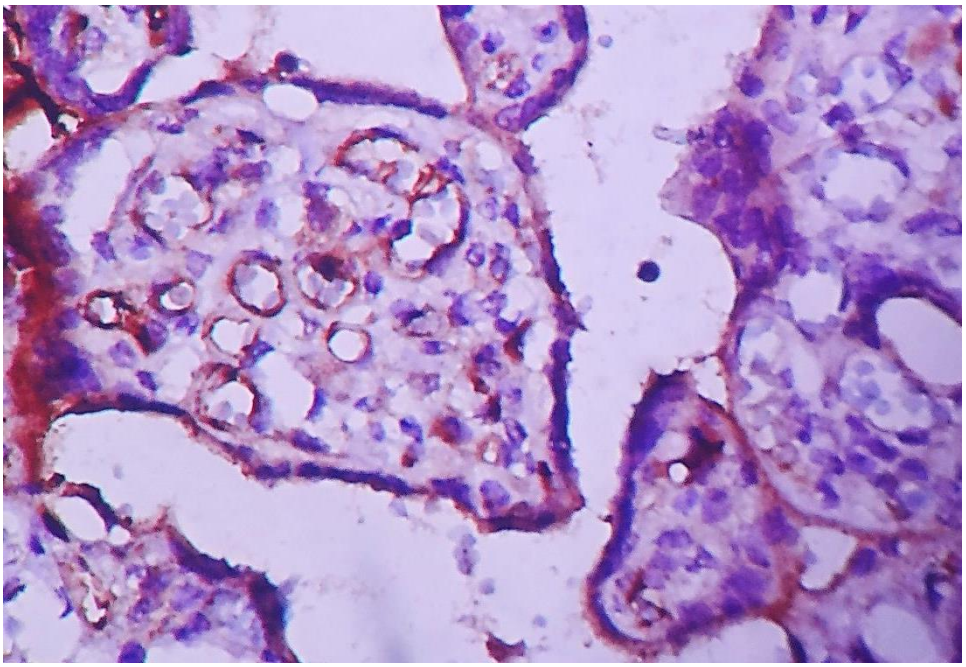


Figure 26 – Moderate intensity staining of VEGF in endothelial cells – 40x view

DISCUSSION

This prospective study was carried out in the Department of Pathology, Govt. Stanley Medical College, in collaboration with the Department of Obstetrics & Gynaecology, RSRM Govt. Stanley Hospital. A total of 80 specimens were taken for this study which included 40 placentas from diabetic mothers, gestational or overt, considered as study group and 40 placentas from non-diabetic mother, with no other pregnancy associated disorders like eclampsia, preeclampsia and anaemia considered as normal (control) group.

1) Age of the mother:

In our study the cases in both the groups showed age ranging from 19 to 38 years of age.

In the year 2004, Emmanuel Odar observed in his study that the age group at risk of getting gestational diabetes was between 20-39 years in 96.8% of cases.[59]

In our study 97.5% of cases in study group was between 20-39 years of age, with a mean age of 26.5 years.

2) Parity:

In the year 2003, A. Ben-Haroush, observed that high parity was one of the risk factor for GDM [92]. In the year 2004, a study done by Ma'asoumah A. Makhseed et al also revealed that the percentage of

multiparity was higher, with 60% of cases in impaired gestational glucose tolerance group.[93]

In our study 65% of cases in study group were multigravida.

3) Shape of placenta:

In 1951, Hamilton showed that the term placenta is circular to oval in outline and is determined by the form of villi left on chorionic sac.[30]

According to the study conducted by Muhammad Ashfaq in the year 2005, shape of the placentas in diabetic and non-diabetic group were roughly oval or round except one placenta which was bilobed.[94]

In our study 50% of cases in the diabetic group had round shape of placenta, and 40% had oval shape which was almost similar to the result we got for the normal group. In diabetic group we got 4 placentas i.e. 10% with an irregular shape, out of which 3 were having succenturiate lobe.

Placenta extrachorialis, which is a morphological abnormality of the placenta, is defined as "a condition in which the transition from a membranous to villous chorion occurs at some variable distance within the circumference of the placenta and not at the placental edge" and hence the basal plate is larger than the chorionic plate.[95]

Extrachorial placentas are of 2 types – Circum-marginate placenta and circumvallate placenta.

In Circum-marginate placenta the margins of the chorionic plate appears as a raised, thin fibrous rim where the fetal vessels appear to terminate.

Circumvallate placenta is a thickened membranous rim, which is composed of a double fold of chorion and amnion with fibrin and degenerated decidua in between, and is folded inward towards the centre.

According to a study done by Wilson et al in 1967 and by Kasturi Lal in the year 1973, extrachorial placenta is a serious clinical problem associated with increased incidence of antepartum and postpartum haemorrhage.[96, 97]

In diabetic group we got 2 circummarginate placenta **(Figure 27)** i.e. 5% cases but no complications were seen in the mother.

4) Site of cord insertion:

In our study there was not much difference in the distribution of site of insertion of umbilical cord between the two groups which was similar to a study done by Pathak et al in the year 2010 and by Soma Saha et al in the year 2014.[98,99]

But in the study conducted by Soma Saha et al, the most common site of insertion of umbilical cord was marginal observed in

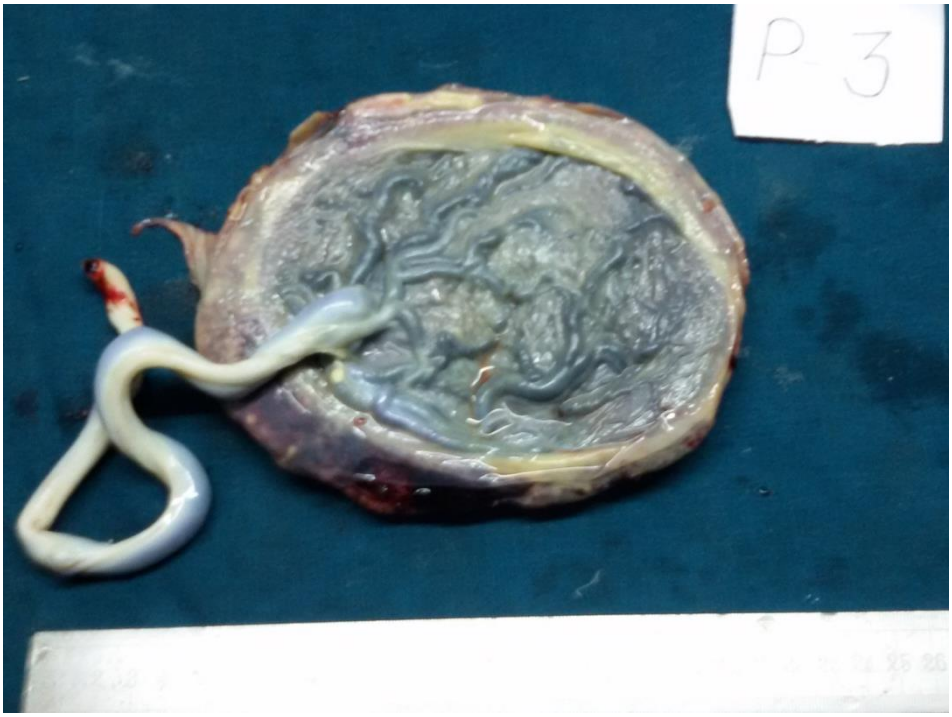


Figure 27 – Circum-marginate Placenta

33.8% of cases, while in our study it was central, observed in 35% of cases.

5) Weight of the placenta:

In our study, the minimum placental weight in diabetic group was 340gms, maximum was 800gms and the mean placental weight was 563.75±96.78gms, which showed a statistically significant difference with the mean weight of normal group which was 469.63±88.93gms.

Table no. 12 – Comparison of placental weight in different studies

S. NO	Studies	Mean placental weight(gms)±SD
1.	Soma Saha et al, 2014[99]	565.75±41.04
2.	Rafah Hady Lateef Al-Mamori 2014[100]	590.00
3.	Muhammad Ashfaq, 2005[94]	656
4.	Present study	563.75±96.78

A significant increase in the fetal and placental weights were found in the diabetic group compared to the normal group in a study conducted by Jauniauxa, and Burton in the year 2006.[101]

The increased placental weight in diabetes may be because of reactionary hyperglycemia in foetuses of diabetic mothers which leads to compensatory hyperplasia of the villous structure and fetal macrosomia. Another factor which leads to villous hyperplasia could be because of

vascular compromise sin diabetes mellitus which causes low oxygen tension in chorionic villous blood.[102]

Teasdale stated that the cause of heavier placenta in gestational diabetes is mainly because of significant accumulation of non-parenchymal tissue and a moderate increase in parenchymal tissue.[51]

6) Placental diameter, Circumference and Area:

In our study, the mean placental diameter, circumference and area of placentas in diabetic group was 18.15±1.59cms, 56.98±5.02cms and 258.87±44.76sq.cm respectively, which didn't show statistically significant difference with the normal group for which the placental diameter, circumference and area of placenta were 17.62±1.61cms, 55.390±5.05cms and 243.43±45.93sq.cm respectively.

Table no. 13 – Comparison of diameter, circumference and area of placenta in different studies

S.no	Study	Mean Diameter (cms) ±SD	Mean Circumference (cms) ±SD	Mean area (sq.cm) ±SD
1.	Ma'asoumah A. Makhseed et al, 2004[93]	17.79±2.09	66.48±6.80	278.5±53.8
2.	Soma Saha et al, 2014[99]	16.66±1.18	52.32±3.7	219.65±31.34
3.	Present study	18.15±1.59	56.98±5.02	258.87±44.76

7) Central thickness of the placenta:

In our study the mean central thickness of placenta in diabetic group was 2.51±0.57cms while in the normal group it was only 1.79±0.37cms. The difference in the central thickness between the two groups was statistically significant.

Table no.14 – Comparison of mean central thickness of placenta in various studies

S.no.	Study	Mean central thickness (cms)±SD
1.	Ma'asoumah A. Makhseed et al, 2004 [93]	1.90±0.42
2.	Soma Saha et al, 2014[99]	3.15±0.4
3.	Present study	2.51±0.57

The thickness of the placenta depends mainly on the length of stem villi.

8) Birth weight of baby:

Weight of the newborn baby depends directly on the environment it experienced during the intrauterine life. In case of gestational diabetes mellitus, glucose crosses the placental barrier and causes fetal hyperglycaemia which in turn stimulates the pancreatic islet cells and leads to fetal hyperinsulinemia, and as insulin itself is an anabolic hormone.

In our study, the minimum birth weight of the newborn in diabetic group was 2.500 kg, while maximum birth weight was 3.700 Kg with a mean birth weight of 3.143±0.35kg. In control group, the minimum birth weight of the newborn was 2.090 Kg, maximum was 3.493 Kg with a mean birth weight of 2.824±0.36Kg. The difference in the birth weight of 2 group was statistically significant in our study.

Table no. 15 – Comparison of mean birth weight of newborn in different studies

S.no.	Study	Mean birth weight of newborn(Kgs)±SD
1.	Ma'asoumah A. Makhseed et al, 2004 [93]	3.56±0.61
2.	Sanjoy kumar, 2010[103]	3.26±0.40
3.	Present study	3.14±0.35

9) Fetal/placental ratio:

The ratio of weight of newborn fetus and placenta is called as fetal/placental ratio. The normal fetal/placental ratio is 4:1 to 6:1.

In our study the mean value of fetal/placental ratio in diabetic group was 5.68±0.75 and there was a statistically significant difference from that of normal group.

Table no. 16 – Comparison of fetal/placental ratio in different studies

S.no.	Study	fetal/placental ratio
1.	Soma Saha et al, 2014[99]	5.80±0.33
2.	Rafah Hady Lateef Al- Mamori, 2014[100]	5.81
3.	Present study	5.68±0.75

10) Light microscopic findings:

In the present study, various parameters like synctial knots, villous edema, villous fibrosis, fibrinoid necrosis and presence of chorangiosis were noted on light microscopic examination using H&E stain.

Increased syncytial knots:

The villous tree is covered by syncytiotrophoblastic cells which shows considerable variation in thickness, structure and distribution of nuclei. These syncytiotrophoblasts are arranged in a mosaic like pattern with extremely thin anuclear areas called as epithelial plate and accumulation of nuclei called as syncytial knots. Increased number of syncytial knots, bridges and sprouts are called as **Syncytial knotting or Tenny-Parker changes.** These features of increased syncytial knotting should be interpreted with care because they are influenced by the thickness of the sections.

Burstein et al in the year 1963, observed that placentas from the diabetic patients had marked increase in syncytial knotting.[104]

Various studies had revealed that the two dimension view does not always reflect the original three dimensional structure which exist in vivo. Kustermann in 1981 showed that most of the nuclear accumulation or the syncytial knots are nothing but the flat sections of irregularly shaped villous surface by using the reconstruction of serial paraffin sections which was corroborated by Burton(1986a,b), who used plastic serial sections and Cantle et al(1987), who compared the scanning electron microscopy and light microscopic findings of villous sections[105,106,107, 108]. Thus an increased incidence of syncytial knotting points towards increased bulging and branching of the villi which leads to abnormal villous shape and is interpreted as sectional artifact by Kustermann and others.

In the year 1987, Kaufmann et al mentioned in their study that despite the interpretation of increased synctial knots as an artifact, the diagnostic value of it was still useful as it points towards a characteristic deformation of the terminal villi and is usually caused by abnormal placenta oxygenation that leads to abnormal villous angiogenesis.[109]

In a study, done by Vineeta Tewari et al in the year 2011, as compared with the normal placenta, placentas from the diabetic mothers showed an increased syncytial knots in 80% of cases.[62]

In the year 2012, Lavinia Gheorman et al studied 19 cases of pregnant women with diabetes and revealed an increased incidence of syncytial knots in these cases.[63]

In 2014, Rafah Hady Lateef Al-Mamori in his study showed that there was an increased number of syncytial knots in the terminal villi in the placentas of diabetic mothers controlled byinsulin.[100]

In the present study, we find an increased incidence of synctial knots in placentas of diabetic patients as compared to normal group and it was statistically significant.

Villous edema:

Villous edema is defined as accumulation of fluid in the interstitium of the villi with disruption and replacement of intravillous cellular architecture. As hyaluronic acid molecules have the property to retain water, it was concluded that, the presence of abnormal deposits of mucopolysaccharides in the villous stroma can lead to the appearance of the true villous edema in placentas of diabetic mothers.[110]

In the year 1994, Majid S. Al-Okail et al mentioned in his study that villus oedema was slightly observed in well controlled diabetic placentas but it was very clearly observed in gestational diabetic placentas.[56]

In the year 2011, Vineeta Tewari et al, In 2012, Lavinia Gheorman et al, and in 2014, Rafah Hady Lateef Al-Mamori in their respective studies showed that there was an increased incidence of villous edema in placentas of diabetic patients as compared to normal patients.[62,63,100]

In a study done by Soad A. Treesh et al in the year 2015 on 13 placentas of diabetic mothers, showed focal distribution of villous edema on the distal villi in most studied cases.[111]

In the present study, we found an increased incidence of villous edema in placentas of diabetic patients as compared to normal group and it was statistically significant.

Villous fibrosis:

Fibrosis of the stem villi is a normal phenomenon in the placenta and it is a good indicator of placental maturation. Fibrosis usually starts at about 15th week postmenstruation, usually around the stem vessels and completes a few weeks before term. Stromal fibrosis is considered abnormal when it is not restricted to the stem villi. It has been speculated that, in diabetic patients there is an increased villous stromal oxygen partial pressure, in the face of inadequate uptake by the fetal capillaries, which stimulates the synthesis of collagen.[112]

In the year 2010, Verma R et al noticed increased villous fibrosis in GDM controlled by insulin, but such observation was not observed in GDM controlled by diet and in control patients.[61]

In the year 2011, Vineeta Tewari et al observed in their study an increase in villous fibrosis in 60% of diabetic cases.[62]

In the year 2012, Lavinia Gheorman et al noticed an increase in the incidence of villous fibrosis in 47% of diabetic cases.[63]

In the year 2015, Soad A. Treesh et al also mentioned in their study, an increase in the incidence of villous stromal fibrosis which was demonstrated with the help of Masson Trichrome stain.[111]

In the present study, we find an increased incidence of villous fibrosis in placentas of diabetic patients as compared to normal group and it was statistically significant.

Fibrinoid necrosis:

Fibrinoid is a non-cellular homogenous eosinophilic material seen in placenta. It is divided into 2 types - perivillous fibrinoid and intravillous fibrinoid.[113]

Perivillous fibrinoid is mostly a blood clotting product and has a lamellar structure. It is usually found in defects of the villous trophoblastic cover. It is considered to be a normal phenomenon which occurs in all placentas and the amount of fibrinoid increases with advancing pregnancy. Usually these perivillous fibrinoid necrosis has no pathological significance.[113]

Intravillous fibrinoid is also referred as fibrinoid necrosis and is a fibrinoid patch that replaces the villous stroma predominantly, the chorionic villi.[113]

In the year 2010, Verma R et al noticed an increased incidence of fibrinoid necrosis in GDM controlled by insulin and GDM controlled by diet but not in control patients.[61]

In the year 2011, Vineeta Tewari et al noticed an increase in intravillous fibrinoid necrosis in 80% of diabetic cases.[63]

In the year 2012, Lavinia Gheorman et al noticed an increased incidence of fibrinoid necrosis in 47% of diabetic cases.[64]

In 2015, Soad A. Treesh et al noted an increase in both extravillous and intravillous fibrinoid necrosis. Intravillous fibrinoid appearing in the subtrophoblastic space which finally occupies the whole villous stroma.[111]

In the present study, we find an increased incidence of both intravillous and perivillous fibrinoid necrosis in placentas of diabetic patients as compared to normal group and it was statistically significant.

Chorangiosis:

In the year 1958 Hormann, coined the term "chorioangiosis" [114], But it was Altshuler who did a detailed study on this entity in the year 1984, and diagnosed this entity mainly by low power examination of the

histological sections. He mentioned that "when a low power examination, showed 10 villi, each with 10 or more vascular channels in ten or more non infarcted and non ishemic zones of at least three different placental areas" the term chorangiosis can be used.[115]

In cases of uncontrolled diabetes, placental venous congestion is very prominent and if not careful, it may mask the lesion of chorangiosis, but there is an obvious increase in number of vessels per villus in cases of chorangiosis.

Earlier it was considered that chorangiosis was significantly associated with perinatal death and congenital anomalies as studied by Keenan & Altshuler in 1975[116], and was thus considered to be an important signal for scrutiny particularly in cases of placentomegaly. However most of the fetuses whose placentas were showing diffuse chorangiosis were not affected.

In the year 2012, Lavinia Gheorman et al noticed in their study, an increased incidence of chorangiosis in diabetic cases.[63]

In the present study we noted chorangiosis in 6 out of 40 diabetic cases while no cases of chorangiosis were seen in normal group. We did not encountered any congenital anomaly or perinatal death in 6 cases which we reported as chorangiosis.

11) Glycogen deposition & PAS

In routine sections of the placenta stained with H&E, it is difficult to see the trophoblastic basement membrane of a chorionic villi and hence either special stain like PAS or immunohistochemistry for collagen IV or laminin should be applied.

Liebhart, in the year 1971, noted that there was marked thickening of basement membrane in diabetic placentas. The reason for this thickening of basement membrane was probably because the secretory products of trophoblastic cells constitute the basal lamina.[117]

In the year 1987, Iioka et al studied that, as compared to normal pregnancies, the basement membrane of the diabetic placenta may be thicker because of higher degree of non-enzymatic glycosylation.[118]

A finding which was seen in placentas of diabetic patient was an increased deposition of glycogen, which was noted by Desoye et al in the year 1992and which was in contrast to what we see in all the other organs of diabetic patients.[119]

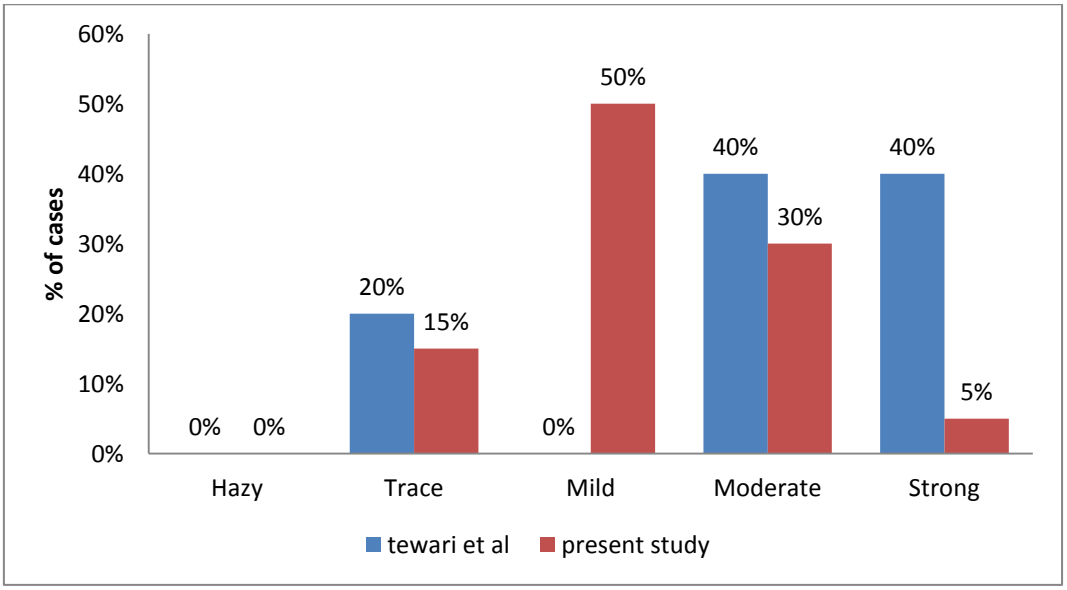
All the above studies were having qualitative data which showed an increase in the basement membrane thickness probably because of increased glycogen deposition.

The only study in the literature which has given descriptive analysis of the PAS staining, was that of Tewari et al in the year 2011. [62]

In the present study, we have also quantified our data for PAS staining into 5 categories and following is the table**[Table no. 17]**for comparison.

Table no. 17 – Comparison of intensity of PAS staining between 2 studies

		Hazy	Trace	Mild	Moderate	Strong
Tewari etal[62]	Diabetic	0%	20%	0%	40%	40%
	Normal	40%	40%	20%	0%	0%
Present study	Diabetic	0%	15%	50%	30%	5%
	Normal	42.5%	45%	12.5%	0%	0%



Graph no. 18 – Comparison of 2 studies depending upon the intensity of PAS staining in diabetic group

The above graph[**Graph no.18]** shows the comparison between the diabetic group in study done by Tewari et al and present study. In both the studies none of the diabetic cases showed hazy positivity, 20% of diabetic cases showed trace positivity in the study done by Tewari et al while the present study showed 15% of diabetic cases with trace positivity. In the study done by Tewari et al maximum number of diabetic cases showed moderate and strong staining in 40% of cases each, while in the present study maximum number of diabetic cases i.e. 50% showed mild staining, while 30% diabetic cases showed moderate and only 5% of diabetic cases showed strong staining.

In the year 2015, a qualitative study was done by Soad A. Treesh et al who also used PAS staining and showed a marked thickening of the basement membrane in diabetic placentas.[111]

The accumulation of glycogen in the placentas of diabetic mothers occurs in marked contrast to other tissues, such as maternal liver, from which glycogen disappears. Glycogenesis and glycogenolysis occurring in the muscle and the liver are under the control of insulin, which regulate the activity of phosphorylase and glycogen synthase. However, in diabetic mothers the glycogen accumulation in the placenta is not dependent on insulin and is related to the extent of maternal hyperglycemia. The increased capacity of placental cells for glucose uptake in diabetes could be related to the expression of GLUTs (glucose carrier transporter isoforms), especially GLUT1[62]

In the present study only few cases showed strong and moderate staining in diabetic group, as compared to the study done by Tewari et al but the difference between the diabetic group and control group was significant and thus present study showed there was basement membrane thickening in diabetic group because of increased glycogen deposition.

12) VEGF expression:

Vascular disorders of any type has the capacity to change the placental function and hence can compromise the development of foetus[120,121].In diabetic mother, there is an increase in circulating concentration of glucose, that alters the metabolism of placental lipids, carbohydrate and protein, simultaneously because of reduced pancreatic function, there is a decrease in circulating levels of insulin which can adversely affect the metabolism in the foetus[122]. A rise in blood glucose concentration promotes atherosclerosis which may impair the uteroplacental circulation and can lead to ischemia and hypoxia, creates excessive syncytial knots and can lead to endothelial dysfunction.[123]

In the year 1996, Carmeliet et al observed in their study that VEGF and its receptors are essential for the development of embryonic vasculature as embryonic death can result from the loss of even a single VEGF allele[124]. VEGF is a potent inducer of endothelial cell proliferation, activation and migration.

The following table[**Table no.18]** shows the result of study done by Satu Helske et al in the year 2001, who compared the VEGF expression in placenta of normal and diabetic patients.[88]

Table no.18 – Result of study done by Satu Helske et al[88]

	Negative	Light	Moderate	Strong
Normal	0%	50%	50%	0%
Diabetic	0%	62%	13%	25%

The above study is difficult to compare with the present study as Satu Helske at el had not clearly mentioned in their study about the sites of expression of VEGF but had mentioned that the strongest reactivity for VEGF was located in the endothelial cells of the villous capillaries.

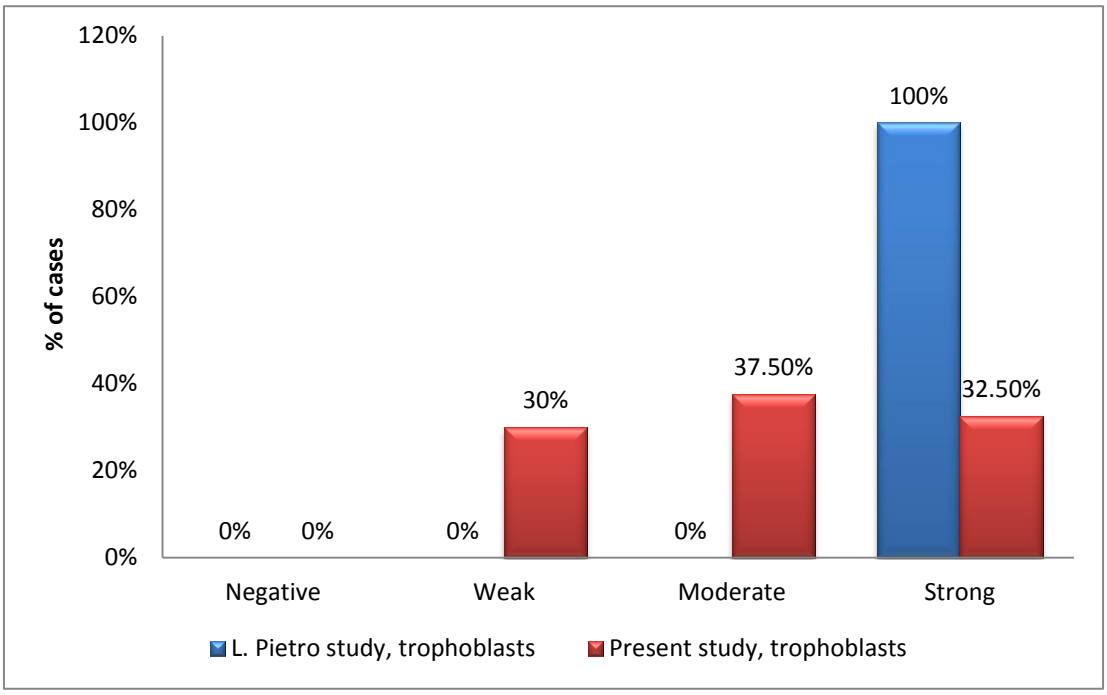
In the present study we have considered endothelial cells of fetal capillaries and trophoblastic cells for the expression of VEGF using immunohistochemistry and for each site we have given an intensity score.

In the year 2010, L. Pietro et al studied VEGF and its receptor expression in normal and hyperglycaemic patients [90]. The following table**[Table no. 19]** shows the comparison of L. Pietro study and the present study.

Table no. 19 – Comparison of VEGF expression between study done by L. Pietro and present study

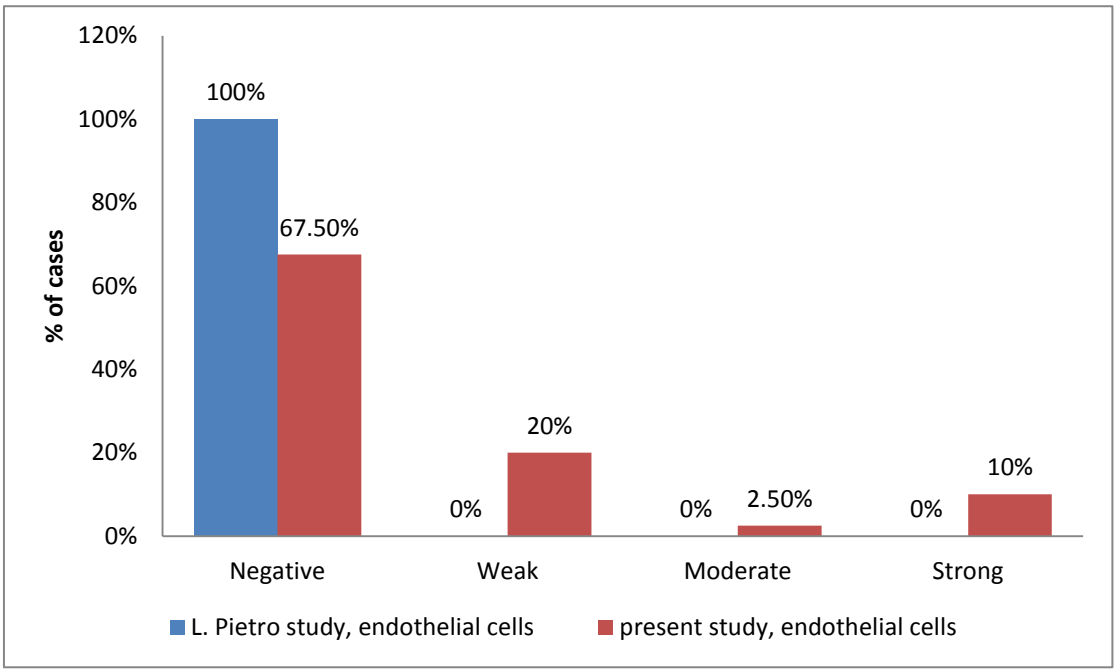
	Site of expression	Negative	Weak	Moderate	Strong
L. Pietro[90]	Trophoblast	0%	0%	0%	100%
	Fetal endothelial cells	100%	0%	0%	0%
Present study	Trophoblast	0%	30%	37.5%	32.5%
	Fetal endothelial cells	67.5%	20%	2.5%	10%

In the present study the expression of VEGF was reduced at both the sites in diabetic patients with the trophoblastic cells showing strong positivity in 32.5% of cases, and the fetal endothelial cells showing strong positivity in only 10% of cases.



Graph no. 19 – Comparison of L. Pietro study and present study for VEGF expression in trophoblasts in diabetic group

The above graph**[Graph no. 19]** shows the comparison of expression of VEGF in the trophoblasts in a study done by L. Pietro et al and the present study, which showed 100% cases showing strong positivity for VEGF in trophoblastic cells as compared to the present study which showed only 32.5% of cases showing strong positivity, 37.5% of cases showing moderate positivity and 30% cases showing weak positivity. None of the cases in both the studies showed negative staining for VEGF in trophoblasts.



Graph no. 20 - Comparison of L. Pietro study and present study for VEGF expression in fetal endothelial cells

The above graph**[Graph no. 20]** shows the comparison of expression of VEGF in the fetal endothelial cells in the study done by L. Pietro et al and the present study, which showed 100% cases showing negative staining for VEGF in the fetal endothelial cells. The present study showed 67.5 % of cases showing negative staining for VEGF, 20% showing weak positivity, 2.5% showing moderate positivity and 10% cases showing strong positivity.

Apart from the fetal capillary endothelial cells and trophoblastic cells, few cases from both the groups showed weak staining of the villus mesenchymal cells, some previous studies had also mentioned about the staining of the villus mesenchymal cells.

The major problem of the comparison between the study done by L. Pietro et al and the present study was that, the sample size for L.Pietro study was very small and hence had low sensitivity.

The difference in the expression of VEGF in the present study between the 2 groups might be because of hyper- or hypoglycaemia, which is reported to cause dysregulation of angiopoietins expression [125]. This dysregulation of angiogenesis in diabetes might be present only in non-compensated patients with severely affected metabolic status[126] and hence few cases showed strong intensity for VEGF in trophoblastic and endothelial cells while other cases showed mild or even negative staining.

SUMMARY AND CONCLUSION

This prospective study was carried out in the Department of Pathology in collaboration with the Department of Obstetrics and Gynaecology over a period of one year from September 2014 to August 2015, after obtaining the approval from the Institutional Human Ethical Committee of Government Stanley Medical College, Chennai.

Total of 80 cases were included in the study which includes 40 cases with gestational or overt diabetes and 40 cases with normal pregnancy. In this study we compared the placentas of 2 groups on the basis of gross features, histopathological features, glycogen deposition with the help of PAS stain and expression of VEGF by using immunohistochemical technique as well as the fetal weight in both the groups.

The following are the observations:-

- There is an increased incidence of gestational diabetes in the age group of 20-39 years, with a mean age of 26.5 years.
- Multiparous women have been found to be more prone for gestational diabetes mellitus as compared to the primigravida.
- The predominant shape of the placentas in the diabetic group is round, with 2 cases of circummarginate placenta while in normal group it is oval with 3 cases showing succenturiate placenta.

- The most common site of cord insertion is central in diabetic group while it is moderately eccentric in normal group.

- The placentas of diabetic mothers are more heavier and more thicker than the placentas of normal pregnancy.

- The values of the diameter, circumference and areas of placentas in both groups did not show much difference.

- The newborn of diabetic mothers are heavier than that of normal non-diabetic mothers.

- Histopathologically most of the diabetic cases shows an increase in syncytial knots, villous fibrosis, villous edema and fibrinoid necrosis as compared to the normal placenta, with few of the diabetic cases showing features of chorangiosis.

- There is an increase in glycogen deposition in placentas of diabetic mothers with basement membrane thickening as demonstrated by PAS staining, with 15% of diabetic cases showing trace staining, 50% showing mild staining, 30% showing moderate staining and 5% cases showing strong staining.

- There is reduced expression of VEGF in the fetal endothelial cells and trophoblastic cells in placentas of diabetic mother as compared to the normal placentas.

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ANNEXURE I

PROFORMA

AGE OF THE MOTHER:

PARITY:

NORMAL: ☐ DIAEBTIC: ☐

IF DIABETIC:

ON INSULIN: ☐ ON MEAL PLAN: ☐

FBS ON DAY OF DELIVERY:

PP2BS ON DAY OF DELIVERY:

VAGINAL DELIVERY: CESAREAN SECTION:

WEIGHT OF THE BABY:

SEX OF THE BABY:

சுயஒப்புதல் படிவம்

நீரழி வு நோயாளிகள் நஞ்சுகொடியினை திசு பரிசோதனைக்கு உட்படுத்தி நீரழிவு நோயினால் கருவிற்கு ஏற்படும் விளைவுகளைக் கண்டறிதல்.

ஆராய்ச்சிநிலையம்:நோய்க்குறியியல்துறை

ஸ்டான்லி மருத்துவக் கல்லூரி

சென்னை-600001.

பங்கு பெறுபவரின் பெயர் :

பங்கு பெறுபவரின் எண் :

மருத்துவ ஆய்வின்விவரங்கள் எனக்குவிளக்கப்பட்டுள்ளது.

எனது ஆய்வுபற்றிய சந்தேகங்களை கேட்கவும் அதற்கான தகுந்த விளக்கங்களைப் பெறவும்

வாய்ப்பளிக்கப்பட்டது.

நான் எனது இரத்தமாதிரிகள் மற்றும் நஞ்சுகொடியினை பரிசோதனைக்கு உட்படுத்த முழுமனதுடன் சம்மதிக்கிறேன்.எக்காரணத்தாலும் எந்த கட்டத்திலும் எந்த சாட்சிகளுக்கும் உட்படாமல் நான் இவ்வாய்வில் விலகிக்கொள்ளலாம் என்றும் அறிந்து கொண்டேன்.

இந்த ஆய்வு மூலம் கிடைக்கும் தகவல் களையும் பரிசோதனை முடிவுகளையும் மருத்துவர்மேற் கொள்ளும் ஆய்வில்பயன்படுத்தி கொள்ளவும் அதை பிரசுரிக்கவும் நான் முழுமனதுடன் சம்மதிக்கிறேன்.

எனக்கு கொடுக்கப்பட்ட அறிவுரைகளின்படி நான் நடந்து கொள்வதுடன் இந்த ஆய்வுமேற் கொள்ளும் மருத்துவருக்கு உண்மையுடன் இருப்பேன் என்றும் உறுதி அளிக்கிறேன்.

பங்குபெறுபவரின் பெயர்மட்டும்,விலாசம் :

பங்குபெறுபவரின் கையொப்பம் :

இடம் :

நாள் :

கட்டைவிரல் ஒப்பம் :

தகவல் படிவம்

தங்களது இரத்த மாதிரிகள் மற்றும் நஞ்சுகொடியினை பரிசோதனைக்கு உட்படுத்தி நீரழிவு நோயினால் கருவிற்க்கு ஏற்படும் விளைவுகளை கண்டறிய ஆய்வு மேற்கொள்ளப்பட உள்ளது. இதில் தங்களது நோய் குறித்த விவரங்கள் இதர ஆராய்ச்சி முடிவுகளை தங்கள் சம்மதத்துடன் இவ்வாய்வில் பயன்படுத்தவிரும்புகிறோம்.பின்னாளில் மீண்டும் ஆய்வில் பங்கேற்க்கவும் தங்கள் முழுசம்மதம் பெற்ற பின்னர் மட்டும் மேற்கொள்ளப்படும்.

தாங்கள் விரும்பினால் இவ்வாய்வில் இருந்து எப்பொழுது வேண்டுமானாலும் விலகிக் கொள்ளலாம்.

இவ்வாய்வின்மூலம் கிடைக்கும் தகவல்களும்,பரிசோதனை முடிவுகளும் தங்களின் ஒப்புதல் மூலம் மட்டுமே ஆய்வில் பயன்படுத்தப்படும்.

ஆய்வாளரின்பெயர் :

ஆய்வாளரின்கையொப்பம் :

இடம் :

நாள் :

MASTER CHART

S. NO.	AGE	PARITY	SHAPE	CORD	PLACENTAL WEIGHT	CENTRAL THICKNESS	DIAMETER	CIRCUMFERENCE	AREA	BABY WT.	FETAL/PLACENTAL RATIO	N/DM
1	23	G2P2L1	OVAL	CENTRAL	390 gms	1.1 cm	17.0 cm	53.38 cm	223.725 sq.cm	2.370 Kg	6.07:1	N
2	27	G1P1	OVAL	HIGHLY ECCENTRIC	540 gms	1.4 cm	20.0 cm	62.80 cm	306.935 sq.cm	2.830 Kg	5.24:1	D
3	19	G1P1	OVAL	MODERATELY ECCENTRIC	440 gms	1.1 cm	15.0 cm	47.10 cm	173.48 sq.cm	2.695Kg	6.12:1	D
4	26	G1P1	ROUND	MODERATELY ECCENTRIC	520 gms	1.3 cm	19.0 cm	59.66 cm	278.478 sq.cm	3.040 Kg	5.84:1	N
5	23	G1P1	ROUND	MODERATELY ECCENTRIC	530 gms	1.6 cm	18.0 cm	56.52 cm	253.550 sq.cm	3.045 Kg	5.74:1	N
6	19	G3A2L0	ROUND	MODERATELY ECCENTRIC	600 gms	1.5 cm	18.0 cm	56.52 cm	253.555 sq.cm	3.430 Kg	5.71:1	N
7	25	G2P2L1	ROUND	HIGHLY ECCENTRIC	440 gms	1.3 cm	16.5 cm	51.81 cm	211.950 sq.cm	2.980 Kg	6.77:1	N
8	28	G4P4L3	OVAL	CENTRAL	530 gms	2.2 cm	17.7 cm	55.73 cm	229.610 sq.cm	3.045 Kg	5.74:1	N
9	19	G1P1	OVAL	MARGINAL	430 gms	1.9 cm	14.7 cm	46.32 cm	153 .075 sq.cm	2.515 Kg	5.84:1	N
10	23	G2P2L1	ROUND	CENTRAL	750 gms	2.5 cm	18.3 cm	57.46 cm	262.504 sq.cm	3.493 Kg	4.65:1	N
11	37	G2P2L1	ROUND	HIGHLY ECCENTRIC	450 gms	1.5 cm	16.3 cm	51.03 cm	206.847 sq.cm	2.815 Kg	6.25:1	N
12	20	G1P1	OVAL	HIGHLY ECCENTRIC	480 gms	2 cm	15.3 cm	47.89 cm	176.625 sq.cm	2.890 Kg	6.02:1	N
13	20	G1P1	OVAL	CENTRAL	440 gms	1.6 cm	19.7 cm	62.01 cm	303.795 sq.cm	3.195 Kg	7.26:1	N

S. NO.	N/DM	VILLOUS EDEMA	VILLOUS FIBROSIS	INC. SYNCTIAL KNOT	FIBRINOID	CHORIOANGIOSIS	PAS	VEGF IN TROPHOBLAST	VEGF IN ENDOTHELIAL CELLS
1	N	0%	0%	15%	1%	ABSENT	HAZY	MODERATE INTENSITY(1)	STRONG INTENSITY(2)
2	D	8%	12%	45%	12%	PRESENT	MODERATE	STRONG INTENSITY(2)	MODERATE INTENSITY (1)
3	D	10%	14%	55%	10%	ABSENT	MODERATE	STRONG INTENSITY(2)	STRONG INTENSITY(2)
4	N	7%	5%	10%	4%	ABSENT	HAZY	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
5	N	4%	2%	18%	1%	ABSENT	TRACE	STRONG INTENSITY(2)	STRONG INTENSITY(2)
6	N	3%	4%	10%	5%	ABSENT	HAZY	STRONG INTENSITY(2)	MODERATE INTENSITY (1)
7	N	8%	2%	15%	7%	ABSENT	TRACE	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
8	N	1%	1%	15%	1%	ABSENT	TRACE	STRONG INTENSITY(2)	STRONG INTENSITY(2)
9	N	1%	1%	30%	3%	ABSENT	TRACE	STRONG INTENSITY(2)	STRONG INTENSITY(2)
10	N	4%	5%	25%	5%	ABSENT	MILD	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
11	N	2%	2%	15%	5%	ABSENT	TRACE	STRONG INTENSITY(2)	STRONG INTENSITY(2)
12	N	2%	1%	20%	7%	ABSENT	TRACE	WEAK INTENSITY(0.5)	MODERATE INTENSITY (1)
13	N	1%	5%	20%	8%	ABSENT	TRACE	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
14	N	20%	1%	5%	1%	ABSENT	TRACE	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
15	N	1%	1%	40%	1%	ABSENT	MILD	MODERATE INTENSITY(1)	NEGATIVE (0)
16	N	1%	1%	30%	1%	ABSENT	MILD	STRONG INTENSITY(2)	STRONG INTENSITY(2)
17	N	1%	1%	15%	1%	ABSENT	HAZY	STRONG INTENSITY(2)	STRONG INTENSITY(2)
18	N	1%	1%	10%	5%	ABSENT	TRACE	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
19	N	2%	1%	10%	2%	ABSENT	TRACE	STRONG INTENSITY(2)	STRONG INTENSITY(2)
20	N	2%	1%	20%	4%	ABSENT	HAZY	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
21	N	1%	1%	10%	2%	ABSENT	HAZY	STRONG INTENSITY(2)	STRONG INTENSITY(2)
22	N	1%	1%	20%	1%	ABSENT	TRACE	STRONG INTENSITY(2)	STRONG INTENSITY(2)
23	N	1%	1%	25%	4%	ABSENT	HAZY	STRONG INTENSITY(2)	STRONG INTENSITY(2)
24	D	20%	0%	35%	10%	ABSENT	TRACE	WEAK INTENSITY(0.5)	NEGATIVE (0)
25	N	1%	1%	15%	1%	ABSENT	HAZY	STRONG INTENSITY(2)	STRONG INTENSITY(2)
26	N	1%	1%	25%	5%	ABSENT	HAZY	STRONG INTENSITY(2)	STRONG INTENSITY(2)
27	D	14%	10%	30%	10%	ABSENT	STRONG	WEAK INTENSITY(0.5)	WEAK INTENSITY(0.5)
28	D	15%	15%	80%	10%	ABSENT	MODERATE	STRONG INTENSITY(2)	NEGATIVE (0)
29	N	1%	1%	25%	1%	ABSENT	TRACE	STRONG INTENSITY(2)	MODERATE INTENSITY (1)
30	N	10%	1%	25%	6%	ABSENT	MILD	STRONG INTENSITY(2)	STRONG INTENSITY(2)
31	N	1%	15%	10%	2%	ABSENT	HAZY	STRONG INTENSITY(2)	STRONG INTENSITY(2)
32	D	20%	10%	30%	5%	PRESENT	MODERATE	STRONG INTENSITY(2)	NEGATIVE (0)
33	N	2%	2%	15%	5%	ABSENT	HAZY	STRONG INTENSITY(2)	STRONG INTENSITY(2)
34	N	1%	1%	10%	2%	ABSENT	TRACE	STRONG INTENSITY(2)	STRONG INTENSITY(2)
35	N	10%	2%	15%	4%	ABSENT	HAZY	MODERATE INTENSITY(1)	WEAK INTENSITY(0.5)
36	N	10%	2%	20%	5%	ABSENT	HAZY	WEAK INTENSITY(0.5)	MODERATE INTENSITY (1)
37	N	5%	2%	20%	4%	ABSENT	TRACE	STRONG INTENSITY(2)	STRONG INTENSITY(2)
38	N	4%	2%	25%	5%	ABSENT	TRACE	STRONG INTENSITY(2)	STRONG INTENSITY(2)
39	D	20%	5%	45%	8%	ABSENT	MILD	STRONG INTENSITY(2)	STRONG INTENSITY(2)
40	N	2%	2%	15%	2%	ABSENT	MILD	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
41	N	2%	1%	20%	1%	ABSENT	HAZY	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
42	N	8%	2%	15%	4%	ABSENT	TRACE	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
43	D	15%	3%	20%	5%	ABSENT	TRACE	MODERATE INTENSITY(1)	NEGATIVE (0)
44	N	2%	1%	10%	5%	ABSENT	HAZY	STRONG INTENSITY(2)	STRONG INTENSITY(2)
45	D	5%	2%	30%	10%	ABSENT	TRACE	WEAK INTENSITY(0.5)	NEGATIVE (0)
46	N	5%	1%	20%	8%	ABSENT	HAZY	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
47	D	15%	2%	40%	12%	ABSENT	TRACE	STRONG INTENSITY(2)	NEGATIVE (0)
48	N	2%	1%	10%	5%	ABSENT	HAZY	MODERATE INTENSITY(1)	MODERATE INTENSITY (1)
49	D	10%	1%	20%	5%	PRESENT	MILD	WEAK INTENSITY(0.5)	NEGATIVE (0)
50	D	15%	2%	20%	8%	ABSENT	MILD	WEAK INTENSITY(0.5)	NEGATIVE (0)
51	D	5%	2%	35%	15%	ABSENT	MILD	MODERATE INTENSITY(1)	WEAK INTENSITY(0.5)
52	D	10%	2%	32%	10%	ABSENT	MILD	MODERATE INTENSITY(1)	WEAK INTENSITY(0.5)
53	D	20%	3%	30%	8%	ABSENT	MILD	WEAK INTENSITY(0.5)	NEGATIVE (0)
54	D	8%	1%	25%	8%	ABSENT	MILD	MODERATE INTENSITY(1)	WEAK INTENSITY(0.5)
55	D	10%	10%	32%	18%	PRESENT	MODERATE	MODERATE INTENSITY(1)	WEAK INTENSITY(0.5)
56	D	10%	5%	30%	15%	ABSENT	MODERATE	WEAK INTENSITY(0.5)	NEGATIVE (0)
57	D	15%	4%	35%	14%	ABSENT	MILD	WEAK INTENSITY(0.5)	NEGATIVE (0)
58	D	11%	3%	45%	8%	ABSENT	MILD	MODERATE INTENSITY(1)	NEGATIVE (0)
59	N	10%	2%	10%	2%	ABSENT	TRACE	MODERATE INTENSITY(1)	STRONG INTENSITY(2)
60	N	5%	1%	15%	3%	ABSENT	TRACE	WEAK INTENSITY(0.5)	STRONG INTENSITY(2)
61	D	30%	4%	34%	10%	ABSENT	MODERATE	MODERATE INTENSITY(1)	WEAK INTENSITY(0.5)
62	D	15%	10%	40%	15%	ABSENT	MODERATE	STRONG INTENSITY(2)	NEGATIVE (0)
63	D	10%	2%	55%	15%	ABSENT	MILD	STRONG INTENSITY(2)	NEGATIVE (0)
64	D	10%	3%	20%	10%	ABSENT	MILD	STRONG INTENSITY(2)	NEGATIVE (0)
65	D	8%	2%	35%	15%	ABSENT	MODERATE	MODERATE INTENSITY(1)	WEAK INTENSITY(0.5)
66	D	2%	1%	8%	2%	ABSENT	TRACE	MODERATE INTENSITY(1)	NEGATIVE (0)
67	D	12%	8%	45%	12%	ABSENT	MILD	MODERATE INTENSITY(1)	NEGATIVE (0)
68	D	1%	1%	40%	14%	ABSENT	MILD	MODERATE INTENSITY(1)	NEGATIVE (0)
69	D	1%	2%	30%	5%	ABSENT	TRACE	MODERATE INTENSITY(1)	NEGATIVE (0)
70	D	4%	10%	55%	13%	ABSENT	MILD	STRONG INTENSITY(2)	NEGATIVE (0)
71	D	4%	2%	30%	12%	ABSENT	MILD	STRONG INTENSITY(2)	NEGATIVE (0)
72	D	15%	8%	40%	15%	ABSENT	MODERATE	STRONG INTENSITY(2)	NEGATIVE (0)
73	D	10%	3%	35%	16%	ABSENT	MILD	MODERATE INTENSITY(1)	NEGATIVE (0)
74	D	15%	5%	30%	14%	ABSENT	MODERATE	WEAK INTENSITY(0.5)	NEGATIVE (0)
75	D	5%	5%	37%	13%	ABSENT	MODERATE	WEAK INTENSITY(0.5)	STRONG INTENSITY(2)
76	D	10%	8%	25%	9%	ABSENT	MILD	WEAK INTENSITY(0.5)	STRONG INTENSITY(2)
77	D	12%	2%	55%	12%	PRESENT	MILD	WEAK INTENSITY(0.5)	NEGATIVE (0)
78	D	14%	5%	32%	14%	ABSENT	STRONG	MODERATE INTENSITY(1)	WEEK INTENSITY(0.5)
79	D	20%	8%	25%	15%	PRESENT	MILD	STRONG INTENSITY(2)	NEGATIVE (0)
80	D	12%	2%	22%	5%	ABSENT	MILD	MODERATE INTENSITY(1)	NEGATIVE (0)