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IMMUNOHISTOCHEMICAL EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR VEGF-A, VEGFR-2 AND TOPOGRAPHICAL FEATURES OF SCANNING ELECTRON MICROSCOPY OF PLACENTAS IN GESTATIONAL DIABETES AT DELIVERY

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ABSTRACT

The aim of this study was to notice different histopathological changes in light and SEM of diabetic placentas and Immunohistochemical expression of vascular endothelial growth factor VEGF-A, VEGFR-2. Gestational diabetes mellitus result in the metabolic and hormonal alteration in the fetus. fetal hyperglycaemia will result in fetal hyperinsulinemia and stimulation of fetal metabolism. Lower fetal oxygen content associated by higher lactate concentrations reflect better fetal metabolism as a result of hyperglycaemia and hyperinsulinemia. So, the growth of fetal oxygen stresses will worsen the chronic fetal hypoxia. The hyperglycemia of GDM an effect on placental angiogenesis and microvascular remodeling. VEGF/VEGFR-2, which was designated as descriptive factors related to the placental blood barrier and angiogenesis in this study, is important in indorsing vascular endothelial cell growth and in increasing the number of vessels and capillaries, the suitable supply of nutrients to the fetus. Pregnant women were screened for diabetes. They were subsequently divided into normoglycemic (40 cases), GDM (40 cases). Placentas collected were subjected to histopathological examination. VEGF expressions were studied by immunohistochemistry expression. Placental villi in a case of fetal death associate to complications of pregnancy as preeclampsia severe, megaloblastic macrocitic anemia, infection by Zika virus and marginal insertion of umbilical cord were showed in woman pregnancy of 26 years old at 22 weeks of pregnancy causing severe degenerative changes. Is our objective to explore the cell surface of the trophoblast identifying the changes observed with scanning electron microscopy. Samples of normal trophoblast were compared with samples of the case with the four complications. This case was correlated besides with the features found with light microscopy according with a preliminary study previously published. Groups of villi were seen not following their normal morphological pattern. Immature globular terminal villi rush out of mesenchymal villi in irregular aspect. This experimental study revealed the reduced expressions of VEGFA and VEGFR2 in the placental tissues obtained from women with GDM. It should be noticed for both clinicians and GDM women that the reduced VEGF/ VEGFR2 may affect the placental blood barrier and angiogenesis, and resulting placental function, in the pregnancy with GDM. Histological finding of GDM group showed increased thickening of Placental barrier, massive fibrinoid necrosis and chorangiosis . Histopathological changes in SEM of diabetic placentas found bad development of villi with curvilinear with reduced cylindrical microvilli in the surface of terminal villi.

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Keywords: GDM , Immunohistochemical expression of vascular endothelial growth factor VEGF-A, VEGFR-2 histopathology, Scanning electron microscopy.

INTRODUCTION:

Pregnancy is a diabetogenic state characterized by reduced insulin sensitivity. The human placenta is a hemochorial placenta, which means that maternal blood is in direct contact with fetal trophoblast. Terminal villi were evaluated with respect to the placental blood barrier (thickness of the vasculo-syncytial membrane, thickness of the syncytiotrophoblast (ST) basal membrane (BM) and thickness of the endothelial BM). The major factors conducive to GDM are the placental hormones, such as human placental lactogen, progesterone, cortisol, growth hormone and prolactin. These hormones cause the decreased phosphorylation of insulin receptor substrate-1 (IRS-1), resulting in profound insulin resistance. IRS-1 decreases 50–60 % of insulin sensitivity and 50 % of beta cell function in a normal pregnancy. Beta cell function declines in GDM Gestational diabetes mellitus (GDM) is one of the most common medical complications of pregnancy, and has important health implications for mother and child. Mothers with GDM have an excess of hypertensive disorders during pregnancy, recurrence of gestational diabetes , post-partum diabetes and cardiovascular disease after. VEGF is a homodimeric disulfide-linked glycoprotein involved in both angiogenesis (growth of new blood vessels from existing ones) and vasculogenesis (de novo formation of blood vessels).(1) . Vasculogenesis is characterized by in situ differentiation of hemangiogenic stem cells derived from the pluripotent mesenchyme, followed by proliferation of angioblastic cells which give rise to precursor cells. This is followed by angiogenesis. Extensive vascular remodeling and stabilization of the vascular bed occur in second half of gestation..The process of angiogenesis involves proliferation, migration, and maturation of both maternal and fetal endothelial cells.(2). Placentas from gestational diabetes mellitus (GDM) are often hypervascularized; contribution of vascular endothelial growth factor (VEGF) and its receptors in this placental adaptation is unclear. Immunohistochemistry of placental blood vessels were performed . VEGF-A promotes angiogenesis, induces the growth of vascular endothelial cells, reduces apoptosis, and increases permeability. Hypoxia is a potent stimulus for the expression of VEGF-A. Vascular disorders can alter placental function and cooperation pregnancy outcomes. Diabetes leads to atherosclerosis that may damage uteroplacental blood flow, produces perivillous fibrin deposition, excessive syncytial knots, and Proliferation of capillaries can be explained by stimulation of endothelial cells by VEGF through VEGFR-2 as a result of low oxygen level. Villous degeneration without angiogenesis is generally associated with severe hypoxia despite stimulation by VEGF. VEGF family members include VEGFA, (PlGF, VEGFB, VEGFC, and VEGFD).(3)

VEGFs initiate cellular responses by interacting with tyrosine kinase receptors on the cell surface. Tyrosine kinase receptors are a specific type of protein kinase receptor that functions by phosphorylating the substrate to stimulate cellular responses. The VEGF receptor (VEGFR) consists of an extracellular domain, a single transmembrane straddling region, and an intracellular component containing a tyrosine kinase domain. There are three main VEGF-R subtypes: VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4). VEGFA binds to and VEGFR2; VEGFB, to VEGFR1; and VEGFC and VEGFD, to VEGFR2 and VEGFR. (4)

MATERIALS AND METHODS

The study was assumed in a tertiary care hospital in the branch of pathology with teamwork from department of obstetrics. The selected pregnant women signed an informed consent. A cross-

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sectional case-control study from March 2019 to March 2021. A total of 80 pregnant women 40 fore each group(normal& GDM) were from full-term (39-41 weeks' gestation). Placentas were collected after delivery from full-term within 36th to 40th week of gestation, were included in this study of normal and gestational diabetic groups we private the present study to women gained GDM and excluded any woman who already had any type of DM before pregnancy . Patients and normal pregnant women, who were attended a pretested guided questionnaire was managed through the prepared set of questionnaires to collect data of family history of diabetes, complete information regarding maternal age, parity, miscarriages, and complications of pregnancy. The data was entered one by one by observing at each questionnaire. Diagnosis of GDM was made based on the American Diabetes Association (2010) .(5)

Eighty placentas were collected immediately after delivery of both groups. The control women were within the accepted normal ranges of blood glucose level from to 160 mg/dL while above 160mg/dl was GDM. Fasting blood glucose were determined by enzymatic methods using Envoy® 500 reagents (Vital Diagnostics, USA). Immunohistochemistry Paraffin blocks made from placenta specimen were collected and the sections were used for hematoxylin and eosin staining and for immunohistochemical (IHC) examinations. VEGF-A immunostaining: Slides were de-waxed, rehydrated, antigen retrieved and bathed in hydrogen peroxide for 30 mins. IHC was performed using immunohistochemistry (IHC) Kit for Detection of (Abcam, England). Ab64259- mouse specific HRP/DAB immunohistochemistry (IHC). Detection kit (Abcam, England) was done according to standard protocols. Monoclonal Anti-VEGFA antibody. The immunohistochemistry detection Kit is a reliable and convenient tool to identify specified gene expression on tissues. A biotinylated, cross-adsorbed and affinity purified primary anti-mouse and rabbit specific HRP/DAB was used to detect primary antibody-antigen complexes adhered to a glass microscope slide, following reaction with an enhanced detection reagent. This kit is compatible with mouse and rabbit specific HRP/DAB secondary antibodies and can be used for paraffin-embedded tissue sections (Cat. Number: - Ab64259, Abcam, England**).**

SEM examination

Scanning electron microscopy is a standard method of biological investigation used for the explore cell surface. Placental tissue was obtained of full-term placenta in GDM and normal pregnancy for light and Scanning electron microscopic examination. SEM shows to us the examine a panoramic impression large areas of external surface in trophoblastic tissue. The placenta normal was obtained at 38 weeks of patient maternal surface selected at random from the region central parabasal in the vertical plane. Specimens were observed with scanning electron microscope according to conservative stains of SEM. The placentae were obtained small cubes of tissue (0.5 cm3) were cut from the parenchyma of centrally situated lobules, but not including, the maternal surface. The tissue pieces were fixed in buffered glutaraldehyde and for SEM, immersed in 2% formaldehyde according.(6)

RESULT

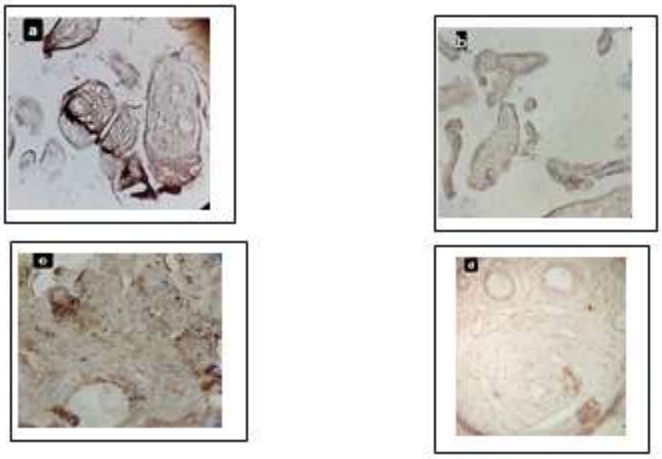
The result of Immunohistochemical staining to study the cellular localization of VEGFA and VEGFR2 proteins in the GDM and control placentas. (Fig.1 a) showed the representative images of VEGFA immunoreactivity in the Syncytiotrophoblasts ST layer of term control tissues more than term GDM tissues (Fig.1 a). the immunoreactivity revealed that the VEGFA protein level was lower in the placentas of the GDM group than in those of the control group but with no visible difference in protein localization. (Fig.1 b) showed the localization and distribution of the VEGFR2 protein in the endothelial cells of fetal vessels in the placentas of the

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control group, (Fig.1 b right) revealed weaker staining in the endothelial cells of fetal vessels in the placentas of the GDM group than normal group but no visible difference in the localization of VEGFR2 was noted.

Histopathological changes observed our finding in figure (2). The GDM placenta showed perivillous fibrinoid degeneration, edema and areas of fibrosis left (4x). Infraction of the villi completely surrounded by fibrinoid dispassion, Hofbauer cells & edematous spaces in core of villous, syncytial knots and calcification (10x) right with H&E stain. Figure (3) The GDM placenta showed massive fibrinoid dispassion, fibrinoid necrosis, edematous spaces, Fetal vessel thrombosis and proliferation left (4x), More magnification display (angiogenesis) increased thick walled vessels, edematous spaces and thickening of Placental barrier (vasculosyncytial membrane) right(10x) H&E stain. Figure (4) showed increased thickening of Placental barrier (vasculosyncytial membrane), massive fibrinoid dispassion, chorangiosis and syncytial knots.

Result of SEM Our finding showed that distinct alternations of scaning electron microscopic in GDM. Figure (5) peripheral ramifications of the mature villous tree, consisting of a stem villus (100 µm) left and stem villi with it's large arteries (500 µm) right Figure (6) Bad development of villi with curvilinear and proliferation of terminal villi in their extremity (300 µm)left and perivillous fibrinoid deposition at intervillous spaces with reduced cylindrical microvilli in the surface of terminal villi (50 µm) right . Figure (7) Increase the distance between fetal blood vessels with fibrinoid deposition inside villi and basement membrane of placental barrier of trophoblast (100 µm). Figure(8) Globular villi cover by fibrinoid exhibited on the surface of many villi (100 µm) left and the globular villi bearded irregular cylindrical microvilli (10 µm) right . Figure (9) Deep depressions are display in the trophoblast of placental villi with fibrinoid deposition (50 µm) left. More magnification of figure revealed deep depressions of the cell surface of the trophoblast are showed with fissure (30 µm) right.



(Fig.1 a) showed the illustrative images of VEGFA immunoreactivity in the Syncytiotrophoblasts ST layer of term control tissues more than term GDM Syncytiotrophoblasts ST layer (Fig. 1b). (Fig.1 c) showed the localization and distribution of the VEGFR2 protein in the endothelial cells of fetal vessels in the placentas of the control group. **(Fig.1 d) revealed weaker staining in the endothelial cells of fetal vessels in the placentas of the GDM group than normal group (40x).**

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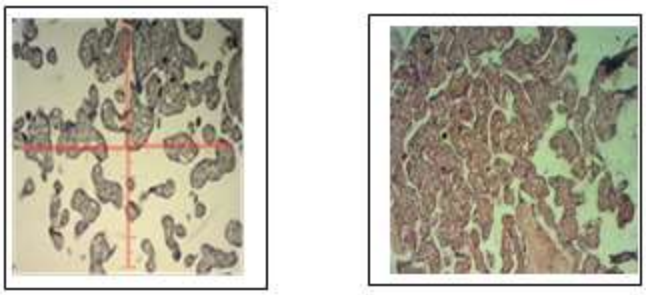


Figure (2) Display diameter of blood vessels of TV were measured In GDM group using eyepiece graticule scale (4x) right. Increase number of blood vessels (angiogenesis) with narrowing lumine (4x) with H&E stain.

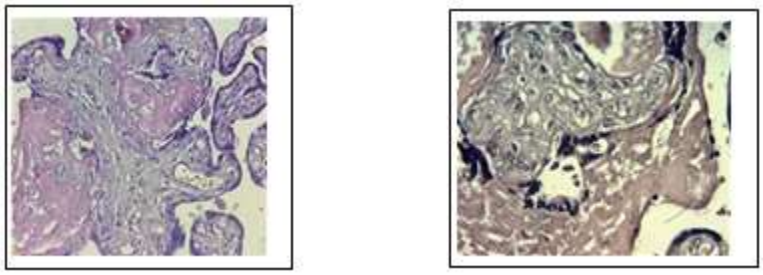


Figure (3) The GDM placenta showed intravillous fibrinoid degeneration, Hofbauer cells and edematous spaces in core of villous left (40x). The villi completely surrounded by intervillous spaces of fibrinoid dispassion with syncytial knots and infraction of the villi (40x) right with H&E stain.

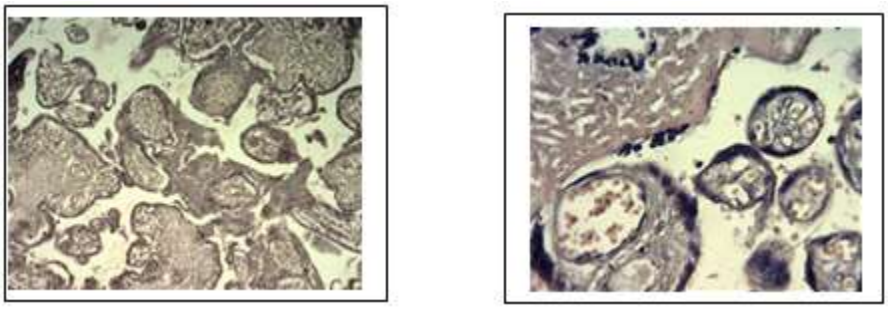


Figure (4) The GDM placenta showed massive fibrinoid dispassion, fibrinoid necrosis, edematous spaces, and proliferation of blood vessels left (4x). More magnification display (angiogenesis) increased thick walled vessels, Fetal vessel thrombosis and thickening of Placental barrier (vasculosyncytial membrane) right(10x) H&E stain.

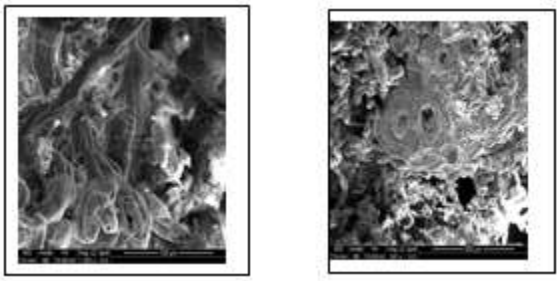


Figure (5) peripheral ramifications of the mature villous tree, consisting of a stem villus (100 μm) left, stem villi with it's arteries (500 μm) right.

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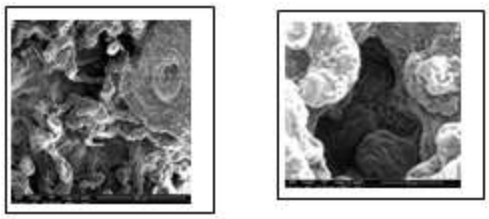


Figure (6) Bad development of villi with curvilinear and proliferation of terminal villi in their extremity (300 µm)left. The intervillous space is obliterated in many villi of fibrinoid deposition that are in intimate association and lack of circulation in hypoxic condition (50 µm) right.

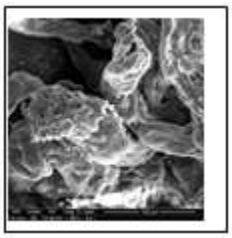


Figure (7) Increase the distance between fetal blood vessels with fibrinoid ideposition inside villi and basement membrane of placental barrier of trophoblast (100 µm).

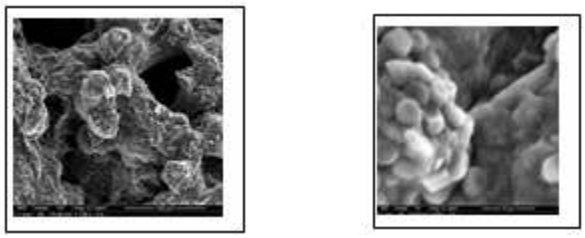


Figure (8) Globular villi cover by fibrinoid exhibited on the surface of many villi (100 µm) left and the globular villi bearded irregular cylindrical microvilli (10 µm) right .
Figure (9) Deep depressions are display in the trophoblast of placental villi with fibrinoid deposition (50 µm) left. More magnification of figure revealed deep depressions of the cell surface of the trophoblast are showed with fissure (30 µm) right.

DISCUSSION

The hyperglycemia of GDM develops during pregnancy only in the late second trimester and an impact on placental processes occurring in later stages of pregnancy, as angiogenesis and microvascular remodeling. VEGF/VEGFR-2, which was factors related to the placental blood barrier and angiogenesis, is helping vascular endothelial cell growth and in increasing the number of vessels and capillaries and give nutrients to the fetus. We found that the expressions of VEGFA and VEGFR2 was significantly lower in the placentas of women with GDM. VEGF expression and protein production are oxygen-dependent, which are regulated by hypoxia. chronic fetal hypoxia as the importance of maternal diabetes stimulate placental vasculogenesis and angiogenesis by increasing the growth factors expression in the placenta and fetus(1)
The distribution of VEGF was observed in the placentas of GDM group, which a decrease in VEGF production. VEGF was mainly detected in the ST layer of term control tissues and term GDM tissues.(7)
The target cells for VEGF in the chorionic villi were determined based on the immunolocalization of VEGFR-2, which expression was mainly low in the placental capillary

endothelial cells of gestational diabetes, in difference to normoglycemic women. The binding between VEGF and VEGFR-2 inductions a signaling pathway that negatively regulates angiogenesis.

Cetinkaya et al. found that VEGF expression increased in diabetic placentas in comparison to normal placentas. They found VEGF expression mainly in the syncytiotrophoblastic layer of the placental villi in normoglycemic placentas. They reported that GDM placentas displayed the strongest VEGF expression.(8) Changes in VEGF expression contribute to various histopathological changes in diabetic placentas. VEGF expression pattern is altered in GDM.(2)

The result of Histopathological study agreement with study said histopathological study showed the placental villous edema was recognized vascular endothelial tight junctional complexes with loss of protein and fluid transport regulation which leads to extravasation of fluid and protein by finding of open spaces within the cytoplasm of intervillous cells and in the interstitial of the villi. The edema fluid interrupted a barrier to gas exchange between mother and fetus. The capillaries were blocked by density leading to reduction in blood flow through the villi. These abnormalities, reduce gas exchange. It is elective that hypoxia could partially be prevented by preventing the growth of placental villous edema.(9)

The effects of hypoxia lead to increase numbered of the placental terminal villi called angiogenesis. Hyperplasia of the terminal chorionic villi due to the low partial pressure of oxygen (pO2) in the blood of the chorionic villi. This decrease in pO2 is reduced maternal blood supply flowing into the intervillous space and resulting in damage to the endothelial cells followed by their proliferation, which can lead to a narrowing of the maternal blood vessel lumens, reducing the uteroplacental circulation. Another cause of low pO2 in the chorionic villus blood the excessive thickness of the placental barrier. (10)

The distance between maternal and fetal circulation is increased because of an increase so greater thickness of the syncytiotrophoblast basal membrane due to an increased type IV collagen deposition. The stroma between the villi is edematous, which modifies the metabolic and endocrinal function of these placentas. A greater penetration of these vessels within the villi. Low oxygen, that would produce hyperplasia of terminal chorionic villi. (11)

The placenta in mothers with diabetes is affected by generalized fetal macrosomia observed in GDM. Compensatory hyperplasia of the terminal chorionic villi due to the low partial pressure of oxygen (pO2) in the blood inside the chorionic villi. The result of a reduced maternal blood supply flowing into the intervillous space, leading to the development of diabetic endarteritis and resulting in damage to the endothelial cells followed by their proliferation, which can lead to a narrowing of the maternal blood vessel lumens, reducing the uteroplacental circulation. Another cause of low pO2 in the chorionic villus blood the excessive thickness of the placental barrier. The basal lamina of the chorionic capillaries is part of the placental barrier; therefore, the increase in its thickness will make the placental barrier thicker overall, which can lead to a reduction in the oxygen transport and other nutrients through the barrier. In response to this reduction in pO2 , the terminal villi exhibit a hyperplasia ,edematous and immature with an increase in mature and immature intermediate villi and a relatively reduced number of terminal villi with the formation of syncytial knots and the increase in angiogenesis.(12)

Hydropic villi were diagnosed when large terminal villi were present with edematous fluid and villous macrophages. Fetal vessel thrombosis was diagnosed when a large fetal stem villous vessel was partially or completely occluded by a thrombus. Avascular villi were diagnosed when a group of at least five fibrotic avascular villi without inflammation were seen. an increase in the

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number of vessels, a finding that has as a mechanism for maintaining homeostasis at the maternal-fetal interface.(2)

The GDM placenta also showed increased syncytial knots, thickening of vasculosyncytial membrane, vast fibrinoid necrosis and chorangiosis. The areas of BV were increased (Vascular Hyperplasia or Chorangiosis) than the control. But diameters of BV were found to be decreased in TV of GDM than control. (13)

The endothelium of the human fetoplacental vasculature is a monolayer directly facing fetal blood and corresponds to the epithelium underlying the syncytiotrophoblast layer. GDM appears to effect the microvascular remodeling at angiogenesis at 3rd trimester of pregnancy, insulin as a key factor playing a modulatory role in GDM-associated altered angiogenesis. (14) In maternal diabetes, the structure of the placental villi was focally edematous and reduced number of terminal villi with the formation of syncytial knots. The most common Hofbauer cells can detected within the connective tissue matrix of the placenta are present throughout pregnancy. These cells are concerned in functions important for a successful pregnancy including placental morphogenesis, immune regulation, control of stromal water content, and the transfer of ions and serum proteins across the maternal-fetal barrier. Derangements in Hofbauer cell homeostasis are associated with placental pathologies involving infection, inflammation, GDM and inadequate placental development. (15)

Placental Hofbauer cells (HBCs) even in low-grade inflammatory states such as GDM . It is concerned in placental vasculogenesis and angiogenesis. This indicates a regulatory, tissue remodeling rather than an inflammatory macrophage phenotype, play a role in maternal immunological tolerance against the fetus examined by hematoxylin and eosin staining in the cases of GDM. (16)

The result of Immunolocalization of VEGF-A and VEGFR-2 agreement with study said Immunolocalization of VEGFR-2 (KDR) Reactivity to VEGFR-2 was observed in the GDM group and the control, with intense staining in the syncytiotrophoblast cytoplasm . In endothelial cells, staining was observed in the capillaries, larger vessels and mesenchymal cells of normoglycemic and hyperglycemic women. In pregnant women with gestational diabetes, staining was restricted to the syncytiotrophoblast cytoplasm. These results were a response to placental hypoxia induced by hyperglycemia. High circulating blood glucose concentrations and deficiency in insulin production leads to changes in the structure of hemoglobin, with increased glycosylation of this protein that can lead to erythrocyte deformation and increased blood viscosity. These factors lead to reduce the affinity of hemoglobin for oxygen, leading to poor oxygen transport and local hypoxia. (7) Differential activation of KDR by VEGF in endothelial cells . (17) GDM features as a pro-angiogenic placental state. These results appear increased activity of KDR. Gestational diabetes mellitus is associated with increased pro-migratory activation of vascular endothelial growth factor receptor 2. (18)

. The result of SEM agreement with study said the placental barrier represents the site of interface between the maternal and fetal circulations. In the control group, it was formed of Sy, a thin layer of Cy and a thin layer of vascular endothelial cells with a thin BM together with a villous core made up of stroma. IT is suitable to ensure proper oxygenation and nutrition of the embryo which are essential for a successful pregnancy . The placenta barrier was composed of ST, endothelium and the space between them. ST was a continuous syncytial layer with multiple nucleus and numerous apical microvilli in normal group. (19)

The result agreement with study said deposition of fibrinoid is exhibited on the cell surface of many villi. Bad development of villi with fibrinoid deposition are originating villi news and bad

development of villi which have motivated high risk for the interchange of gases or nutrients lead to the fetal death. (6) Another study said Microvilli of the syncytiotrophoblast have been observed reduced in number of placental villi associated with obesity and hypertensión. (20)

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