Role of vascular endothelial growth factor and natural killer cells in histological changes of human placenta in pre-eclampsia

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Abstract: ***Background*:** Placenta provides the means for physiological exchange of blood gases, nutrients and wastes between the fetal and the maternal circulation. Normal fetal growth and survival depend on the proper placental development and function. Preeclampsia is a condition characterized by hypertension and proteinuria after 20th week of gestation. ***Aim of the work*:** This study was designed to investigate the role of VEGF and NK cells in histological changes in pre-eclamptic placentae. ***Subjects and methods:*** A total of 60 placental specimens were collected from 3rd trimester deliveries. Placental tissues were divided into two study groups, 20 specimens from normal pregnancies and 40 specimens from pregnancies complicated by pre-eclampsia. Paraffin sections were stained with Hematoxylin and Eosin and immunohistochemically for VEGF-A and CD56. Quantitative morphometeric studies followed by statistical analysis of the data were done. ***Results:*** Light microscopic examination showed that syncytial nuclei were aggregated into knots with the presence of sprouts and strands. Villous connective tissue core was condensed with apparently decreased fetal capillaries. Immunohistochemically, VEGF-Aimmunoexpression and CD56 +ve NK cells were significantly higher in placental biopsies from pre-eclamptic patients when compared to that of the control.
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Keywords: Pre-eclampsia, Placenta, VEGF, NK cells and CD56.

1.Introduction:

Placenta is a highly vascularised organ responsible for maintenance of the pregnancy and normal foetal development. It has been described as a "diary of intrauterine life" and has the potential to elucidate many aspects of the processes going on during pregnancy ***(Akhlaq et al. 2012).***

Placenta provides a link between the maternal circulation and that of the foetus and serves as the organ for exchange of nutrients, gases and waste products through diffusion ***(Paria et al. 2000 and Guttmacher et al. 2014).*** Placenta also has metabolic and endocrine activity: producing hormones such as progesterone which is important in maintaining the pregnancy; somatotropin which acts to increase the amount of glucose and lipids in the maternal blood; oestrogen, responsible for foetal weight; relaxin for relaxation of the cervix during parturition and human chorionic gonadotrophin (hCG) ***(Wang et al. 2004).***

Pre-eclampsia is generally defined as the development of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive woman ***(Huppertz, 2008)***. The risk of developing this systemic syndrome is about 2–8%. Pre-eclampsia, especially in the developing countries, is a major cause of maternal morbidity and mortality. Worldwide it is one of the leading contributors to adverse perinatal outcomes, such as prematurity and intrauterine growth retardation ***(Khan et al. 2006 and Duley, 2009)***. In Egypt, the national survey for maternal mortality ranked pre-eclampsia as the second cause of maternal death accounting for 16.7% of maternal deaths ***(El-Moselhy et al. 2011)***.

In pregnancy, VEGF participates in the proliferation, migration, and metabolic activity of trophoblasts, it is expressed by human villous and extravillous trophoblasts. Conclusive evidence indicates that it regulates trophoblast function by stimulating release of nitric oxide. VEGF promotes endothelial cell proliferation, migration and survival, it also promotes neovascularization, reduces blood pressure and is crucial for the formation and maintenance of the glomerular filtration barrier ***(Barut et al. 2010).*** Uterine NK cells play an important role in regulating the maternal immune response to the foetal allograft, controlling the trophoblast growth and the invasion and the development of the placenta during human pregnancy through release of many factors, such as vascular endothelial growth factor (VEGF) and placental growth factor (PGF) to promote endometrial angiogenesis. ***(French and Yokoyama 2004).***

Aim of the work

The present study was designed to investigate the role of vascular endothelial growth factor and natural killer cells on the histological changes of placentae of pre-eclamptic pregnant women.

2. Material and methods

a) Subjects:

A total of 60 placentae were obtained from pregnant women, aging 25-35 years average (30.6 ys), admitted to terminate their pregnancy by caesarean section at Obstetric Department of Kasr El –Ainy Hospital, Cairo University, in the period from October 2012 to May 2013. Twenty placentae were taken from normal pregnancies (Control group) and fourty placentae were taken from pregnant women presenting with pre-eclampsia (Pre-eclamptic group). Medical files of all participants were reviewed and their written consents and approval of the ethical board of Obstetrics and Gyneacology were obtained.

•Selection criteria of the control group:

1-Age: 25-35 years old.

2-Gestational age: 34-40 weeks.

3-Number of viable fetuses: one.

4-Blood pressure: less than 140/90 in two separate occasions 6 hours apart, after 26 weeks gestation.

5-Medical history: No past or present history of chronic hypertension, pre-eclampsia, gestational hypertension, diabetes mellitus, bronchial asthma, hepatic or renal diseases or any disease that may interfere with results of this study.

6-No history of drug intake during pregnancy.

7-Urine analysis: Free, no protein (albumin).

• Selection criteria of the pre-eclamptic group:

1-Age: 25-35 years old.

2-Gestational age: 34-40 weeks.

3-Number of viable fetuses: one

4-Blood pressure: more than 140/90 in two separate occasions 6 hours apart, after 26 weeks gestation.

5-Medical history: No past history of chronic hypertension or any disease that may interfere with results of this study.

6-No history of drug intake during pregnancy.

7- Urine analysis: Shows protein (albumin) at least +1 on a urine dipstick test.

b) Methods:

After delivery, the placentae were examined grossly from the maternal surface to avoid sampling from areas of obvious infarctions or calcifications. Samples (1.5 X1.5 X 1 cm in size) were taken from the central part of the maternal surface opposite to the site of insertion of the umbilical cord in the fetal surface of the placenta.

Tissue processing: The obtained placental specimens were fixed in 10% buffered formalin solution for 24-48 hours, dehydrated in ascending grades of ethanol, cleared in xylol and then embedded in paraffin wax.

Serial sections of 7µm thickness were subjected to the following:

1.Hematoxylin& Eosin staining (H&E).

2.Immunohistochemichal staining for vascular endothelial growth factor (VEGF) using anti VEGF-A antibody obtained from Neo Markers Lab Vision Corporation laboratories (Westinghouse, California, USA).(Cat.# RB-9031-P).

3.Immunohistochemical staining for CD56 to detect natural killer cells using anti CD56 antibody obtained from Neo Markers Lab Vision Corporation laboratories (Westinghouse, California, USA). (Cat.# MS-1149-P).

4.Morphometric study, using "Leica Qwin 500 C" image analyzer computer system (Cambridge, England) to detect:

a)The mean number of the syncytial knots in placental villi (in Hematoxylin and Eosin stained sections).

b)The mean area percent of +ve VEGF-A immunoreactivity in placental villi (in VEGF-A immunostained sections).

c)The mean number of CD56 immunopositive NK cells in placental basal plate (in CD56 immunostained sections).

Statistical analysis

Statistical analysis of the obtained data were done using Statistical Package for the Social Science (SPSS) version 19 software (Chicago,USA).

3.Results

Examination of Hematoxylin and Eosin stained sections from control placentae group showed villi ranging from large stem to intermediate and small terminal villi. The villous structure appeared with central connective tissue core containing fetal vessels and was covered with a continuous syncytial layer. Villi were separated from each other by intervillous space filled with maternal blood. The villous core was rich in vascularity with thin walled peripherally located fetal blood vessels ***(Fig.1)***. Syncytial knots were occasionally seen as an aggregation of dark small apoptotic syncytial nuclei in groups within the syncytial layer ***(Fig.2).*** The placental basal plate was covered with amnion which appeared as a thin layer of cubical cells. The underlying connective tissue contained a mixture of cells including fetal extravillous trophoblasts demonstrated as medium sized acidophilic cells with central dark nuclei. Few large vaculated extravillous trophoplasts were also noted, together with small spindle shaped decidual stromal cells with flat nuclei ***(Fig3.).***

Examination of Hematoxylin and Eosin stained sections from pre-eclamptic placentae group revealed several changes especially in the syncytial layer of placental villi; many syncytial knots appeared as groups of aggregated small dark pyknotic syncytial nuclei within the syncytial layer were detected. The

syncytial layer continuity was lost in some areas. Syncytial sprouts were observed as aggregated syncytial cells protruding from the syncytial layer into the intervillous space, long slender syncytial strands were also seen as strands of syncytial cells bridging the IVS connecting villi together. The connective tissue core of villi appeared condensed ***(Fig.5)***. The wall of fetal vessels showed moderate to marked thickening in some sections giving the characteristic shape of onion-like appearance ***(Fig.4).***Patchy areas within C.T appeared acidophilic, hyaline and non-cellular mainly at the periphery, together with vessel wall degeneration were detected***(Fig.4).***

Regarding the basal plate in sections of pre-eclamptic placentae group, there was an obvious increase in the number of large vaculated extravillous trophoblast ***(Fig.6).***

Examination of VEGF-A immunostained sections from control placentae group showed positive VEGF-A immunoreactivity in the syncytial layer of different villi ***(Fig.7)***. Also, in the extravillous trophoblasts of the basal plate ***(Fig.8)***.Examination of VEGF-A immunostained sections from pre-eclamptic placentae group revealed widely distributed positive VEGF-A immunoreactivity in the syncytial layer of different villi together with the cells and endothelial lining of the blood vessels in the villous connective tissue core ***(Fig.9).***Also, revealed an increase in the positive VEGF-A immunoexpression in the extravillous trophoblasts of the basal plate ***(Fig.10).***

Examination of CD56 immunostained sections from control placentae group revealed positive CD56 immunoreactivity in the decidual natural killer cells of the basal plate ***(Fig.11)***.Examination of CD56 immunostained sections from pre-eclamptic placentae group showed an increase in the number of the immunopositive CD56 decidual natural killer cells in the basal plate ***(Fig.12).***

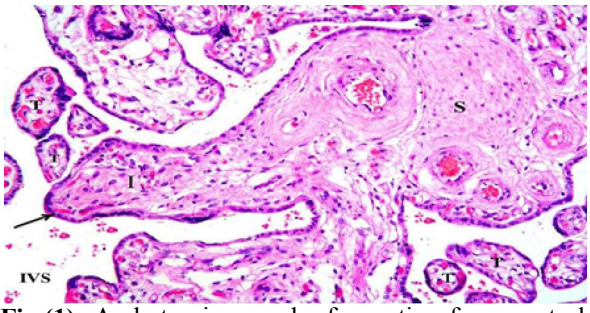


Fig.(1): A photomicrograph of a section from control placenta group showing a stem villous (S), intermediate villous (I) and terminal villi (T) with connective tissue core containing fetal blood vessels. Villi are covered with a continuous syncytial layer (arrow) and separated by an intervillous space (IVS) containing maternal blood (H&E X 200).

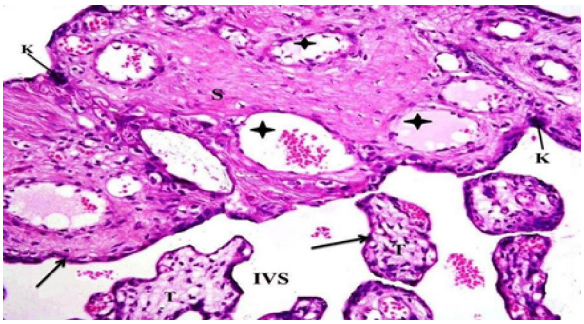


Fig.(2): A photomicrograph of a section from control placenta group showing a stem villous (S) with a connective tissue core containing many thin walled and peripherally located fetal vessels (stars). The villous is covered by a continuous syncytial layer (arrows). Few syncytial knots could be observed (K). Terminal villi (T) appeared separated by intervillous space (IVS) containing maternal blood.(H&E X 200)

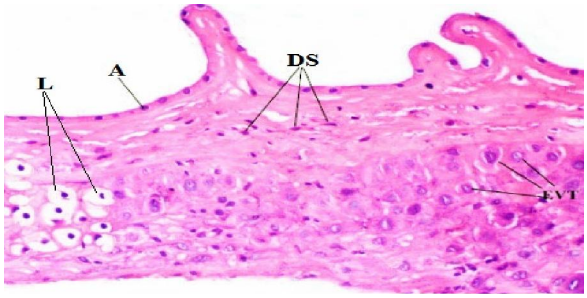


Fig.(3): A photomicrograph of a section from control placenta group showing a placental basal plate with a mixture of medium sized acidophilic extravillous trophoblasts (EVT) and small spindle shaped decidual stromal cells (DS). The basal plate is covered with amnion (A) appearing as a thin layer of cubical cells. Note the presence of few large vaculated extravillous trophoblasts (L) (H&E X 200).

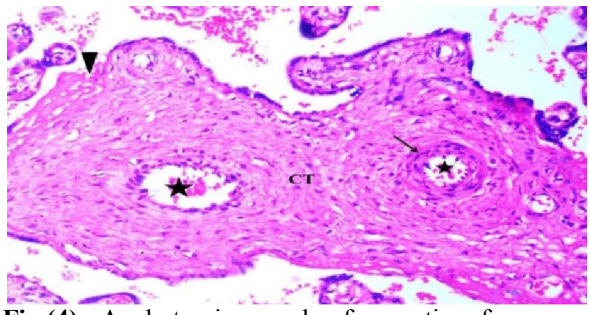


Fig.(4): A photomicrograph of a section from pre-eclamptic placenta group showing a villous with apparently condensed connective tissue core (CT) and scanty fetal vessels (stars) with thickening of the wall (arrow) giving onion-like appearance. Note the presence of an area of apparent degeneration at the periphery of the villous with discontinuity of the syncytial layer (arrow head). (H&E X 200)

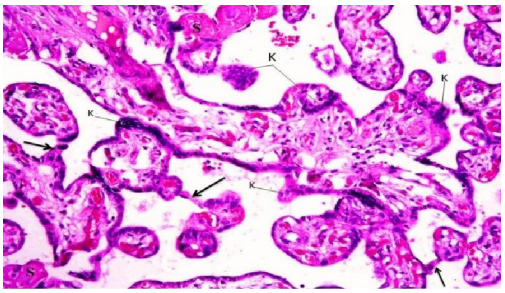


Fig.(5): A photomicrograph of a section from pre-eclamptic placenta group showing many syncytial knots (K), syncytial sprouts (S) and long slender syncytial strands (arrows) bridging the intervillous space connecting villi together. Note the absence of the villous core in syncytial strands and sprouts. (H&E X 200)

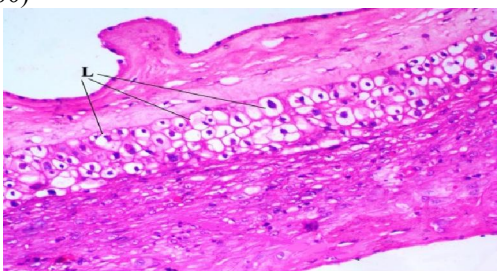


Fig.(6): A photomicrograph of a section from pre-eclamptic placenta group showing placental basal plate with an obvious increase in the number of the large vaculated extravillous trophoblasts (L). (H&E X 200)

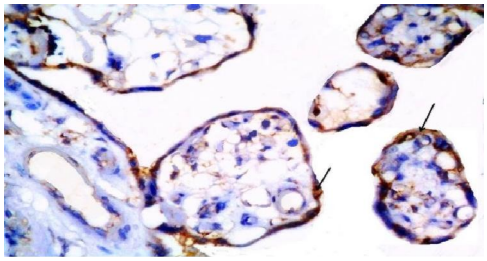


Fig.(7): A photomicrograph of a section from control placenta group demonstrating positive VEGF-A immunoreactivity limited to the syncytial layer of different villi (arrows). (anti VEGF-A X 400)

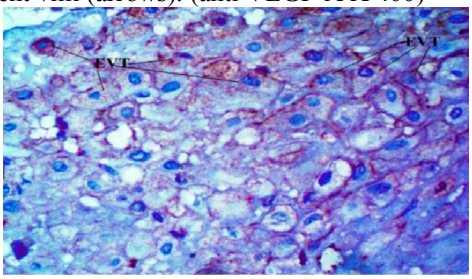


Fig.(8): A photomicrograph of a section from control placenta group showing basal plate demonstrating positive cytoplasmic and membranous VEGF-A immunoreactivity within the extravillous trophoblasts (anti VEGF-A X 400)

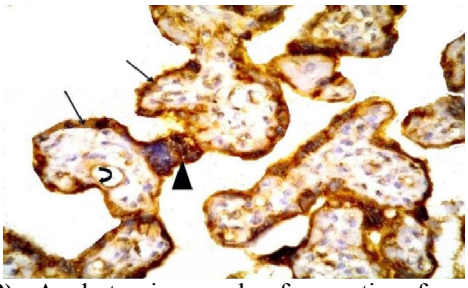


Fig.(9): A photomicrograph of a section from pre-eclamptic placenta group showing villi demonstrating widely distributed positive VEGF-A immunoreactivity in the syncytial layer (arrows), syncytial bridge (arrow head) as well as the endothelial lining fetal blood vessels (curved arrow). (anti VEGF-A X 400)

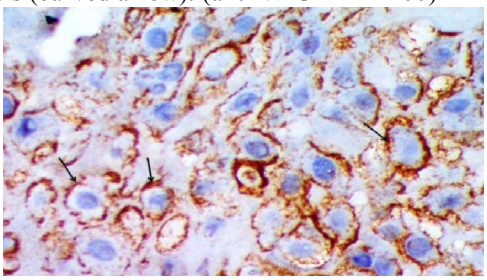


Fig.(10): A photomicrograph of a section from pre-eclamptic placenta group showing basal plate demonstrating increased VEGF-A immunoexpression within the extravillous trophoblasts (arrows). (anti VEGF-A X 400)

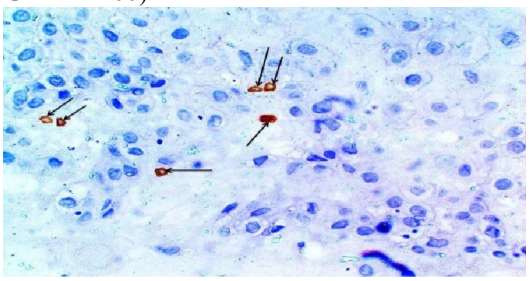


Fig.(11): A photomicrograph of a section from control placenta group showing the basal plate with positive CD56 immunoreactivity in decidual natural killer cells(arrows). (anti CD56 X 400)

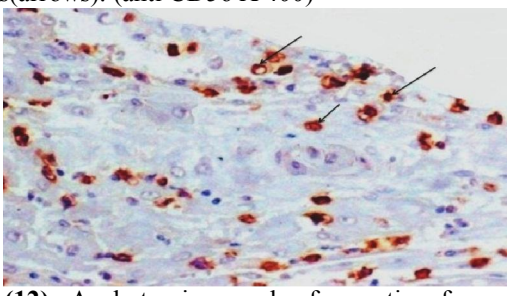
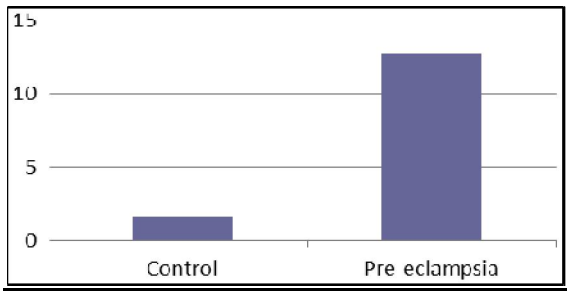


Fig.(12): A photomicrograph of a section from pre-eclamptic placenta group showing increased number of positive CD56 immunoreactive decidual natural killer cells (arrows) in the basal plate. (anti CD56 X 400)

Table(1):

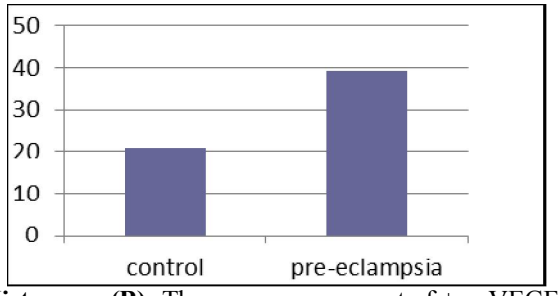
Group	Mean number of syncytial knots ± SD
Control	1.6± 0.25
Pre-eclampsia	12.7± 1.34



Histogram (A): The mean number of syncytial knots in control and pre-eclampsia groups.

Table (2):

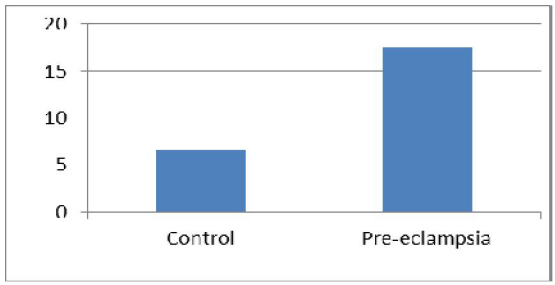
Group	Mean area percent of +VEGF ± SD
Control	20,7± 1.6
Pre-eclampsia	39.3± 1.8



Histogram (B): The mean area percent of +ve VEGF-A immunoreactivity in control and pre-eclampsia groups.

Table (3)

Group	Mean number of CD56 +ve NK cells ± SD
Control	6.64± 1.53
Pre-eclampsia	17.52±1.96



Histogram (C): The mean number of CD56 +ve NK cells in control and pre-eclampsia groups.

4. Discussion

The exact etiology of pre-eclampsia remains unclear, it has been suggested that uteroplacental ischemia, endothelial dysfunction, increased trophoblast apoptosis/necrosis, oxidative stress and exaggerated systemic inflammatory response may play major roles in its development ***(Varughese et al. 2010)***.

In the present study, examination of **Hematoxyline and Eosin** stained sections from control placentae group revealed syncytial knots which were occasionally seen as an aggregation of dark small pyknotic syncytial nuclei within the syncytial layer. It has been suggested that syncytial knots increase with the increasing gestational age and may be used to evaluate villous maturity ***(Roberts and Post 2008)***.

In the present study, examination of **Hematoxyline and Eosin** stained sections from pre-eclamptic placentae group revealed histological changes in the syncytial layer; the syncytial cells aggregated and protruded from the syncytial layer into the intervillous space forming syncytial sprouts and long slender syncytial strands were found bridging the intervillous space to connect villi together and give the villous tree pseudolabyrinthine appearance. This might be an attempt to increase surface for maternofetal exchange as a trial to compensate ischaemia.

The above findings were in accordance with ***Ishihara et al. (2002) and Kadyrov et al. (2006)***. The absence of villous core in sections of syncytial strands or sprouts, agreed with that of ***Saleh and Dkhil (2008).Shin et al. (2008), Barut et al. (2010) and Furuya et al. (2011),*** reported similar results, they suggested that the syncytial nuclei found aggregating and protrouding into the itervillous space forming syncytial knots are markers for uteroplacental ischaemia which are known as " Tenny-Parker changes".***Salgado and Salgado (2011)*** observed a similar increase in the number of the syncytial knots in conjugation with increased severity of the pre-eclampsia.

Straszeweski-Chavez et al. (2005) and Shin et al. (2008) explained the syncytial knots finding as follows: placental hypoxia in preeclampsia induces apoptosis of the trophoblastic cells which in turn produce clusterin protein that play a role in cell adhesion and aggregation. Moreover, ***Matthiesena et al. (2005)*** also reported that increased apoptosis of syncytiotrophoblasts may increase the amount of syncytiotrophoblast debris and syncytial knots that leak into the maternal circulation and generate an exaggerated systemic endothelial activation.

In addition, connective tissue core of some villi was condensed causing regression of fetal capillaries.

This connective tissue condensation could be an attempt for regeneration following the degeneration that occurred as a result of placental ischaemia.The above mentioned findings were in accordance with that of ***Allaire et al. (2000), Walford et al. (2005) and Saleh and Dkhil (2008)*** who reported a regression of fetal capillaries up to complete disappearance due to connective tissue condensation in pre-eclamptic placental villi.

Concerning the blood vessels in the present work, marked thickening of the arterial wall giving onion-like appearance was observed. The marked thickening of the arterial wall might be due to hypertrophy and hyperplasia as a result of ischemia and associated hypoxia, this hyperplasia could explain the associated narrowing of the lumen and the hyaline degeneration could be a sequalae of ischemia in an area that couldn't undergo hypertrophy and hyperplasia.

Muntefering et al. (2004) observed similar changes in placental vessels together with endothelial atrophy and disruption.

Concerning the basal plate, Hematoxylin and Eosin stained sections from pre-eclamptic placentae group showedan increase in the extavillous trophoblast giant cells. ***Redline et al. (2004) and Kotani et al. (2009)*** in similar studies defined the increase of giant cells as the presence of tightly cohesive groups of 10 to 20 (or more) vacuolated immature EVT arranged in sheets or clusters in the superficial basal plate.

In the present study, examination of the **VEGF-A immunostained sections** from control placentae group gave positive VEGF-A immunoreactivity limited to the syncytial layer of different villi together with extravillous trophoblasts of the basal plate.VEGF is likely to be involved in the uterine vessels remodeling and in the angiogenesis, which occurs during the growth of the placenta throughout pregnancy. Another important role has been postulated for VEGF in the regulation of the trophoblast invasion, proliferation and differentiation ***(Levine et al. 2006 and Wang et al. 2009)***.

In the present study, examination of the **VEGF-Aimmunostained sections** from pre-eclamptic placentae group revealed a widely distributed positive VEGF-A immunoreactivity in the villous trophoblast, connective tissue core and endothelial lining of blood vessels together with EVT of the basal plate.The area percent occupied by +ve VEGF immunoreactivity increased significantly in pre-eclamptic placentae when compared to the control. This high VEGF-A expression in pre-eclamptic placentae may be a compensatory mechanism in an attempt to restore placental blood flow to normal as VEGF is a well-known marker for angiogenesis. VEGF-A mediates many functions in endothelial cells, it promotes angiogenesis, induces the growth of vascular endothelial cells and reduces apoptosis. In addition, ***Zhou et al. (2003)*** reported that VEGF-A promotes vasodilatation via the endothelial-derived nitric oxide pathway. Studies on placental VEGF-A expression using immunohistochemistry have reported a conflicting data, ***Simmons et al. (2000), Chung et al. (2004) and Akercan et al. (2008)***reportedan increase in the VEGF-A expression in pre-eclampsia when compared with that of normal pregnancy.

Zhou et al. (2003) and Cirpan et al. (2007) have reported a decrease in the VEGF-A immunostaining in pre-eclampsia when compared to controls. This discrepancy is believed to be due to the type of VEGF-A measured. It is proposed that total VEGF-A may be increased in pre-eclamptic pregnancies and that the free level is reduced due to binding to VEGF-1 receptor (FLT-1).

The present work involved the detection of uterine NK cells using anti CD56 antibody as Uterine NK cells play an important role in regulating the maternal immune response to the foetal allograft, controlling the trophoblast growth and the invasion and the development of the placenta during human pregnancy ***(French and Yokoyama 2004)***. For examination of decidual NK cells, biopsies from placental bed should be obtained from the uterine cavity, because of the invasiveness of this procedure, in our study we examined decidual NK cells in the placental basal plate. In a previous study, it has been proved that both types of decidua collection (placental bed and basal plate) revealed identical cell populations when performed on the same patient ***(Rieger et al. 2009)***. Also, ***Akhlaq et al. (2012)*** has studied decidual NK cells in the basal plate.

Examination of CD56 immunostained sections from control placentae group revealed positive CD56 immunoreactivity in the decidual natural killer cells of the basal plate.The present finding is in agreement with the view that that decidual NK cells are present at term but that their numbers are maximal during the period of trophoblast invasion and spiral artery remodeling which is a characteristic feature of the first 20 weeks of pregnancy and followed by gradual decrease ***(Bachmayer et al. 2006, Rieger et al. 2009 and Akhlaq et al. 2012)***.

Laresgoiti-Servitje et al. (2010) have reported that CD56+ve decidual NK cells decline after the first trimester to disappear completely at full term. In the current work a significant increase in the number of CD56 +ve natural killer cells in the placental basal plate in the pre-eclamptic group was detected when compared to the control group. Such increase in number might be to overcome the pre-eclampsia associated ischemia, oxidative stress and inflammation

as decidual NK cells release factors, such as vascular endothelial growth factor (VEGF) and placental growth factor (PGF) to promote endometrial angiogenesis. Our findings agree with that of ***Bachmayer et al. (2006) and Akhlaq et al. (2012)*** who reported increased numbers of CD56 +ve NK cells in pre-eclamptic decidua compared with control ones.

In contrast, ***Wiliams et al. (2009)*** demonstrated a significant reduction of CD56+ in the decidua of preeclamptic patients by using immunohistochemistry. This observation is rather surprising, since inflame

mation is thought to be an important component of clinically manifest pre-eclampsia.

Placenta oxidative stress and inflammation induce secretion of mediators like soluble fms-like tyrosine kinase 1 (sFlt-1) leading to an enhanced maternal systemic inflammatory reaction which may ultimately cause the clinical signs of PE such as hypertension and proteinuria ***(Smith et al. 2009 and Hazan et al. 2010)***. These findings point to a role for NK dysregulation in the pathogenesis of preeclampsia. However, because the decidua and trophoblasts had been examined at term, it is still unknown whether NK dysregulation is a cause or an effect of preeclampsia.

Conclusion:

It might be concluded that in pre-eclampsia tissue hypoxia occurs leading to an increase in natural killer cells which produce angiogenic factors including VEGF to overcome tissue hypoxia via neoangiogenesis, so VEGF might have a role in prevention or treatment of pre-eclampsia.

Recommendations:

Further studies are recommended regarding the role of VEGF and NK cells in the pathogenesis of pre-eclamsia during the first trimester of pregnancy. Also, further studies are recommended about the usage of other angiogenic factors in prevention or treatment of pre-eclampsia.

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