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Decidual Natural Killer Cells (Cd56+) Population in the Placental Bed in Accidental Hemorrhage

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ABSTRACT

Background: Accidental hemorrhage is defined as abnormal complete or partial separation of normally implanted placenta after 20 weeks of gestation and prior to birth. Risk factors which have been found associated with accidental hemorrhage include maternal age, parity, smoking, hypertension, past history of accidental hemorrhage, thrombophilic disorders, abdominal trauma and polyhydramnios. **Aim of the Work:** The aim of our study was to find if there is any role for decidual natural killer cells in cases with placental abruption. **Patients and Methods:** This is a case control study in which 60 pregnant women recruited from Obstetric Department of Ain Shams University Maternity Hospital. Patients were subjected to emergency cesarean section. Multiple tissue biopsies (5 mm thick) were taken from the decidua basalis of the placental bed and immunostained for decidual CD 56+ve bright natural killer cell marker.

Results: Our results show that there was a highly statistically significant difference between the study and control group as regard immunohistochemical scores (according to CD 56+ %), In control group: score 0 = 0.0%, score 1+ = 0.0%, score 2+ = 18.5%, score 3+ = 29.6%, score 4+ = 51.9% while in the study group: score 0 = 25.9%, score 1+ = 40.7%, score 2+ = 29.6%, score 3+ = 3.7%, score 4+ = 0.0%; (Chi square 42.133 FE) and (P. value = 0.000). Also there was a highly statistically significant difference between the study and control group as regard dNK cells density (uNK cells as a percentage of total stromal cells), in control group: low density = 18.5% and high density = 81.5% while in the study group: low density = 96.3% and high density = 3.7%; (Chi square 33.400 FE) and (P. value = 0.000). **Conclusion:** These findings suggest that low dNK score and density was associated with cases of placental abruption.

Keywords: Placental abruption, Natural killer cells, CD56+^{bright}, Cesarean section, Immunohistochemistry.

INTRODUCTION

Accidental hemorrhage is defined as abnormal complete or partial separation of normally implanted placenta after 20 weeks of gestation and prior to birth[(1)]. Risk factors which have been found associated with accidental hemorrhage include maternal age, parity, smoking, hypertension, past history of accidental hemorrhage, thrombophilic disorders, abdominal trauma and polyhydramnios. Placental abruption seems to be multifactorial. Its etiology is not fully understood but impaired placentation, placental insufficiency, intrauterine hypoxia, and uteroplacental underperfusion are considered the key mechanisms causing abruption[(2)]. Abruption results from a rupture of maternal decidual artery causing dissection of blood at the decidual-placental interface, around placental margin, or behind the membranes. Acute vasospasm of small vessels may be one event immediately preceding placental separation. Thrombosis of the decidual vessels with associated decidual necrosis and venous hemorrhage also are often present[(3)].

Immunological defects may well play a role in the origin of placental abruption[(4)]. These defects may lead to maternal inflammatory response with cytokine release resulting in a chain of events such as shallow trophoblast invasion, defective spiral artery remodeling, placental infarctions, and thrombosis[(5)].Normal placentation requires trophoblast invasion of maternal spiral arteries, and development of a high-flow, low-resistance uteroplacental circulation[(6)].

Abruption is often discovered when bright red or dark clotted blood is discharged from the vagina. However, bleeding from the vagina is not always present [(7)]. Bleeding and pain consist the classical symptoms of placental abruption but the clinical picture varies from asymptomatic, in which the diagnosis is made by inspection of the placenta at delivery, to massive abruption leading to fetal death and severe maternal morbidity[(8)].

Natural killer (NK) cells are part of the innate immune system, and are found in both peripheral blood and endometrium. Although both peripheral NK (pNK) and uterine NK (uNK) cells express the surface antigen CD56, pNK cells are phenotypically and functionally different from uNK cells and <10% of pNK cells resemble uNK cells[(9)],Furthermore 90% of pNK cells are CD56dim and CD16+ whereas 80% of uNK cells are CD56bright and CD16− [(10,11)].

The maternal immunologic state is maintained by local secretion of T helper-2 (Th2) cytokines while the pregnancy complications such as spontaneous miscarriage, preterm delivery and preeclampsia, seem to be associated with a

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predominance of T helper-1 (Th1) reactivity in the mother[12].

These cytokines activate the NK phenotype cells (CD56+CD16+) and transform them into LAK (lymphokine activated killer), powerful cells able to damage the trophoblast[13].

Decidual natural killer (dNK) cells comprise approximately 70% of the decidual leukocyte population and, unlike peripheral blood NK cells, are a cytokine-producing cell type with limited cytotoxic capacity. dNK cells accumulate around spiral arteries, are present ahead of trophoblast cell invasion and continue to be present during the remodelling process[14]. It has been suggested that dNK cells might be involved in angiogenesis during decidualisation[15,16], regulation of trophoblast invasion[17] and spiral artery remodeling[18].

AIM OF THE STUDY

The aim of our study was to find if there is any role for decidual natural killer cells in cases with placental abruption. There is considerable evidence that dNK play a role in the implantation and early invasion of trophoblast.

PATIENTS AND METHODS

This case control study was carried out on 60 pregnant women recruited from Obstetric Department, Ain Shams University Maternity Hospital, between (April/ 2016) and (April/ 2017). They were divided into two groups:

- **Group A:** including 30 singleton pregnancies complicated by accidental hemorrhage (study group).
- **Group B:** including 30 singleton normal pregnancies (control group).

Inclusion criteria

Singleton pregnancy, after 28 weeks of gestation, maternal age between 20 and 35 years.

Exclusion criteria

1. Vaginal or cervical cause as erosions, polyps or cancer, vasa praevia and placenta praevia, placenta accrete, increta and percreta.

2. Diseases or conditions which alter the count of decidual natural killer cells during pregnancy:

- High risk pregnancy as hypertension, diabetes mellitus and pre eclampsia[19].
- Intrauterine growth restriction[20].
- Patients with immunotherapy such as prednisolone or intravenous immunoglobulin (IVIG)[21].
- Smoking[22].
- Chorioamnionitis.
- Antiphospholipid syndrome, thrombophilia and collagen disease as SLE.

METHODS

Written informed consents were obtained from all participants and the study were approved by the Hospital Ethical & Research Committee.

All participants were subjected to the following:

1) ***Complete history taking:*** Detailed history of present pregnancy as maternal age, gestational age, parity, smoking and pregnancy induced hypertension, past history of chronic hypertension, diabetes mellitus, autoimmune diseases, previous cesarean section, previous placental abruption, previous premature rupture of membranes (PROM), previous abortion, family history of similar conditions.

2) **General examination:** Blood pressure, pallor, jaundice, chest and heart auscultation and lower limb edema.

3) **Abdominal examination:** Uterine tenderness and/or abdominal pain, Fundal level and gestational age: equal to period of amenorrhea if revealed and above if concealed, Fetal distress or death[23].

4) **Local examination:** Vaginal bleeding (dark brown), speculum examination to exclude vaginal or cervical cause.

5) **Investigations:**

Lab investigations: Complete blood count, blood grouping and Rhesus factor, urine analysis, liver functions, renal functions, random blood sugar, coagulation profile.

Obstetric U/S: Retroplacental hematoma: hypoechoic area between the placenta and uterine wall. Placental site to exclude placenta previa (the most important), vasa praevia: fetal vessels at the internal cervical os. Other value: confirm the gestational age and exclude IUGR.

Tests of fetal wellbeing: C.T.G.

Patients were recruited after admission to the hospital for emergency cesarean section. During cesarean section, the diagnosis can be confirmed by detection of retroplacental hematoma then multiple tissue biopsies (5 mm thick) were taken from the decidua basalis of the placental bed according to the technique described by[24]: the site of placental insertion were identified by the surgeon after the birth of the fetus, then wedge-shaped were obtained with a scalpel blade. When necessary, held a hemostatic point with absorbable suture. The biopsy fragment must be immediately fixed in 10% formalin and then embedded in paraffin blocks for haematoxylin and eosin (H&E) followed by immunohistochemical staining.

Tissue biopsies from all patients were immunostained for decidual CD 56+ve bright natural killer cell marker. It is a rabbit monoclonal

antibody. The immunohistochemical technique were performed by using the supersensitive avidine–biotin detection kit. After microwave antigen retrieval in citrate buffer (PH = 8) was performed, the slides were treated with 3% hydrogen peroxide in methanol to block the endogenous perioxidase activity. The slides were examined by Dr. Nahla Mohammed Awad consultant of pathology in Ain Shams University (histopathology department) who is unaware of the clinical history. The dNK positive cells were detected as positive membranous and/or cytoplasmic staining. The average cell number per 10 HPF (high power field) were calculated using these scores: 0 = lack of positive cells, 1+ = 1–5 positive cells, 2+ = 6–10 positive cells, 3+ = 11–20 positive cells, 4+ = > 20 positive cells.

The scoring system were classified according to density of NK cell population into:

- Low density: 0, 1+ and 2+.
- High density: 3+ and 4+.

Sample Size Justification

Group sample size of 30 and 30 achieve 85% power to reject the null hypothesis of equal means when the population mean difference is u1-u2=2.0-3.5=1.5 with a standard deviation for both groups of 1.0 and with a significance level (alpha) of 0.050 using a two – sided two – sample equal – variance t-test.

Data Management and Analysis

Data was entered into excel sheet, cleaned and edited. Statistical analysis was done using SPSS ver 21 software.

Quantitative data was presented as range, mean and standard deviation. Comparison between mean of two groups was carried out using independent t – test, while comparison of means of more than two groups was done using one-way ANOVA. I appropriate post hoc comparison between each pairs (post hoc pairwise comparison) was done using Bonferroni t-test.

Qualitative data was presented as number and proportion. Comparing of qualitative data across the groups was done using chi-square test.

P- value: level of significance: P>0.05: Non significant (NS), P< 0.05: Significant (S), P<0.01: Highly significant (HS).

RESULTS

Table (1) shows that there was a statistically insignificant difference between cases and controls as regard Age, gestational age and BMI (P>0.05).

Table (1): Comparison between cases and controls as regard Age, Gestational Age and BMI

Variables	Group				Independent sample t-test	P-value
	Control		Case			
	Mean	Standard Deviation	Mean	Standard Deviation		
	28.85	4.40	28.74	4.37	0.093	0.926
Gestational Age	38.52	1.55	35.93	2.40	4.765	0.999
	28.23	3.54	28.89	2.92	-0.746	0.459

Table (2) shows that there was a statistically insignificant difference between cases and controls as regard Parity, previous abortion and Previous CS (P>0.05).

Table (2): Comparison between cases and controls as regard Parity, Previous Cs and Previous Abortion

Variables		Group				Chi square	P-value
		Control		Case			
		No	%	No	%		
Parity	**0**	6	22.2%	5	18.5%	0.526 FE#	0.962
	1	5	18.5%	4	14.8%		
	2	7	25.9%	7	25.9%		
	3 or more	9	33.3%	11	40.7%		
Previous Abortion	**0**	15	55.6%	15	55.6%	2.074 FE#	0.782
	1	12	44.4%	10	37.0%		
	2	0	0.0%	1	3.7%		
	3 or more	0	0.0%	1	3.7%		
Previous CS	**0**	12	44.4%	11	40.7%	2.792	0.441
	1	6	22.2%	6	22.2%		
	2	7	25.9%	4	14.8%		
	3 or more	2	7.4%	6	22.2%		

(#) Fisher Exact Test was used as (20.0%) of the cells or more have an expected count less than 5

Table (3) shows that there was a highly statistically significant difference between cases and controls as regard Immunohistochemical scores (P<0.01).

Table (3): Comparison between cases and controls as regard Immunohistochemical scores

Immunohistochemical scores	Group				Chi square	P-value
	Control		Case			
	No.	%	No.	%		
0	0	0.0%	7	25.9%	42.133 FE#	.000**
1+	0	0.0%	11	40.7%		
2+	5	18.5%	8	29.6%		
3+	8	29.6%	1	3.7%		
4+	14	51.9%	0	0.0%		

(**) Highly statistically significant at P<0.01
(#) Fisher Exact test was used as (20.0%) of the cells or more have an expected count less than 5

Table (4) shows that there was a highly statistically significant difference between cases and controls as regard dNK Cell Density (P<0.01).

Table (4): Comparison between cases and controls as regard dNK Cell Density

dNK Cell Density	Group				Chi square	P-value
	Control		Case			
	No.	%	No.	%		
Low	5	18.5%	26	96.3%	33.400	0.000**
High	22	81.5%	1	3.7%		

(**) Highly statistically significant at P<0.01

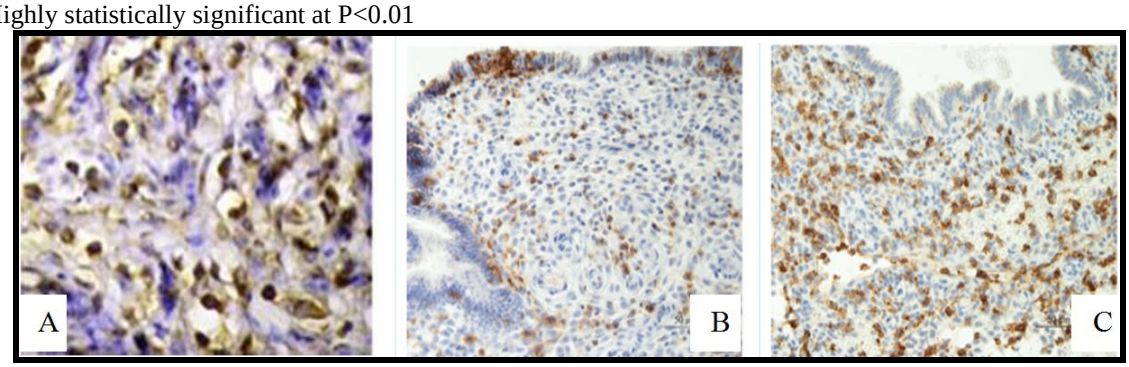

Figure (1): (A, B, C) Immunohistochemical stain of sections from decidua basalis in normal placenta (control group), show dense infiltration with CD56+dNK cells (CD56 x 400) (Positive cells appear brown).

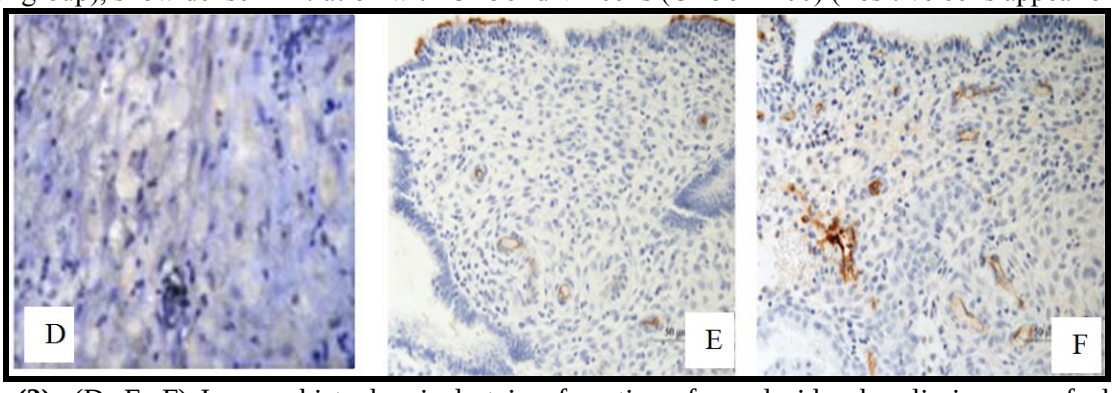

Figure (2): (D, E, F) Immunohistochemical stain of sections from decidua basalis in case of placental abruption (study group) show very scanty positive cells (CD56 x 400) (Positive cells appear brown).

DISCUSSION

The aim of our study was to find if there is any role for decidual natural killer cells in cases with placental abruption. There is considerable evidence that dNK play a role in the implantation and early invasion of trophoblast.

The current study carried out on sixty pregnant women from Obstetric Department, Ain Shams University Maternity Hospitals (six participants were excluded; three patients from the control group have no decidual tissue detected by haematoxylin and eosin (only myometrium) and the remaining three patients from the study group 1 show no retroplacental hematoma during cesarean section and 2 show no decidua. The remaining 54 participants between 20 -35 years old and after 28 weeks of gestation were categorized as follows: 27 consecutive patients considered as study group (singleton pregnancy complicated by placental abruption) and another consecutive 27 women considered as control group (singleton normal pregnancy). Patients were recruited after admission to the hospitals for emergency cessarian section.

All of patients fulfilling our inclusion and exclusion criteria. The stratification of the patients enrolled on the study was one of the strong points in this research. In addition, the exclusion criteria were done strictly to avoid any factors that may affect the distribution of dNK cells in different patients. Furthermore, the semiquantitative scoring analysis of dNK cells provided a more accurate estimation of dNK cells population density in those patients. Demographic data were collected from both groups as regarding: age, parity, BMI, gestational age, previous abortion and previous cesarean sections.

In our study we found that there was no statistically significant difference between the study and control group as regard age; with mean of 28.85, in control group and 28.74, in study group (t- test 0.093, p-value= 0.926), BMI; with mean of 28.23 in control group and 28.89 in study group (t-test 0.746, p-value= 0.459), gestational age; with mean of 38.52 in control group and 35.93 in study group (t-test 4.765, p-value 0.999), parity; with P- value = 0.962 and Chi square 0.526 FE, previous abortion with p-value= 0.782 and chi square 2.074 FE and previous cessarian sections; with P- value = 0.441 and chi square 2.792 FE (P. value > 0.05), this might be an evidence that it was not only the previous demographic data determined the pathogenesis of placental abruption but other immunological factors might play a crucial role as back stop in the process of placental invasion.

Also our results show that there was a highly statistically significant difference between the study and control group as regard immunohistochemical scores (according to CD 56+ %), In control group: score 0 = 0.0%, score 1+ = 0.0%, score 2+ = 18.5%, score 3+ = 29.6%, score 4+ = 51.9% while in the study group: score 0 = 25.9%, score 1+ = 40.7%, score 2+ = 29.6%, score 3+ = 3.7%, score 4+ = 0.0%; (Chi square 42.133 FE) and (P. value = 0.001). Also there was a highly statistically significant difference between the study and control group as regard dNK cells density (uNK cells as a percentage of total stromal cells), in control group: low density = 18.5% and high density = 81.5% while in the study group: low density = 96.3% and high density = 3.7%; (Chi square 33.400 FE) and (P. value = 0.001).

This lowest dNK cells scores and density in placental abruption might indicate that dNK cells played a role in abnormal placental invasion and pathgenesis of placental abruption. These cells appear to protect against undesired deep invasion of extravillus trophoblast (EVT) into the uterine wall. The scanty dNK cells may allow the EVT to invade deeply into the uterine tissues.

To our best of knowledge, this study is the first case-control study that focused on the possible role of the decidual natural killer cells in the pathogenesis of placental abruption.

In our research the decreased expression of dNK cells in patients with placental abruption is in accordance with the results of[25] who studied 76 participants categorized as follows: 26 patients as study group (morbidly adherent placenta previa), 25 patients as comparison group (non adherent placenta previa) and 25 women as control group (normally implanted placenta). The study group was further subdivided into two subgroups according to their postoperative outcome; the first subgroup (A) included 10 patients who underwent a cesarean hysterectomy due to failure of placental separation. The histopathological examination of their uteri revealed invasion of the placental villi deep into the uterine muscle layer (placenta increta). The second subgroup (B) included 16 patients with delayed placental separation and

uterine saving. The immunohistochemical scores of the dNK cells were highest in the control and the comparison groups compared to the other two study subgroups (P < 0.001). The study subgroup (A) showed the lowest dNK cells population density, while dNK cells population scores were higher in the study subgroup (B) compared to the study subgroup (A) (P = 0.002). Moreover, there was no statistically significant difference between the comparison and control groups regarding the dNK cells scores (P = 0.1). They found that CD56+ cells in patients with placenta increta were significantly lowered than in comparison and control group.

Also, our results support the results of[26] as placental bed biopsies from 12 women undergoing elective Caesarean section with no hypertension or foetal growth restriction (FGR), 8 women with FGR without maternal hypertension and 12 women with pre-eclampsia (PE) were used to quantify decidual CD56+ uterine NK cells, CD14+, macrophages, CD3+ T lymphocytes and CD8+ lymphocytes. In PE The numbers of all cell types were reduced compared with third trimester controls; CD3: study 20.4 ± 1.9 versus control 40.3 ± 6.7; P < 0.01; CD56: 18.3 ± 1.9 vs 29.0 ± 3.3; P = 0.01; CD14: 8.3 ± 0.7 vs 17.3 ± 1.2; P < 0.0001.

In FGR without maternal hypertension, there was a similar reduction in the numbers of the three main leucocyte types (CD3: 24.0 ± 2.0; CD56: 14.1 ± 2.7; CD14: 12.0 ± 0.5) compared with control decidua. This reduction was, however, only significant for CD56+ uNK cells (P < 0.05), reductions in CD3+ (P = 0.07) and CD14+ (P = 0.06) just failing to reach statistical significance. No significant differences were identified between decidual leucocyte populations in PE and FGR.

Furthermore, our results is discordant with the result of[27].In that study; the uNK cell density ranged from 0.3 to 19.9% of stromal cells in the recurrent miscarriage group and from 0.3 to 17.1% of stromal cells in the recurrent implantation failure group. Also there was a positive correlation between the number of endometrial arterioles stained and the density of CD56 immunopositive uNK cells. There was also a positive correlation between the number of endometrial lymphatics and uNK cell density. In that study, they found that uNK cell density was positively correlated with endometrial angiogenesis, oedema and a decreased uterine artery resistance to blood flow. An interpretation of these data is that uNK cell function in preimplantation endometrium is to promote angiogenesis and thus endometrial blood flow. Thus, these data provide a potential mechanism by which the increased endometrial uNK cell density noted in multiple studies of recurrent reproductive failure contributes to increased preimplantation angiogenesis leading to early maternal circulation and miscarriage by the final common pathway of excessive oxidative stress.

The exact explanation for the remarkable difference in dNK density in different groups will need further genetic and immunological studies. In addition other lymphocytic subpopulations infiltration in the uterine decidua may be a matter of future research.

CONCLUSION

Decidual natural killer cells density and scoring in deciuda basalis is significantly decreased in cases of pregnancy complicated by placental abruption in contrast to normal pregnancy without complications. So, we believe that further studies are needed to support these results.

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