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Assessment of Decidual Natural Killer Cells CD56^{+} Population in Placental Bed in Fetal Growth Restriction

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ABSTRACT

Aim: this study to compare the density of decidual natural killer cells (CD56) in deciduas of placental bed in patients with Intrauterine Growth Retardation (IUGR) and women with normal pregnancy.

Patients and Methods: in the study 23 pregnant women were recruited and divided into 2 groups: The study group (A): included 14 patient females which diagnosed as IUGR by ultrasound of the third trimester and the control group (B): included 14 normal females at third trimester with no antenatal complications recruited from the antenatal care clinics. All patients underwent elective cesarean delivery. Decidual biopsies were taken during the operation. An immunohistochemical staining for decidual natural killer cells (dNK, CD56+^{bright}) and a semi quantitative scoring were done.

Results: In this study it was found that the Immunohistochemical scores and dNK cell population density is statistically higher in controls compare with IUGR group ($P<0.01$)

Conclusions: findings reinforce the concept of pregnancy as a controlled systemic inflammatory state that if altered can have adverse consequences for the mother and the fetus.

Keywords: Natural killer CD56, intrauterine growth retardation, decidua, immunohistochemical stain.

INTRODUCTION

The most commonly adopted definition to describe a fetus that has not reached its target weight based on sonographic estimated fetal weight (EFW) for a certain gestation is an abdominal circumference (AC) or EFW measurement <10th centile. The American Congress of Obstetricians and Gynecologists (ACOG) and Royal College of Obstetricians and Gynecologists (RCOG) agree that at this cutoff the risk of perinatal morbidity and mortality increases [1].

Altered NK cell numbers and activity have been associated with a variety of clinical conditions such as endometriosis, recurrent pregnancy loss, IUGR, and preeclampsia. Uterine NK cells have a unique phenotype compared to blood NK cells and this is likely due to the specific tissue environment in which they reside. Specific chemokines produced by human endometrium and trophoblasts have been identified that maybe responsible for recruitment of NK cells. Uterine NK cells can produce cytokines and may be an important part of vascular remodeling during placental development [2].

The numbers and proportions of immune cells also differ between blood and decidual tissue, as well as between different phases of gestation [3].

Invasive trophoblasts are the key to the modulation of the state of the uterine vessels. These unique cells leave the placenta, penetrate the endometrium and upper layers of the myometrium, selectively permeate the uterine spiral arteries, and modify these vessels to yield widened, low-resistance vascular channels that carry the markedly increased maternal blood flow to the placenta. Enacting this scenario takes a very delicate balancing of conflicting biological needs between the mother and fetus. The fetus, on the one hand, requires its invasive trophoblasts to penetrate the mother's uterus aggressively in search of vessels to modify. The mother, on the other hand, must protect herself from the invasive trophoblasts, lest they completely penetrate her uterus, causing her to hemorrhage and bleed to death [4].

Aim: This study aims to compare the density of decidual natural killer cells (CD56) in deciduas of placental bed in patients with Intrauterine Growth Retardation (IUGR) and women with normal pregnancy.

PATIENTS AND METHODS

This case control study was conducted at Ain-Shams University maternity hospital starting from April 2016 till January 2017.

In the study 30 pregnant women were recruited and divided into 2 groups as following:

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The study group (A): included 15 patient females which diagnosed as IUGR by ultrasound of the third trimester, and the control group (B): included 15 normal females at third trimester with no antenatal complications, written consent was obtained from all women.

The inclusion criteria: women aged between 20-40 years, Singleton pregnancy, Gestational age of 28-40 weeks, and Reliable dates by early antenatal ultrasound.

The exclusion criteria: Congenital fetal malformation, Fetal hydrops, Multiple pregnancy, Post-term pregnancy, Fetal macrosomia, Intrauterine fetal death, High risk pregnancy (as preeclampsia and DM), autoimmune diseases (as systemic lupus erythematosus SLE), renal and liver diseases, Patients on immune therapy (as steroids therapy or IVIG), Intrauterine infection, Immunocompromised Patients (as human immunodeficiency virus HIV), Prolonged preterm premature rupture of membranes, Morbidly adherent placenta, Patients starting uterine contractions.

Detection of IUGR by ultrasound using fetal abdominal circumference or Expected Fetal Weight less than the 10th percentile for gestational age [5].

During the cesarean section, several tissue biopsies were taken from the decidua basalis of the placental bed from all patients, immediately fixed in 10% formalin and then paraffin blocks were prepared.

Paraffin embedded tissue sections (4 μm thick) from all patients were selected histologically by conventional microscopic examination of H&E stained slides (the selected cases contain enough viable decidual tissue, were tissues contain blood clots or placental tissues have been excluded). The selected cases were immuostained for decidual CD 56+ve bright natural killer cell marker. It was Mouse Monoclonal Antibody ***CD56 (NCAM: 123C3.D5).***

The immunehistochemical technique was performed in the following steps:

Cut 3-4 μm section of formalin-fixed paraffin-embedded tissue and place on positively charged slides; dry overnight at 58°C.

Deparaffinize, rehydrate, and epitope retrieve; using EDTA Ph8 in the microwave oven until boiling. Upon completion, rinse with 5 changes of distilled water.

Place slides in peroxide block for 10 minutes; rinse. then apply the Anti-CD56 is a mouse monoclonal antibody from supernatant diluted in tris buffered saline, pH 7.3-7.7, with protein base, and preserved with sodium azide and incubate for 30 - 60 minutes; rinse. Then apply the link and incubate for 10 minutes; rinse. Then apply the label and incubate for 10 minutes; rinse. Then apply ample amount of chromogen and incubate for 1 - 10 minutes; rinse and then Dehydrate and cover slip**.**

The following scores were used: Low density dNK cell population includ: 0 = lack of positive cells, 1+ = 1–5 positive cells, 2+ = 6–10 positive cells. And High density dNK cell population includ: 3+ = 11–20 positive cells, 4+ = more than 20 positive cells.

The results were calculated by a semiquantitative method described by Wickerek and Galazka [6]. The dNK positive cells were detected as positive membranous and/or cytoplasmic staining. Histopathological examination and immunostaining evaluation was done.

The study was done after approval of ethical board of Ain Shams university and an informed written consent was taken from each participant in the study.

Statistical analysis

The collected data was revised, coded, tabulated and introduced to a PC using statistical package for social sciences (SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, 2001). Data was presented and suitable analysis was done according to the type of data obtained for each parameter. Chi square test was used to examine the relationship between two qualitative variables but when the expected count is less than 5 in more than 20% of the cells; Fisher's Exact Test was used.

P-value: Level of significance as: P>0.05: Non significant, P<0.05: Significant, P<0.01: Highly significant.

RESULTS

In the study 28 pregnant women were recruited and divided into 2 groups as following:

The study group (A): included 14 patient females which diagnosed as IUGR by ultrasound of the third trimester (one patient was excluded because she was in labor).

The control group (B): included 14 normal females at third trimester with no antenatal complications (one female was excluded because she underwent to normal vaginal delivery).

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

Variables	Group				Independent sample t-test	P-value
	Group A		Group B			
	Mean	Standard Deviation	Mean	Standard Deviation		
Age	29.33	±6.33	31.73	±3.56	1.280	0.214
Gestational Age	36.87	±3.14	38.60	±1.50	0.917	0.321
BMI	29.73	±3.10	30.07	±2.99	0.300	0.767

(*) Statistically significant at P<0.05

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

Table (2): Comparison between cases and controls as regard Systolic, Diastolic B.P and HB

variables	Group				Independent sample t-test	P-value
	Group A		Group B			
	Mean	Standard Deviation	Mean	Standard Deviation		
Systolic BP	118.67	±8.34	119.33	±7.99	0.224	0.825
Diastolic BP	76.67	±7.24	78.00	±7.75	0.487	0.630
	9.89	±0.39	9.88	±0.44	-0.044	0.966

(**) Highly statistically significant at P<0.0

Table (2) shows that there is a highly statistically insignificant difference between cases and controls as regard **Systolic, Diastolic BP and HB** (P>0.05).

Table (3): Comparison between cases and controls as regard Birth weight and Apgar score at 5 minutes

	Group				Independent sample t-test	P-value
	Group A		Group B			
	Mean	Standard Deviation	Mean	Standard Deviation		
Birth weight	2.14	±0.55	3.12	±0.76	6.897	0.001**
APGAR score	7.53	±1.60	8.47	.92±0	1.963	0.062

(**) Highly statistically significant at P<0.01

Table (3) shows that there is a highly statistically significant difference between cases and controls as regard **Birth Weight (P<0.01); In addition to that** there is a statistically insignificant difference between cases and controls as regard **APGAR score at 5 minutes**(P>0.05).

Table (4): Comparison between cases and controls as regard Immunohistochemical scores

Immunohistochemical scores	Group				Chi square	P-value
	Group A		Group B			
	No.	%	No.	%		
0	4	28.5%	0	0.0%	20.152 FE#	0.001**
1+	3	21.4%	3	21.4%		
2+	7	50.0%	1	7.1%		
3+	0	0.0%	6	42.8%		
4+	0	0.0%	4	28.5%		

(**) 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 is a highly statistically significant difference between cases and controls as regard **Immunohistochemical scores** (P<0.01).

The following scores were used:

Low density dNK cell population:

- 0 = lack of positive cells,
- -1+ = 1–5 positive cells,
- -2+ = 6–10 positive cells.

High density dNK cell population:

- 3+ = 11–20 positive cells
- 4+ = more than 20 positive cells.

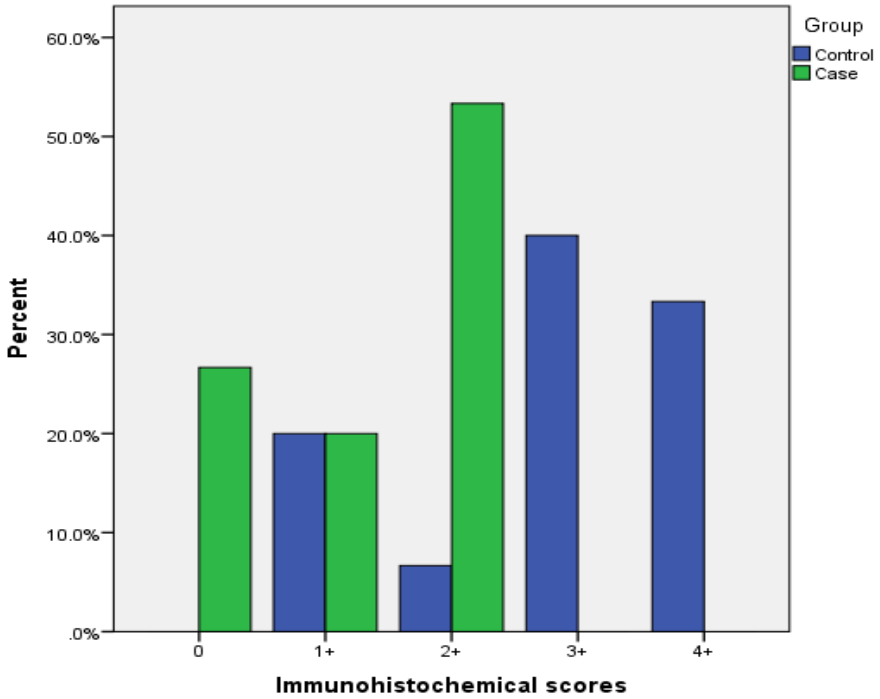

Figure (1): Comparison between cases and controls as regard Immunohistochemical scores.

Figure (1) shows that there is a highly statistically significant difference between cases and controls as regard **Immunohistochemical scores** (P<0.01).

Table (5): Comparison between cases and controls as regard dNK Cell Density

dNK Cell Density	Group				Chi square	P-value
	Group A		Group B			
	No.	%	No.	%		
Low	14	100.0%	4	28.5%	17.368	0.001**
High	0	0.0%	10	71.5%	FE#	

(**) 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 (5) shows that there is a highly statistically significant difference between cases and controls as regard **dNK Cell Density** (P<0.01)**.**

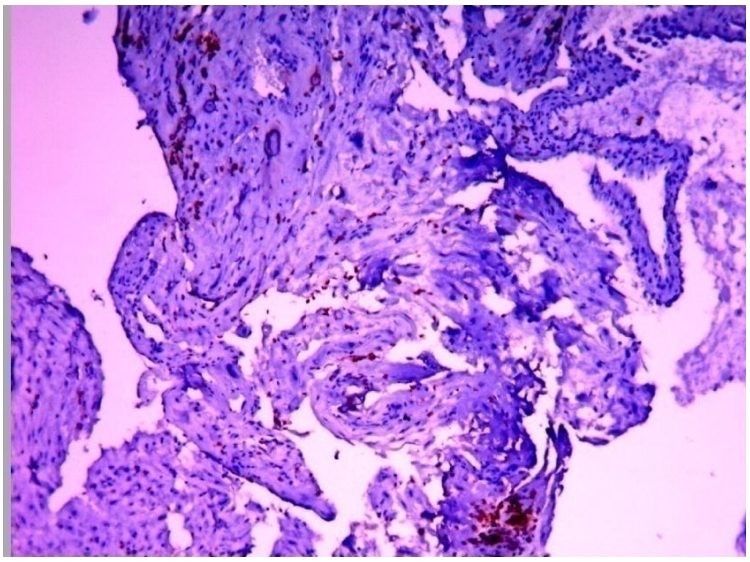

Figure (2):dNK cells CD56in subject from control group shows high density by immunohistochemical score 4+ in HPF x100.

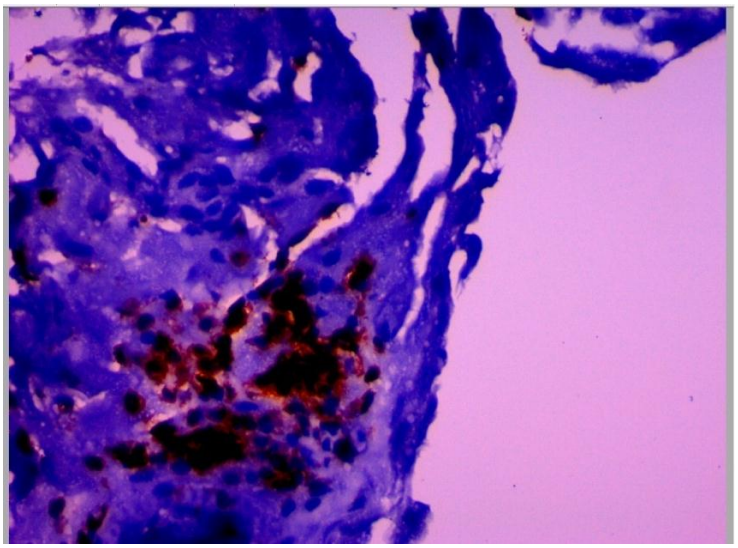

Figure (3):dNK cells CD56 in subject from control group shows high density by immunohistochemical stainin HPF x400.

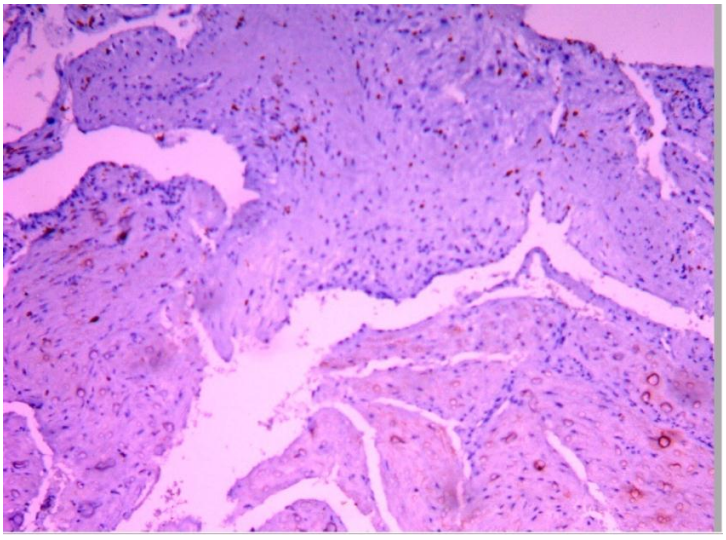

Figure (4):dNK cells CD56 in patient complicated IUGR shows low density by immunohistochemical score 2+ in HPF x100

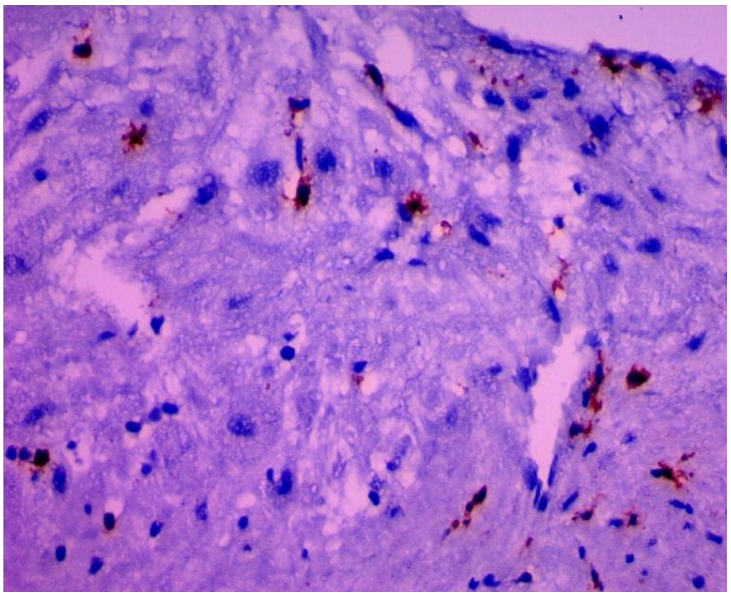

Figure (5):dNK cells CD56in patient complicated IUGR shows low density by immunohistochemical stainin HPF x400

DISCUSSION

Gestational age was matched as closely as possible between subject groups, but the gestational age for the control group was higher than that for IUGR group. Birth weight was significantly lower in the IUGR group than the control group.

In this study it was found that the Immunohistochemical scores and dNK cell population density is statistically higher in controls compare with IUGR group (P<0.01).

In agreement with this study, **Williams *et al.*** [7] in their study which include Placental bed biopsies were performed on 12 healthy women with no hypertension or fetal growth restriction FGR undergoing elective Caesarean section due to breech presentation or previous Caesarean section and 8 women with FGR without maternal hypertension found that In FGR without maternal hypertension, there was a reduction in the numbers leucocyte types (CD56: 14.1 ±2.7) compared with control deciduas (CD56: 29.0 ±3.3). This reduction was significant for CD56+ uNK cells (P<0.05).

Alterations in trophoblast differentiation occur in various pathophysiological situations and may underlie pregnancy disorders, such as preeclampsia and fetal growth restriction (IUGR).

Preeclampsia and IUGR are associated with defects in endovascular extravillous trophoblast EVT invasion, where some spiral arteries are not invaded at all and some are superficially invaded, leading to lack of the normal physiological adaptation of spiral arteries to pregnancy, reduced blood flow into the intervillous space, and relative hypoxia/ischemia. Interstitial EVT density, however, is not different in preeclamptic pregnancy. Minimal EVT apoptosis is seen in normal pregnancy, but 15 to 50 percent of cells are apoptotic in preeclamptic pregnancy, a finding associated with macrophages around spiral arteries [8]**.**

So, in cases of pre-eclampsia, intrauterine growth retardation and stillbirth, inadequate trophoblast conversion of uterine arteries is the primary defect. Only humans have such extensive placental invasion, possibly because of the long intrauterine period that is required for development of the fetal brain [9]**.**

In disagreement with this study, ***Akhlaq et al.*** [10] in their study A total of 150 placentae were collected; of these, 50 belonged to normal pregnancy, 50 belonged to pre-eclamptic women, and 50 were from patients of eclampsia. Placental NK cells were stained the tissue sections for CD56 to identify NK cells using formalin fixed paraffin embedded tissue. The stain was taken up very well using IHC technique, they expressed only in the diseased group According to their observation, NK cells were found to be increased in pre-eclamptic (16.6 ±5) and eclemptic placentae(18±9) in which P value significant 0.008 . It suggested that NK cell functions and interactions with fetal-derived trophoblasts have a profound impact on outcome of pregnancy. Altered NK cell numbers and activity have been associated with recurrent pregnancy loss and pre-eclampsia. It is also suggested that uterine NK cells can produce cytokines that may be an

important part of vascular remodeling during placental development.

Placenta accreta is characterized by lack of intervening decidua which allows direct contact of anchoring villous tissue to underlying myometrium. This leads to excessive invasion, an adherent placenta, and bleeding. The scenario has parallels to tubal pregnancy where the absence of decidua in the fallopian tube is associated with deep invasion of trophoblast into adjacent vessels.

Placental attachment disorders are classified according to the depth of myometrial penetration. In placenta accreta, villi embed on the myometrium in the absence of decidua; in placenta increta, villi embed deeper into myometrium, whereas in placenta percreta, the villi have penetrated through the uterine serosa. Whether the pathology results from lack of decidua or over-invasiveness of trophoblast is controversial.

Studies of EVT invasion in placenta creta are lacking. Conflicting findings have been presented regarding the extent and depth of vascular remodeling by EVT in these pregnancies [11].

Another agreement with this study by ***Laban et al.*** [12] in their study 76 patients from Ain Shams Maternity Hospital between June 2012 to August 2013 were recruited, they were divided into study subgroup (A) which included 10 patients who underwent cesarean hysterectomy due to unseparated placenta accreta, study subgroup (B) included 16 patients with separated placenta accreta, a comparison group included 25 patients with placenta previa and a control group included 25 patients with normally situated placenta. All patients underwent elective cesarean delivery. Decidual biopsies were taken during the operation. An immunohistochemical staining for (dNK, CD56+bright) and the results were The mean dNK cells scores were (0.4 ± 0.5, 1.9 ± 1, 3.3 ± 0.5 and 3.5 ± 0.5) for study subgroups (A), (B) comparison and control groups respectively) with a highly significant statistical difference ($P < 0.001$).

There was a significant statistical difference between study subgroups (A) and (B) $P = 0.002$. These findings suggest that low dNK score was associated with cases of morbidly adherent placenta accreta.

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