

## Natural killer cells, Macrophages and Inflammatory Chemokines in Recurrent Pregnancy Loss: Immunohistochemical Study

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**Abstract: Background:** Recurrent pregnancy loss (RPL) is a common pregnancy complication and is defined as three or more consecutive pregnancies loss before the 20<sup>th</sup> week of gestation. Immunological factors are believed to be a major cause in RPL, where natural killer (NK) cells and macrophages have a crucial role. Their presence in the endometrium at the perigestational period suggests their role in implantation, pregnancy continuation and/or complications. **Objective:** To study the changes of the placental/decidual immune cells in relation to RPL. **Material and Methods:** Placental/decidual samples were collected from 50 cases with RPL (study group) and from another 50 cases with sporadic abortion (control group). Samples were fixed in 10% neutral-buffered formalin and prepared for sections stained with markers for decidual natural killer (dNK) cells CD56, decidual macrophages (dM) CD68, and chemokines (CCL3 and CXCL12). Expression of these markers was detected by using the peroxidase labeled avidin-biotin method. **Result:** Our result indicated that the number of CD56 immunopositive dNK cells was increased significantly in cases of RPL ( $154.80 \pm 81.158$ ) compared to those of sporadic abortion ( $36.80 \pm 16.604$ ),  $P= 0.009$ . In contrast, the optical density of the CD56+ NK cells showed no significant difference between RPL cases ( $0.527059632 \pm 0.112194276$ ) compared to sporadic abortion cases ( $0.4786766 \pm 0.17088177$ ),  $P= 0.117$ . Additionally, the number of decidual CD68 immunopositive macrophages was increased significantly in cases of RPL ( $506.20 \pm 260.522$ ) compared to those of sporadic abortion ( $150.40 \pm 8.532$ ),  $P= 0.009$ . While, the optical density of the CD68+ macrophages showed no significant difference between RPL cases ( $0.4614744 \pm 0.12156944$ ) compared to those of sporadic abortion cases ( $0.4549126 \pm 0.108159912$ ). On the other hand, the number and optical density of CCL3 and CXCL12 immunopositive cells showed no difference between the two study groups. **Conclusion:** Decidual natural killer cells and macrophages have a crucial role in pregnancy continuation. However, great increase in their number could affect the pregnancy outcome, by unclear mechanism's leading to RPL. [Tarek A Atia and Mohamed Abd Elzaher. **Natural killer cells, Macrophages and Inflammatory Chemokines in Recurrent Pregnancy Loss: Immunohistochemical Study.** *Life Sci J* 2014;11(2):134-142]. (ISSN:1097-8135). <http://www.lifesciencesite.com>. 20

**Key words:** RPL, NK cells, decidual macrophages, chemokines, immunohistochemistry

### 1. Introduction

Recurrent pregnancy loss (RPL) or recurrent miscarriage is a common pregnancy complication; where 70% of human conceptions fail to achieve viability. RPL is defined as three or more consecutive pregnancies loss before the 20<sup>th</sup> week of gestation<sup>1</sup>. Epidemiologic studies have revealed that 1-3% of women experience RPL<sup>2</sup>. The risk of miscarriage is 30% after two previous losses and 35% after the third one. This strongly suggests a need for massive evaluation after just two losses in patients with no prior live births. Multiple etiologies have been reported to cause RPL, such as uterine anomalies, coagulation disorders, immunologic defects, endocrine disorders, genetic anomalies and endometrial defects<sup>3</sup>. However, up to 50% of women with RPL are still reported as unexplained, often because of limited clinical investigations<sup>4</sup>.

Immunological factors are believed to be a major cause in RPL. Physiologically the maternal immune response is suppressed selectively during pregnancy, as the fetus represents a foreign graft that the mother tolerates during pregnancy. Feto-placental tissues seem to be immunologically foreign to the maternal host as they contain paternally inherited gene products. Maternal recognition of the paternal antigens plays a fundamental role in the embryo implantation and in the maintenance of pregnancy. Thus pregnancy loss could be due to impaired maternal immune response accounting for 25% of the aetiology<sup>5,6</sup>. T-lymphocytes are the predominant cells in the endometrium and mainly occur at the site of implantation<sup>7</sup>. However, many studies have indicated that T-helper 1 (Th1) cytokines {IL-2, interferon [IFN]- $\gamma$ , and tumor necrosis factor [TNF]- $\alpha$ } and Th2 cytokines {IL-4, IL-5, IL-6, and IL-10} play a fundamental role in continuation or

termination of pregnancy, where Th1 cytokines are harmful and lead to pregnancy loss. Miscarriage is associated with an increased level of IFN- $\gamma$  and decreased level of IL-10, whilst opposite data has been noticed in a control group, supporting the idea that miscarriage is a Th1 phenomenon<sup>8,9</sup>.

Natural killer (NK) cells are predominant in the endometrium. Peripheral blood NK cells usually express CD56 and CD16 receptors on the surface. According to the concentration of the CD56 antigen, NK cells are subdivided into CD56<sup>dim</sup> and CD56<sup>bright</sup> cells. CD56<sup>dim</sup> cells show high cytotoxicity and express high level of CD16. On the other hand, CD56<sup>bright</sup> cells show low cytotoxicity; express low level of CD16, and produce some immunoregulatory cytokines, mainly IFN- $\gamma$ <sup>10,11</sup>. The majority of uterine NK (uNK) cells show high levels of CD56<sup>bright</sup>, but do not express CD16<sup>12</sup>. The number of the uNK cells varies greatly during the menstrual cycle; where their number increases during the secretory phase and the early few weeks of pregnancy, while it decreases greatly after the first trimester and then disappears completely at term. The presence of NK cells in the endometrium at the peri-gestational period suggests their role in implantation and development of trophoblast<sup>13</sup>. The main role of uNK cells is the cytotoxicity and production of cytokines that are controlled by HLA class I antigens. Indeed, women with RPL show an increased number of dNK cells as well as increased number and cytotoxicity of peripheral blood NK cells<sup>14</sup>. On the other hand, the decidual NK (dNK) cells are known to initiate decidualization, regulate trophoblast migration and invasion; and also mediate endometrial angiogenesis. dNK cells express angiogenic growth factors throughout the menstrual cycle, which suggests their role in endometrial angiogenesis and regeneration<sup>12</sup>.

Decidual macrophages (DM) are the second most prominent cells in the maternal-fetal interface after the NK cells. They produce more cytokines as part of their primary role as antigen presenting cells<sup>15,16</sup>. Macrophages are involved in mediating both normal and abnormal placentation as well as in modulating the placental response to infection via regulating T-cell activities. Additionally, macrophages accumulate near the extravillous trophoblasts, phagocytose the apoptotic decidual cells and enhance the extravillous trophoblasts invasion. However, increased number of macrophages and the associated aberrant apoptosis could adverse the pregnancy outcomes<sup>17-19</sup>.

It is currently believed that many adverse pregnancy outcomes are referred to several pathological conditions. Indeed, placental/decidual immune cells mainly NK-cells and macrophages have the potential to illuminate many aspects of these conditions. So, we aim to study the changes and the

immunohistochemical reactivity of these cells in relation to RPL.

## 2. Material and Methods

### Tissue Samples

This prospective study was conducted between June 2011 to August 2013, at the Obstetrics and Gynecology department, at the Salman Bin Abdulaziz University Hospital and King Khalid Hospital, Al-Kharj, KSA. The study protocol was approved by Hospital Ethics Committee. Written informed consent was obtained from all women prior to enrollment in the study. Five hundred women presented with spontaneous miscarriage admitted to our hospital during this period, only 50 women with RPL were eligible for study (study group), another 50 women with sporadic miscarriages and matched for age for those with RPL recruited in the study as control group. Decidual/placental samples have been taken from all cases by dilatation and evacuation without any prior pharmaceutical induction, within the first 24 hours after diagnosis. All enrolled women of the study the following data were collected: mother's age, parity, body mass index, maternal previous diseases, and family history. After that a thorough clinical examination was performed, aiming to exclude apart from common disorders already known as aggravating factors for increased risk for miscarriages. Following evacuation, specimens from both groups were formalin-fixed and paraffin-embedded for further staining.

### Immunohistochemical staining

Decidual/placental samples were fixed in 10% neutral-buffered formalin for 24 h, routinely processed and embedded in paraffin wax, and then sections were mounted onto APES coated slides. Serial sections were stained with markers for uNK cells (CD56), decidual macrophages (CD68), chemokines (CCL3 and CXCL12). Expression of these markers was detected by using the peroxidase labeled avidin-biotin method. Commercially available antibodies for CD56 (Primary Mouse monoclonal antibody anti-CD56 (NCAM) (sc-7326), CD68 (sc-20060), CCL3 (MIP-1 $\alpha$  Antibody (D-3): sc-166942) and CCL12 (SDF-1 Antibody (FL-93): sc-28876) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) were used to recognize these antibodies according to the manufacturer's instructions.

Sequential slides with 4  $\mu$ m-thick tissue sections were deparaffinized and hydrated in sequential treatment of xylene, ethanol and water. Heated citrate buffer (0.01 M citric acid, pH 6.0) was used to retrieve antigens. Endogenous peroxidase activity and non-specific binding were blocked with 3% H<sub>2</sub>O<sub>2</sub>. Sections were then incubated with primary antibodies overnight at 4°C. The following day, biotinylated secondary antibody and streptavidin-horseradish peroxidase were

added. The peroxidase reaction was developed with 3,3'-diaminobenzidine (DAB; Sigma Chemical Co.) to give a brown product. Counterstaining was done lightly with hematoxylin, and the sections were dehydrated in alcohol before mounting. Appropriate positive controls were performed in each staining run, and negative controls were performed for each sample by replacing the primary antibody with mouse IgG.

#### Data registration and Statistical analysis:

The optical density of CD56 immunopositive NK cell, as well as the immunoreactivity of CCL3 and CXCL12 stained cells were assessed using an image analysis technique. Digital images of 10 randomly selected high-power (X400) fields [h.p.f.] were captured and analyzed using Eclipsenet software (Nikon). The numbers of CD56 immunopositive dNK cells, CD68 immunopositive macrophages, CCL3 and CXCL12 immunopositive cells in each field were counted using the manual select tool in the image software.

The results were tabulated and statistically analyzed using a computer program SPSS (statistic a package for social science) version 15 software. The sample mean ( $\bar{X}$ ), standard deviation (SD), and standard error of the mean as well as the range were obtained for numerical variables. For non- numerical

variables, the frequency, distribution and percentage were calculated.

Mann-Whitney U test (nonparametric) was used. The student's (t) test was used to test the significance of the difference between 2 independent means. The Chi square test ( $\chi^2$ ) was used to test whether the distribution of a certain phenomenon among two or more groups was equal or not. The probability ( $P$ ) value was calculated and a  $P$ -value  $< 0.05$  was considered statistically significant.

### 3.Result:

#### 1- Maternal Demographic data

Hundred women were included in the study; 50 women with RPL (Study group) and the other 50 women with sporadic miscarriages and matched for age for those with RPL recruited as (control group). The two groups were similar; there were no significant differences with respect to age ( $p = 0.7$ ), parity ( $p = 0.9$ ), body mass index ( $p = 0.3$ ), previous caesarean section ( $p = 0.8$ ), and gestational age ( $p = 0.4$ ) at time of abortion, but the number of abortions was significantly higher among the study group than among those of control group ( $P = 0.001$ ) (Table 1).

**Table1:** Maternal Demographic data; where BMI= Body Mass Index, C.S= Cesarean Sections

	Study group (n =50)	Control group(n =50)	P value
<b>Maternal age (years <math>\pm</math> SD)</b>	25.4 $\pm$ 4.9	25.1 $\pm$ 3.2	0.7
<b>Parity</b>	2.9 $\pm$ 1.3	2.8 $\pm$ 2.3	0.9
<b>BMI (Kg/m<sup>2</sup>)</b>	24.5 $\pm$ 3.7	23.7 $\pm$ 2.9	0.3
<b>Gestational age at time of abortion</b>	13.8 $\pm$ 4.1	13.1 $\pm$ 3.4	0.4
<b>Number of Abortions</b>	<b>3.5 <math>\pm</math> 0.7</b>	<b>1.3 <math>\pm</math> 0.5</b>	<b>0.001</b>
<b>Previous C.S</b>	9 (18%)	7 (14%)	0.8

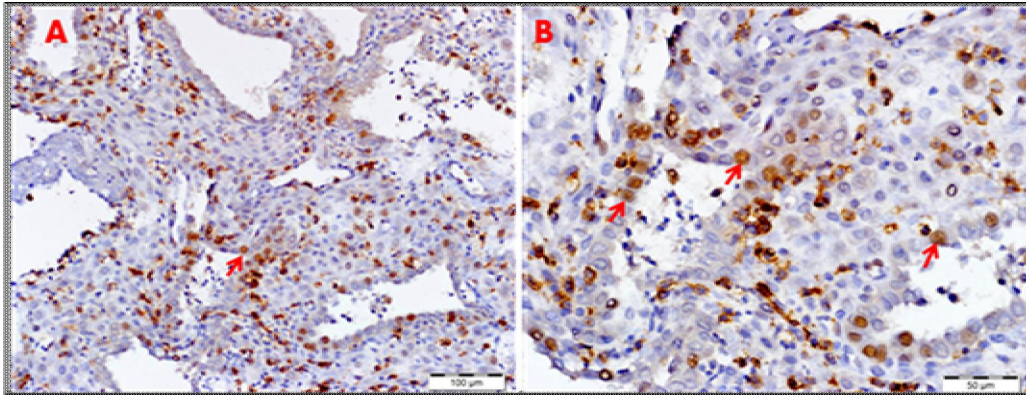
#### 2- Decidual/placental CD56+ NK cells Population and optical density

We have investigated the existence pattern of the decidual/placental NK cells. Tissue samples from cases with RPL and cases with sporadic abortion were immuno-stained against the natural killer cell marker CD56. There was significant increase in CD56+ dNK cells population in RPL cases (**Fig. 1**) compared to sporadic abortion cases (**Fig. 2**); (mean = 154.80  $\pm$  81.158) per 10 h.p.f.; vs. (mean = 36.80  $\pm$  16.604) per

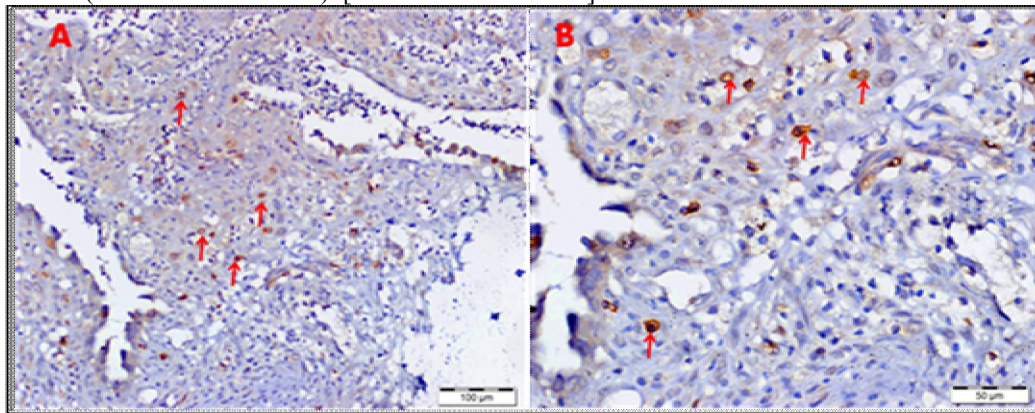
10 h.p.f.; respectively,  $P = 0.009$ . Additionally, there was no correlation between the number of CD56+ NK cells and the maternal age or the number of previous miscarriages.

In contrast, there was no significant difference of the CD56+ NK optical density between RPL cases compared to sporadic abortion cases (mean = 0.527 $\pm$ 0.112 per 10 h.p.f. vs (mean = 0.478 $\pm$  0.170 per 10 h.p.f.; respectively, ( $P = 0.117$ ).





**Figure (1):** Immuno-histogram of decidual tissue of RPL showing the population and the immune-reactivity of CD56+ve NK cells (Brown color reaction). [A: X200 and B: X400]



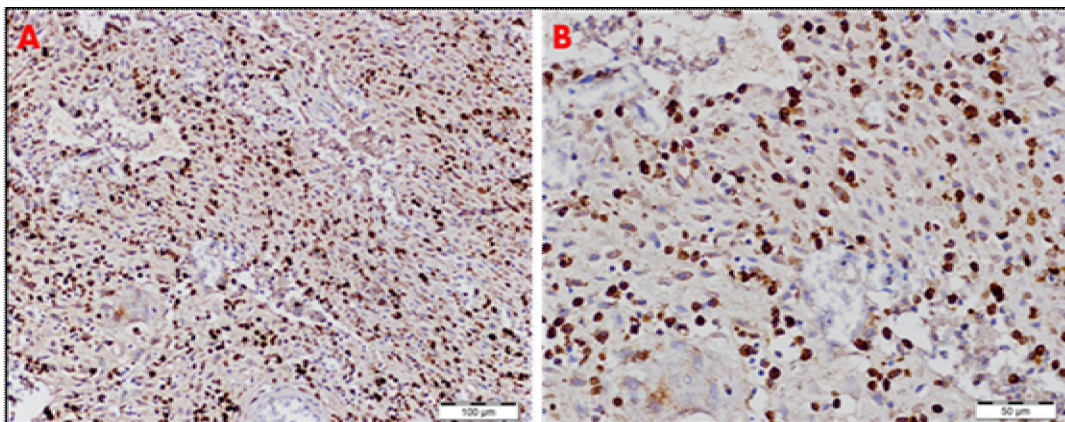
**Figure (2):** Immuno-histogram of decidual tissue of sporadic abortion showing the population and the immune-reactivity of CD56+ve NK cells (Brown color reaction). [A: X200 and B: X400]

### 3- Macrophage Population and optical density:

The decidual/placental macrophages were immuno-stained against the CD68 marker. It was found that the macrophage CD68+ population was significantly increased in RPL cases (**Fig. 3**) compared to sporadic abortion cases (**Fig. 4**); (mean= 506.20 ±

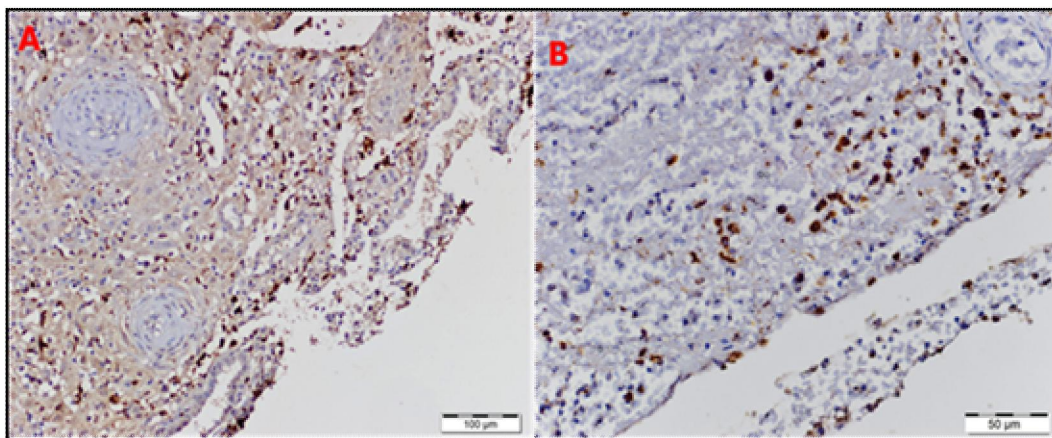
26.522 per 10 h.p.f.) vs. (150.40 ± 8.532) per 10 h.p.f.; respectively, ( $P = 0.009$ ).

In contrast, there was no significant difference of the CD68+ macrophages' optical density between RPL cases compared to sporadic abortion cases (mean = 0.461 ± 0.121 per 10 h.p.f) vs. (mean = 0.454 ± 0.108 per 10 h.p.f.) respectively, ( $P = 0.602$ ).



**Figure (3):** Immuno-histogram of decidual tissue of RPL showing the population and the immune-reactivity of CD68+ve macrophages (Brown color reaction). [A: X200 and B: X400]





**Figure (4):** Immuno-histogram of decidual tissue of sporadic abortion showing the population and the immune-reactivity of CD68+ve macrophages (Brown color reaction). [A: X200 and B: X400]

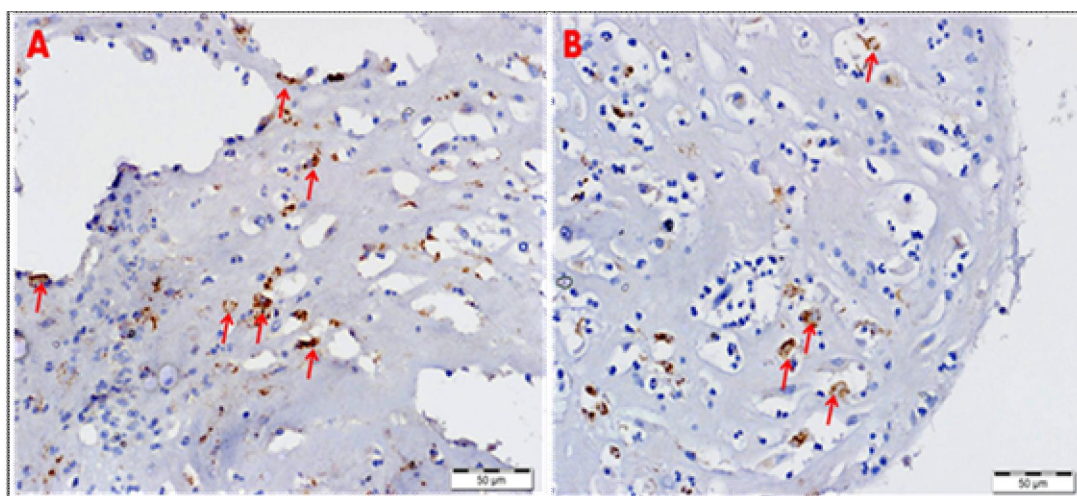
#### 4- Decidual/placental chemokines expression

Chemokines expression in decidual/placental tissue samples from cases with RPL and cases with sporadic abortion were immuno-stained against the CCL3 and CXCL12 markers.

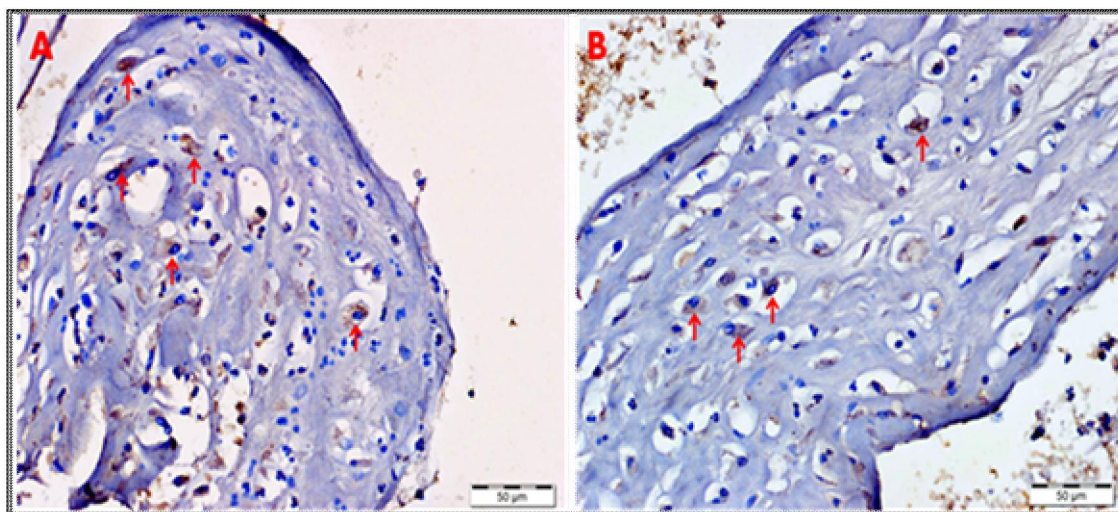
It was found that CCL3 and CXCL12 immuno-positive cell population and their optical density are not significantly changed in RPL cases (**Fig. 5-A; 6-A**) compared to sporadic abortion cases (**Figs. 5-B; 6B**) respectively. Table-2 demonstrates all statistical variations between the study groups.

**Table 2:** Statistical analysis of all cell population the their optical density in cases of RPL vs cases of Sporadic Abortion

	Study group (n=50)	Control group (n=50)	P- value
<b>CD56-Cell Count</b>	154.80 ± 81.158	36.80 ± 16.604	<b>0.009</b>
CD56-Optical dens	0.527059632±0.112194276	0.4786766 ± 0.17088177	0.117
<b>CD68-Cell Count</b>	506.20 ± 26.522	150.40 ± 8.532	<b>0.009</b>
CD68-Optical dens	0.4614744 ± 0.12156944	0.454912624 ± 0.108159912	0.602
CCL3-Cell Count	25.60 ± 12.422	25.20 ± 11.584	0.999
CCL3-Optical dens.	0.4928168 ± 0.11946500	0.4947428 ± 0.11920493	0.602
CXCL12-Cell Count	18.20 ± 9.338	18.00 ± 6.671	0.754
CXCL12-Optical dens.	0.4273994 ± 0.15761949	0.4243105 ± 0.15511662	0.917



**Figure (5):** Immuno-histogram showing the chemokines CCL3 expression in decidual/placental tissue samples (Brown colour reaction) [X400]. A: placental tissue of RPL and B: placental tissue of sporadic abortion.



**Figure (6):** Immuno-histogram showing the chemokine CXCL12 expression in decidual/tissue samples (Brown colour reaction) [X400]. A: placental tissue of RPL and B: placental tissue of sporadic abortion.

#### 4. Discussion:

NK cells; type of granular lymphocytes, involve in the innate immunity. Most dNK cells are phenotypically similar [CD56<sup>bright</sup>+ CD16-] and are highly cytokines secretors<sup>10</sup>. However, the actual origin of uNK cells still unclear, they could originate from the peripheral blood cells, NK cell precursor in the uterine tissue, or could be recruited from other tissues by different mechanisms<sup>20,21</sup>.

Our result indicated that the number of dNK CD56+ cells was increased significantly in cases of RPL compared to sporadic abortion cases ( $154.80 \pm 81.158$  vs.  $36.80 \pm 16.604$ ) respectively, ( $P=0.009$ ). In contrast the optical density of the CD56+ cells showed non-significant differences between the two groups, indicating that most CD56+ cells of both groups are phenotypically similar. Our result were reported by other investigators<sup>10,22,23</sup>, as they had reported a marked increase CD56+ CD16- dNK cells in women who had suffered RPL.

Additionally, other studies had correlated the percentage of dNK cells and the pregnancy outcome, as the increasing percentage of CD56+ dNK cells is usually associated with recurrent miscarriage, recurrent implantation failure or even female infertility<sup>24,25</sup>. Also, Quenby et al<sup>26</sup> has noticed a correlation between the increased number of pre-implantation uNK cells and miscarriage in a subsequent pregnancy. On the other hand, many studies had believed that the dNK cells are originated from the peripheral blood NK cells, and had made a positive correlation between the increasing number of the peripheral blood NK cells and the increasing number of dNK cells in cases of recurrent miscarriage. Moreover, they had shown that

measurement of CD56 NK cells in peripheral blood tend to be a highly specific screening tool for recurrent miscarriage<sup>14,27</sup>. In contrast, Laird *et al.*<sup>28</sup> reported that there was no correlation between the number of dNK CD56+ cells and peripheral blood NK CD 56+ cells in cases of RPL, as both types of cells have different functions. Peripheral blood NK cells are part of the innate immunity, but the functions of uNK cells are variable, and could include a close correlation with MHC complex molecules on the invading trophoblast, production of chemokines and cytokines, angiogenesis or innate immunity<sup>19</sup>.

Decidual NK cells are highly granular cells, active cytokine producers; and they have potent cytolytic potential<sup>29</sup>. Their granules contain granulysin (exhibits potent cytotoxic activity) which is coexpressed with and functionally related to both perforin (apoptotic/cytolytic protein), and granzymes<sup>30</sup>. Nakashima *et al.*<sup>31</sup> noticed that the number of decidual granulysin positive dNK CD56<sup>bright</sup> and granulysin positive decidual lymphocytes were significantly increased in cases of RPL. Also, they speculated that granulysin of dNK CD56<sup>bright</sup> cells together with the action of perforin causing apoptosis of extravillous trophoblasts and subsequent abortion. Additionally, Rodrigues *et al.*<sup>10</sup> reported that increased uNK cell populations is associated with increased angiogenesis and blood flow during peri-implantation, which can lead to early maternal circulation with exposure to excessive oxidative stress that can cause pregnancy failure.

According to macrophages, our result indicated that the number of decidual immunopositive CD68 macrophages was increased significantly in RPL cases



compared to sporadic abortion cases ( $506.20 \pm 26.522$  vs.  $150.40 \pm 8.532$ ) respectively, ( $P=0.009$ ). In contrast the optical density of the CD68+ cells showed non-significant differences between the two groups. Our result was indicated by others<sup>15,31,32</sup>, as they had indicated that there was marked increase decidual CD68+ macrophages in women who had suffered RPL. In contrast, Vassiliadou *et al.*<sup>33</sup> had reported that the increased macrophage cell population was not significant, while Quack *et al.*<sup>34</sup> have demonstrated that there was no difference in the number of macrophages among cases with RPL and spontaneous abortion. However, excess macrophages together with impaired trophoblastic cell apoptosis can exaggerate the maternal inflammatory response to the invading trophoblasts resulting in pregnancy failure. Also, macrophages expression of excess IL-1 $\beta$  and TNF- $\alpha$  associated with infections can cause impaired trophoblasts invasion and abnormal pregnancy outcome. Additionally, excess activated decidual macrophages as well as stimulated first trimester decidual cells by pro-inflammatory cytokines promote extravillous trophoblast apoptosis<sup>35</sup>.

Macrophages have a wide dynamic plasticity; their response to external stimuli is variable and can produce M1 or M2 phenotypes. M1-phenotype is proinflammatory and activated by interferon- $\gamma$ ; while M2-phenotype is anti-inflammatory alternatively activated by IL13 or IL4<sup>36</sup>. M1 and M2 activated macrophages perform different functions; M2 phenotype can be anticipated during normally developing pregnancy; while, the M1-subtype could be a part of mechanism causing pregnancy failure<sup>37</sup>.

Chemokines and chemokine receptors are involved in materno-fetal immune regulations, macrophage migration and angiogenesis<sup>38</sup>. Our result noticed an expression of chemokines CXCL12 and CCL3 in decidual tissues of both study groups. Where, the cell population and the optical density of the immunopositive cells showed no significant difference among both groups. This result was indicated by Park *et al.*<sup>21</sup>, as he had noticed that the immunopositive cell populations and the intensity of CXCL12 and CCL3 reactivity in decidual tissues were not correlated to the number of dNK CD56+ cells. However, other investigators indicated that invasive trophoblast cells have the ability to produce the chemokines CCL3, CCL14, and CXCL12<sup>39</sup>; where their corresponding receptors are found on the decidual macrophages and NK-cells. This in turn indicated that the expression of these chemokines by trophoblast cells plays an important role in the recruitment of specific leukocytes to the decidua<sup>40,41</sup>. Therefore, from this result and others<sup>42</sup> we have speculated that expression of these chemokines regulate leukocytes migration to the site of implantation regardless the pregnancy outcome.

**In conclusion**, decidual NK cell and macrophages play a crucial role in normal pregnancy continuation, while increasing level of these cells could affect the pregnancy outcome. In contrast the decidual chemokines expression seems to be correlated directly to recruitment of leukocytes to the site of implantation regardless the pregnancy outcome or the pathophysiology of the pregnancy itself. Additionally, further studies are needed to investigate other possible causes of RPL such as chromosomal aneuploidy as well as expression of apoptotic markers in decidual tissues.

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