THE ROLE OF IMMUNE INTERACTION BETWEEN NATURAL KILLER CELLS AND HLA-C MOLECULES IN RECURRENT SPONTANEOUS ABORTION IN WOMEN OF BASRAH PROVINCE

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SUMMARY

Pregnancy is a unique symbiotic union between mother and fetus which contradicts many of the general rules of immunology. Clarifying the cellular mechanisms involved in the maternal-fetal cross talk will enable us to establish therapies for gestational problems. Recurrent spontaneous abortion (RSA) one of the most important problems occurs during the first trimester of human pregnancy. RSA with unknown causes is may correlated with immunological alterations. The study aimed to investigate the immune disorders regarded with natural killer cells (NK) and their activation through using the inhibitory and activating receptors associated with human leukocyte antigen-C molecule. The study had been applied to women with recurrent abortion that attending the department of obstetrics and gynecology of al-Fayha teaching hospital and Basrah Maternity and Children hospital from January 2021 until the end of May 2021. A total of 80 samples were included, 55 patients with recurrent miscarriage in the first trimester of pregnancy, their ages ranged from 20-35 years, and 25 healthy pregnant women similar to patients in terms of age and pregnancy period recruited as a control group. The samples were divided into two age groups: group 1 (G1) ranged (20-27) years and group 2 (G2) ranged (28-35) years, the results showed no significant difference (p ≤ 0.928) according to age. II Complete blood count (CBC) was performed to determine total lymphocytes which showed elevated levels in women with RSA in the two age groups. Peripheral natural killer (p NK) cells were measured by flow cytometry and determined as CD3⁻ CD16⁺ which showed a significant difference (p ≤ 0.030) in patients in comparison with control. CD16⁺ CD56⁻ a subset that covers 90% of p NK cells had been evaluated and showed significant rising in aborted women than healthy pregnant women. Flow cytometric evaluation of killer-cell immunoglobulin-like receptor (KIR2DL1) that is expressed on NK cells surface had been determinate and showed high significant elevation in healthy pregnant women in comparison with RSA women. Furthermore, soluble human leukocyte antigen-C (HLA-C) showed high significant levels in the control group in comparison with aborted women. Statistically, a positive correlation between KIR2DL1 (CD158a) and HLA-C molecules cells (p ≤ 0.01) had been found.

The study suggests that natural killer cells correlate with the occurrence of recurrent abortion by their inhibitory receptors associated with HLA-C molecules. Indeed, the elevated levels of the pro-inflammatory cytokines may act as an indicator for early diagnosis of miscarriage.

I. INTRODUCTION

Recurrent pregnancy loss (RPL) is a pregnancy disorder experienced by ~2-3 % of women trying to conceive. RPL is defined as the loss of two or more clinically recognized pregnancies before 20 - 24 weeks of gestation [1]. The etiologies of RPL are complex and multifactorial; including anatomical defects, hormonal imbalance, infections, chromosomal abnormalities, environmental causes and immune factors [2]. Nearly half of the cases of RPL remain unsolved and are treated empirically using supplements with progesterone, anticoagulation, or immunomodulatory methods [3]. It is believed that 50% of the causes of miscarriage due to the rejection of the fetus by the maternal immune system [2]. To prevent the recognition and rejection of the fetus, three major immune escape mechanisms occur during human pregnancy: (a) The separation between the mother and the fetus anatomically by the placenta barrier. (b) Alteration of human leukocyte antigen (HLA) that is expressed on the placental cells. (c) Suppression and modulation of the maternal immune cells [4]. The HLA is a very polymorphic molecule in the immune system that guides the immune response toward the antigens. HLA incompatibility is considered the main cause for
allograft rejection [5]. Human leukocyte antigens sharing between couples have an important role during pregnancy [6]. These molecules interact with natural killer cells (NK), CD8 T lymphocyte cells and CD4 T cells to induce immune cooperation that removes foreign or non-self-antigens [7]. HLA-C can trigger an immune response by maternal NK cells and T cells to establish maternal-fetal immune tolerance which needs to the fetus acceptance [8]. Chapter one Introduction 2 Natural killer cells which are large granular lymphocytes derived from bone marrow [9], are innate immunity effectors that can carry out a cytotoxic activity toward tumor and virus-infected cells and are involved in cytokine production [10]. There are two subtypes of NK cells classified according to their expression of CD16 and CD56 surface markers. Peripheral NK cells (p NK) that are CD16+CD56- and decidual NK cells which express CD16+ CD56+ surface markers [11].The function of the NK cell is regulated and induced by an array of inhibitory and activator receptors on its surface [12]. The activating receptors include natural cytotoxicity receptors (NCRs) (NKp30, NKp44, and NKp46) that induce NK cell cytotoxicity. The inhibitory receptors are the killer-cell immunoglobulin-like receptors (KIRs) which are heterodimers and the ligands for the major histocompatibility complex (MHC1) [13]. If the target cell normally expresses MHC molecules (HLA in humans) on its surface that engaged to the inhibitory receptors on NK cells, this cell will be protected from the lysis by NK cells. In contrast, if these cells lack the expression of HLA, natural killer cells will release their perforin and granzymes granules and destroy the target cells [14]. KIR2DL1 which is also known as CD158a, the inhibitory receptor of NK cell, can interact with its ligand (HLA-C molecule) and inhibit NK cells cytotoxic function [15]. These interactions between KIRs and HLAs in normal conditions give an immune tolerance to the fetus. In the human genome, both KIR and HLA genes are highly polymorphic, thus there are different interactions between KIRs associated with HLAs molecules [16], and It possible that is some of these unique combinations have a powerful effect in autoimmune disorders underlying recurrent miscarriage [17].

The aim of the study
1. Determine the levels of total NK cells and CD16+ CD56- subset in peripheral blood by flow cytometry.
2. The expression of KIR2DL1 receptor by flow cytometry procedure using CD158a.
3. Determination of soluble HLA-C molecules by ELISA.

II. METHODS

Study population
This study had been conducted between January 2021 to May 2021. Venous blood samples were collected from women who were attending the department of obstetrics and gynecology of Al-Fayha Teaching Hospital and Basrah Maternity and Children hospital with ages ranging from 20 to 35 years. A brief caption of the project was explained to the participants before sample collection. Written informed consent has been gained from all participants before their inclusion. The medical histories of the study population and some required data such as age were obtained by direct interview with women by using a questionnaire.

Study design
The studied women were divided into two groups:
1. Aborted women: Fifty-five women with a history of first trimester recurrent spontaneous miscarriage (two or more previous abortions) were admitted to the hospital with vaginal bleeding for evacuation. The women who had uterine abnormalities, thyroid dysfunction, anti-phospholipid syndrome, ovarian cysts and positive TORCH test had been excluded from this study
2. Normal pregnant women: Twenty-five normal pregnant women that had previously at least one lived child with no history of miscarriage, ectopic pregnancy and preterm delivery were regarded as a control group.

Samples collection
Five milliliters (ml) of blood was collected by vein puncture from aborted women at the time of miscarriage at the first trimester and from the normal pregnant women at the same period of pregnancy. Each sample was divided into two parts: Two ml of blood was collected in a tube containing ethylene diamine tetra acetic acid (EDTA) to estimate total natural killer cells, CD16+ CD56- subset and the expression of KIR2DL1 receptor (CD158a) by
flow-cytometry and three ml of blood was collected in GEL tube. The serum was separated by centrifuge for 3 minutes at 1500 rpm to determine the levels of HLA-C molecules.

**Immunological study**

**Flow cytometry**

Total Natural killer cells, CD16+ CD56- subset and KIR2DL1 (CD158a) receptor were determined for 55 patients and 25 healthy control using BD Accuvi C6 flow cytometry. Four monoclonal anti-human fluorescent-labeled antibodies as flow cytometry reagents were used including Anti-Human CD16 (PE), Anti-Human CD56 (PE-Cy), AntiHuman CD3 (APC-H7) and Anti-human CD158a (APC) Natural killer cells determined as CD3+ CD16+, NK cell subset determined as CD3+ CD16+ CD56-, and KIR2DL1 was determined as CD158a+, figure(1).

**ELISA**

Human MHCC/HLA-C (Major Histocompatibility Complex Class I C) ELISA Kit was used to determine soluble HLA-C antigens. The kit based on sandwich enzymelinked immune-sorbent assay technology.

**Statistical Analysis**

The data were statistically analyzed using SSPS software and the significance of the observed differences, associations, or calculations was determined at p-value < 0.05. Chi2 statistical test was used to investigate the significance of associations; Kruskal-Wallis and Mann-Whitney tests were used for differences between the groups of non-parametric data, and Spearman's test to examine nonparametric correlations.

### III. RESULTS

Present study revealed that there were no significant differences (p ≤ 0.928) between patients and control according to age range groups, table (1). Significant elevation was found in the total number of natural killer cells in RSA women in comparison with normal pregnant women. Moreover, CD16+ CD56- showed significant difference between patients and control, table (2). The expression of KIR2DL1 receptor was highly increased in normal pregnant women in comparison with patients. Furthermore, statistical analysis indicated that there was high significant difference in HLA-C molecules between women with RSA and control, table (3).

**Table (1): Distribution of patients and control according to age range groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age range groups (years)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1 (20-27) years</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>patients</td>
<td>28</td>
<td>51 %</td>
</tr>
<tr>
<td>control</td>
<td>13</td>
<td>52 %</td>
</tr>
</tbody>
</table>

* Significant; P-value <0.05

**Table (2): Levels of NK cells and CD16+ CD56- in blood of patients and control**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>NO.</th>
<th>Mean</th>
<th>Range</th>
<th>S.D±</th>
<th>p. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK cells</td>
<td>Patients</td>
<td>55</td>
<td>15.03</td>
<td>32.85</td>
<td>9.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>25</td>
<td>10.28</td>
<td>26.62</td>
<td>6.47</td>
<td></td>
</tr>
<tr>
<td>CD16+ CD56- subset</td>
<td>Patients</td>
<td>55</td>
<td>12.68</td>
<td>28.92</td>
<td>10.46</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>25</td>
<td>7.24</td>
<td>28.62</td>
<td>7.45</td>
<td></td>
</tr>
</tbody>
</table>
Table (3): Levels of KIR2DL1 receptor and HLA-C in patients and control

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>NO.</th>
<th>Mean</th>
<th>Range</th>
<th>S.D±</th>
<th>p. value</th>
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</thead>
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<tr>
<td>KIR2DL1 receptor</td>
<td>Patients</td>
<td>55</td>
<td>30.40</td>
<td>84.38</td>
<td>15.80</td>
<td>0.000</td>
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<td></td>
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<td>62.94</td>
<td>76.5</td>
<td>23.32</td>
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</tr>
<tr>
<td>HLA-C</td>
<td>Patients</td>
<td>55</td>
<td>2.25</td>
<td>9.30</td>
<td>1.92</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>25</td>
<td>7.55</td>
<td>11</td>
<td>2.62</td>
<td></td>
</tr>
</tbody>
</table>

![Figure 1](attachment:image.png)

**Figure 1** NK cells gating by flow cytometry. A-lymphocyte determination B- Total natural killer cells determination C- Determination of CD16+CD56- subset. D- KIR2DL1 receptor (CD158a) expression levels.

**IV. DISCUSSION**

NK cells are critical in the maintenance and establishment of pregnancy because of their functional variety, including their roles in vascular remodeling of placenta and immune-regulation. Accumulating evidence indicates an association between the disorders of NK cells and the occurrence of RSA [18]. Peripheral NK cells circulate around the implantation site then migrate into the endometrium and decidua and make contact with the trophoblast cells [19]. In present study, by flow cytometry we found that women with a history of RSA had a significantly higher NK cells levels in their peripheral blood (p ≤ 0.033) in comparison with normal pregnant women. This result is in agreement with several studies including [20] [21] [22] whom showed that there was an increasing in NK cells in women with recurrent abortion. The function and numbers of pNK cells show broad variability upon the patient's condition, e.g. autoimmunity or tumor, infections, treatment condition (ovarian stimulation), day of the menstrual cycle, time of day, stress and exercises [23].This may be the most acceptable explanation for the difference in NK
cells numbers between the two studied groups. We believe that the high number of circulating NK cells could be a non-specific but important peculiarity of tendency to miscarriage. In fact, in RSA patients, the high NK value is not linked to the condition of being pregnant, but present yet before, as a distinctive mark of their immune system. In contrast, [24] [25] [26] was reported that women with RSA and normal pregnant women had the same ratio of peripheral NK cells.

**CD16+ CD56- subset**

Peripheral NK had been subdivided into two subtypes, 90% are cytotoxic CD16+CD56- NK cells, where the remaining 10% is non-cytotoxic CD16- CD56+ NK cells [27]. Depending on recorded data related with flow cytometric investigation of CD16+CD56-, current work indicated that this subset of pNK significantly increased (p ≤ 0.030) in women with RSA in comparison with control group. This result is agree with other researches like [28] [29] [30] whom found an increasing levels of CD16+CD56- subset in RSA patients in compared with normal pregnant women. According to [30], in addition to the importance of the total ratio of natural killer cells, analysis of NK cell subsets can be useful in gaining a better understanding of the reasons of recurrent abortion. Elevated levels of cytotoxic CD16+CD56- pNK cells results in imbalance of immune-related parameters may alter the tolerance of the maternal immune system to the fetus which can lead to pregnancy loss [31] According to these results, we should mention which immune system suppression is needed for successful pregnancy, absolutely, several studies showed that immunoglobulin treatment does not rise the live birth rate in women with recurrent miscarriage [33] [34]. The relationship of miscarriage with an increased percentage of CD16+ CD56- cells in PB strongly establishes future researches to recognize the value of NK cells subsets in evaluation patients with recurrent abortion.

**Killer cell immunoglobulin-like receptor KIR2DL1 levels**

KIR2DL1, a transmembrane glycoprotein, can practically recognize HLA-C molecule. Inhibitory motifs based on tyrosine in immunoreceptor inside the phosphorylated structure can transport negative regulation signals to inhibit NK cells killing activity [35]. Flow cytometric analysis in our study showed that the expression of KIR2DL1 receptor (CD158a) in peripheral blood of aborted women lower than the control group (p ≤ 0.000). Our results support the findings of other investigators such as [22] [36] whom found similar results. Similarly, [37] [38] [39] reported that there was confined gene expression of the inhibitory KIR2DL1 receptor in women with RSA compared to women with successful pregnancies. Data presented by [40] confirmed that maternal KIR2DL1 inhibitory receptor was correlated with pregnancy disorders which linked to unsuitable placentation and it had an adverse effect on the weight of the fetus.

KIR haplotypes are highly polymorphic, differing not just in nucleotide sequence but also in gene content [41]. This genetic complexity reflect the complications confronted clinically in defining and diagnosing recurrent abortion which can be considered not as a disease or disturbance but “simply the extreme end of a continuum of characteristics common to all pregnancies”[42]. Conversely, some studies such as [43] [28] did not agree with our results, they found that women with RSA had higher levels of KIR2DL1 receptor than normal pregnant women. Whereas [44] [45] found that there was no significant difference between the two groups. The inconsistent results may be due to ethnic origin as well as exclusion and inclusion criteria in the preference of patients and controls [46].

**HLA-C**

Human leukocyte antigen-C is the only classical HLA molecule expressed by extra villous trophoblast that present an extensive variety of peptides to maternal T cells and provides protective immunity. Paternal HLA-C expressed by EVT represents a target for maternal T cells and NK cells, thus HLA-C levels may have an impact on how this response is developed. This bilateral function of HLA-C requires strict transcriptional regulation of its levels to induce a balance of tolerance and immunity [8]. Current study demonstrated that serum soluble HLA-C was diminished in RPL patients compared to normal pregnant women. In a study on Chinese Han population, also it was found that there was decreasing in HLA-C ligands in women with RSA compared with healthy pregnant women [39]. Other studies including [47] revealing similar finding and observed that higher frequencies of HLA-C molecules was found in healthy pregnant women. The analysis of the KIRs and HLA-C molecules allo-recognition has been pursued as a new way in studying the immunological etiologies of pregnancy defects including preeclampsia and recurrent abortion. Some studies reported the correlation of appropriate NK cells activation/inhibition and the outcome of pregnancy [48] [39]. According to our results, we support previous studies
which suggested that decreased ligands (HLA-C molecules) for the inhibitory KIRs could potentially increase NK cell activation, mediated by the activating receptors: As a result, it contributes to the pathophysiology of RSA. In contrast, no significant difference in class I or class II HLA molecules was detected between women with and without recurrent abortion in [49] study. Our findings support the idea hypothesized that interaction between maternal KIRs on NK cells and HLA-C molecules affect the placentation process and have a relationship with the occurrence of RSA which also indicated by the statistical analysis positive correlation between KIR2DL1 receptor and HLA-C molecules.

Conclusion

As there was an increasing in peripheral natural killer cells levels in women with recurrent abortion which conducted in current study, we suggest that NK cells have a correlation with the occurrence of RSA. The relationship of miscarriage with an increased percentage of CD56+ CD16+ cells in blood of aborted women had been indicated in this study. KIR2DL1 (CD158a) receptor expression significantly rising in healthy pregnant women. Furthermore, HLA-C molecules strictly diminished in women with RSA.

REFERENCES

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