ENDOMETRIAL DIFFERENTIATION IN PATIENTS WITH RECURRENT ABORTION: A MORPHOMETRIC AND IMMUNOPHENOTYPIC STUDY

By
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ABSTRACT

Twenty non pregnant women with unexplained recurrent abortion were involved in this study to evaluate endometrial differentiation in the perimplantation phase. A assessment of glandular and stromal compartments was done using morphometric criteria. Also, the endometrial leukocytes were evaluated by flow cytometric analysis to determine their immunophenotypic characteristics. The results were compared with the findings in a control group of 12 normal fertile women.

Retarded endometrial development was found in 9 out of 20 women (45%) in the recurrent abortion group. Measurable differences in glandular development (i.e., numbers of glands and glandular epithelial height) were found in this subgroup. The percentage of endometrial CD8+ suppresser T-lymphocytes (+ve cluster differentiation) was significantly decreased and CD4+: CD8+ ratio was significantly increased in the recurrent aborters (P < 0.05). In contrast, the proportion of B-lymphocytes (CD20+) was significantly increased (P < 0.05). The proportion of natural killer (NK) cells was identical in both groups CD16+ CD56 dim NK cell subset with cytolytic activity was higher in the habitual aborters.

It is concluded that women with unexplained recurrent abortion may be divided into two distinct subgroups on the basis of their endometrial response in the pre-implantation period and that it is reasonable to make the diagnosis of endometrial abnormality based on a single accurately timed endometrial biopsy. Also, the endometrial lymphocytes of recurrent spontaneous aborters have a distinct immunophenotypic profile that antedates implantation and suggests that endometrial immunologic conditions are intrinsically altered in recurrent aborters.

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INTRODUCTION
Several improvements and extensions have been introduced to the previously reported methods for the investigation of the endometrium in recurrent abortion. The previous studies have reported retarded endometrial development in patients with recurrent abortion\(^{(1,2 \text{ & } 3)}\). All the studies used the criteria of\(^{(4)}\) to evaluate the endometrium that may lead to inconsistencies arising from interobserver variation. The use of a late luteal phase biopsy has meant that information about the state of the endometrium at the expected time of nidation is lacking. The use of morphometric analysis of the endometrial glandular and stromal structures becomes more objective and yields detailed information on individual features\(^{(5)}\).

Recent studies of non pregnant endometrium of recurrent aborters observed a distinct immunophenotypic profile of leukocytes that suggest a hostile terrain that may be responsible for fetal loss\(^{(6,7 \text{ & } 8)}\). Flow cytometric evaluation of endometrial leukocytes has the potential to become an important tool in the evaluation of immune mechanisms involved in patients who may have immunologically mediated abortions\(^{(9,10)}\).

The aim of the present study was to evaluate the endometrium of non pregnant women with unexplained recurrent abortion, in the peri-implantation phase, using recent morphometric criteria. Also, we aimed at determining the immunophenotypic characteristics of T and B-lymphocytes, NK cells and monocytes and to compare the results with that of normal fertile control group to investigate the possibility of presence of underlying endometrial morphological or immunological abnormalities.

SUBJECTS AND METHODS
Twenty patients with unexplained recurrent abortion were recruited from the gynecologic outpatients clinic of Benha University Hospitals. All had experienced 3 or more consecutive spontaneous abortions in the first trimester with the same partner. All had no apparent cause and revealed no abnormality by the following tests: hysterosalpingography, antiphospholipid antibodies and peripheral leukocyte karyotyping.
Women with infectious (viral and others) or autoimmune diseases were excluded. A control group consisted of 12 normal fertile women with the following criteria: regular menstrual cycle, a history of two or more successful pregnancies, no use of steroid hormones or intrauterine device for at least 6 months before entry into the study.

Endometrial biopsies were performed 7 days after documented ovulation-based upon ultrasonographic evidence (the approximate time of blastocyst implantation into a receptive endometrium).

The biopsy was performed as outpatient procedure using Novak's curette. The material was divided into 2 portions: one fixed into buffered formalin, then embedded in paraffin, sectioned and stained with hematoxylin and eosin and used for morphological study. The second was put in RPMI 1640 medium and used for immunophenotypic study of endometrial leukocytes (T and B lymphocytes, NK cells and monocytes).

**Morphological study:**

Dating of the endometrial samples was done in relation to the day of ovulation in normal cycles. The endometrial dating was defined as histological dating in days minus chronological dating from the day of sonographic detection of ovulation. A diagnosis of retarded endometrium was made if histologic dating was found to be 2 days or more behind the chronological date(11).

The functional endometrial layer was subjected to semiquantitative morphometric evaluation using an image analysis (IMTEC, EXCELL, Uppsala, Sweden). The morphometric parameters included the following indices: (i) number of glands per mm²; (ii) diameter of gland (um); (iii) glandular epithelial height (um); (iv) number of basal vacuoles per 100 gland cells. Using quantitative assessment (EDIS) program, (IMTEC), the volume densities of endometrial glands and stroma were calculated.

**Immunophenotypic characteristics of endometrial leukocytes:**

This work was carried out using the FAC Scan flow cytometry (Becton & Dickinson).

The portions of endometrial samples that were placed in sterile RPMI 1640 media were first
mechanically grinded. The cell suspension was then filtered in order to generate a leukocyte enriched fraction. Labeling of endometrial leukocytes with a panel of monoclonal antibodies (mAbs) was performed using standard techniques and the immunoflorescence reactivity was determined by flow cytometry analysing $10^4$ cells in each sample\(^{(9)}\).

The following mAbs were used to analysis the surface antigens of endometrial leukocytes: anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD16, anti-CD20 and anti-CD56 \((Dako, \text{ Denmark and Biotest Diagnostics, Germany})\).

Values were given as means ± SD, then compared using Student’s t-test. For mean comparisons, analysis of variance (ANOVA) was performed whenever appropriate.

RESULTS

The mean age of the recurrent aborters was 31.6 ± 4.2 years (range 24-36) which was not significantly different from that of the control group which was 33.2 ± 5.5 years (range 28 – 38). The mean number of abortions in the recurrent abortion group was 4.3 (range 3 to 6). Of these patients, 12 were primary aborters (with no childbirth prior to spontaneous abortions) and 8 were secondary aborters.

Histologic dating and morphological study:

The mean endometrial dating in the recurrent abortion group was -2.4 (range -5 to 10), while it was -0.5 day (range -1.5 to 0.5) in the control group. Retarded endometrial development was detected in 9 patients (45%) in the recurrent abortion group. Based on the morphological study, recurrent aborters were divided into two distinct subgroups: group (1) with retarded endometrial development \((n=9)\) and group (2) with normal endometrial differentiation \((n=11)\).

Tables I and II show comparison of the morphometric and stereological data of the endometrium of the recurrent abortion subgroups and the control group. No significant difference was noted between the control group and the subgroup of recurrent abortion with normal endometrial morphology (group 2). While both of them show significant differences regarding the number of glands per mm\(^2\), glandular epithelial height (um), number of basal vacuoles,
volume density of endometrial glands and volume density of stroma when compared with the subgroup of recurrent abortion with retarded endometrial development (group 1).

The immunophenotypic profile of endometrial leukocytes:

The distribution of endometrial leukocyte populations as determined by two colour flow cytometric analysis of the endometrial samples was shown in figure (1).

**T-lymphocytes:**

The major leukocyte population detected in non pregnant recurrent aborters consisted of T-lymphocytes with 42 ± 5% (mean ± SD) CD3 positive cells and 40 ± 9% in the control group. The proportion of CD8+ cells (suppressor T-cells) was however significantly lower in the recurrent aborters (19 ± 7%) than in normal controls (24 ± 6%), P < 0.05. Remaining T-cells were CD4+ (helper T-cells) with no significant difference between both groups. The CD4+:CD8+ cell ratio in each sample was found to be significantly higher (1.5 ± 0.25) in comparison with ratio below 1 in most of the controls (0.78 ± 0.36), P < 0.05.

**B-lymphocytes:**

The percentage of B-lymphocytes was assessed and an increased proportion of CD20+ cells (17 ± 7%) in the recurrent aborters which is about 3 times higher than normal controls (5.5 ± 6%), P < 0.05.

**Natural killer (NK) cells:**

NK cell percentage was lower in recurrent aborters than in normal controls, but no significant difference was found between both groups. The distribution of NK cell subsets by flowcytometric analysis showed higher incidence of CD16+CD56dim in the recurrent aborters. CD16+ NK cells show increased cytolytic activity than CD16- NK cells.

**Monocyte, macrophage fractions** (CD14+) were the lowest among the evaluated cells with comparable numbers in both groups.

**Primary vs secondary aborters:**

The proportion of each cell population was compared in both groups and no significant difference was found between primary and secondary aborters.
Influence of number of abortions on endometrial lymphocytes:

The proportion of endometrial CD8, CD20, CD56+ and CD16+ cells were plotted according to the number of previous abortions as shown in figure (2). The distribution of immune endometrial cells was not affected by the number of previous abortions.

DISCUSSION

It is more than 35 years since(12) reported 67% incidence of histologically abnormal secretory endometrium in women suffering from recurrent abortion. Previous reports of inconsistent results obtained by(1,2&3) can be explained by lack of precision in the timing of biopsy (that is inaccuracy in chronological dating) together with subjective variation in the histologic dating. Both of these potential sources of errors have been avoided in the present study. Peters et al.(11), found that the accuracy of histologic endometrial dating was best documented by ultrasonographic documentation of ovulation rather than by last menstrual period, next menstrual period, or luteinizing hormone testing in infertile populations and in those with recurrent pregnancy loss.

The morphometric analysis gives an objective morphological evaluation of the endometrium(5&13). Retarded endometrial development was detected in 45% of the patients of unexplained recurrent abortion. Serle et al.(14) found retarded endometrial development in 60% of the patients of recurrent abortion using the morphometric criteria. Also, they found reduced levels of mucin related secretory epitopes in the group of retarded endometrial development, the presence of inadequate endometrial development in the peri-implantation phase might have unfavorable influence on the outcome of implantation.

At present, few laboratory tests are available to investigate patients who may have immunologically mediated abortions. Various indicators of immune functions have been used such as measures of mixed lymphocyte reactivity, blocking antibodies and development of antipaternal antibodies(7). Such tests are difficult to perform on routine basis flow cytometry is an accessible procedure and the methods of isolation, labeling and flow cytometric analysis of endometrial leukocytes are easy techniques.
In this study, flow cytometric analysis showed decreased proportion of CD8\(^+\) cells, increased CD4\(^+\):CD8\(^+\) ratio, increased CD20\(^+\) B cells and altered NK cell subsets in the recurrent abortion group. Similar findings were detected by *Hill et al.*\(^{(15)}\) and *Lachapelle et al.*\(^{(16)}\).

The lower number of CD8\(^+\) cells in the endometrium of recurrent aborters suggests that the suppressor T-cells might play a role in preventing the maternal immune system from rejecting the conspectus and that the presence of these cells is important even before induction of abortion\(^{(17)}\). Also, it was found that the B-cells could be involved in antigen presentation and initiation of cascade of immunologic events leading to fetal demise\(^{(18)}\).

The contribution of NK cells to fetal engraftment has been controversial issues and the detection of decreases in CD16\(^+\) CD56\(^{bright}\) NK cells observed in recurrent aborters is associated with altered cytokine expression profile and the failure to provide the conspectus with proper growth environment. This emphasizes the dual roles of NK cells towards the conspectus in promoting or inhibiting its growth\(^{(19,20,21)}\).

In conclusion, the use of precisey timed endometrial biopsy should be incorporated in the investigation of recurrent abortion to distinguish the patients with retarded endometrial development. Also, the distinct immunophenotypic profile of endometrial leukocytes observed in the non pregnant endometrium suggests the hostile role they might play in recurrent abortion.

Further prospective studies are required to follow the patients with altered endometrial leukocyte immunophenotypic profile to assess the future pregnancy outcome. This is an attempt to determine if endometrial immune disturbances that attended fetal implantation correlate with subsequent abortion.

**REFERENCES**


Table (I): Morphometric data of the endometrium of the recurrent abortion and control groups (values are means ± SD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Recurrent abortion n = 20</th>
<th>Control group n = 12</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 n = 9</td>
<td>Group 2 n = 11</td>
<td></td>
</tr>
<tr>
<td>• No of glands per mm²</td>
<td>16.2 ± 5.4</td>
<td>24.2 ± 4.6</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.1 ± 8.3</td>
<td>NS</td>
</tr>
<tr>
<td>• Glandular diameter (μm)</td>
<td>4.6 ± 15.2</td>
<td>61.2 ± 18.4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68.9 ± 10.6</td>
<td>NS</td>
</tr>
<tr>
<td>• Glandular epithelial height (μm)</td>
<td>16 ± 3.0</td>
<td>21.2 ± 2.3</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.2 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td>• No of basal vacuoles /100 cells</td>
<td>24.3 ± 2.9</td>
<td>0.7 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.6 ± 0.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

- Group 1: Patients with retarded endometrial development group.
- Group 2: Patients with normal endometrial differentiation.
- P₁: group 1 versus controls.
- P₂: group 2 versus controls
- P₃: group 1 versus group 2
- NS = not significant

Table (II): Stereological analysis of the endometrium of the recurrent abortion and control groups (values are means ± SD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Recurrent abortion n = 20</th>
<th>Control group n = 12</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 n = 9</td>
<td>Group 2 n = 11</td>
<td></td>
</tr>
<tr>
<td>• Volume density of endomet. Glands</td>
<td>21 ± 3.8</td>
<td>38 ± 4.1</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36 ± 6.1</td>
<td>NS</td>
</tr>
<tr>
<td>• Volume density of stroma</td>
<td>78 ± 2.2</td>
<td>62 ± 1.1</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63 ± 8.1</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure (1): Flow cytometric evaluation of endometrial leukocytes in recurrent aborters (black columns) and fertile controls (white columns). The percentage = mean of Ag positive cells.

Figure (2): Influence of number of abortions on endometrial Lymphocytes in the recurrent aborters.