Microscopic imaging of the endometrium for assessment of uterine receptivity in women with latent genital tuberculosis

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Genital tuberculosis is one of the major causes of female infertility. The diagnosis of the disease, especially of latent form, is difficult as it exists without any signs and symptoms. The effect of latent genital tuberculosis (LGTB) on the expression of biochemical and morphological markers during implantation window in endometrial tissue of women with LGTB is examined. Expression of molecular markers and pinopodes was analyzed by immunohistochemistry and scanning electron microscopy, respectively in the endometrial tissue collected from 30 women with LGTB and 25 controls. A significant difference in the expression of endometrial receptivity markers including \(\alpha_v\beta_3\) integrin, leukemia inhibitory factor, E-cadherin, MECA-79, Mucin-1 and pinopodes expression were observed in women with LGTB as compared to controls. Clinical translation of this basic scientific knowledge will be an important step in modern reproductive therapy for improving fertility rate.

Keywords endometrial receptivity; implantation window; LGTB; biochemical markers

1. Introduction

Though tuberculosis (TB) was declared a global emergency way back in 1993, genital TB, unfortunately, still remains a much neglected area of research. Latent genital tuberculosis (LGTB) in women is defined as tuberculosis of the genital tract which is not clinically evident either by subjective symptoms or objective signs. Also, the disease is not in the communicable stage. Clinical symptoms of LGTB can vary such as infertility (~in 60% of the cases), pelvic pain and menstrual disorders, e.g. scanty menstruation and amenorrhoea. The incidence of infertility in genital TB worldwide varies from 44 to 74%; in India it is reported to be 18% [1]. However, it is difficult to predict its accurate prevalence because the disease often tends to exist without any clinical signs or symptoms.

Successful implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst developmental stage and a synchronized dialogue between maternal and embryonic tissues [2]. Endometrial receptivity is defined as the capacity of the uterine mucosa to facilitate successful embryonic implantation during day 18-24 of the menstrual cycle. Endometrial receptivity plays an important role in determining the reproductive outcome in IVF program [3]. Rinaldi et al. (1996) have shown that even a good quality embryo can often get rejected due to problems within the endometrium [4].

Establishment of endometrial receptivity is still a biological mystery that remains unsolved despite marked advances in our understanding of endometrial physiology associated with its development and function [5]. A systematic review by Achache and Revel (2006) highlights the role and/or correlation of various receptivity markers during implantation [6]. Cell adhesion molecules (CAM) are known to play an important role in blastocyst attachment to the proper endometrial surface. Pinopodes, whose expression is limited to the period of implantation window, have been proposed to be useful morphological markers of endometrial receptivity [7]; nevertheless, controversies still exist [8]. Our earlier study suggests implantation failure following IVF in women with apparently normal pelvic and non-endometrial tubal factors and absence of adhesions could be attributed to LGTB considering the fact that the Indian subcontinent is a zone prone to tuberculosis in any form [9].

The possible link between LGTB and endometrial receptivity, one of the key factors determining implantation, has not been studied so far. The present study, therefore, aims to evaluate the expression of various biochemical and morphological markers (pinopodes) of endometrial receptivity during implantation window in endometrial tissue of women with LGTB. The biochemical markers examined in the present study are the endometrial adhesion molecules (\(\alpha_v\beta_3\) integrin, E-cadherin, L-selectin ligand/ MECA-79), an anti-adhesion molecule mucin 1 (MUC-1) and an interleukin-6 class cytokine, leukemia inhibitory factor (LIF).

2. Materials and methods

2.1 Endometrial tissue collection and sample preparation
Endometrial tissue samples of 30 women classified as unexplained infertile cases having tested PCR positive for *M. tuberculosis* (gives a 123-bp product of *M. tuberculosis* DNA using IS6110 primers) and reporting at the Institute of Reproductive Medicine (IRM), Salt Lake, Kolkata for infertility treatment were included as the study group. These women also tested positive for BACTEC culture. 25 normal healthy fertile women undergoing sterilization were considered as controls. Institutional ethical committee approval was obtained prior to the commencement of the study. The inclusion criteria ensured that the women were <40 years, were having regular menstrual cycles (25-32 days), euthyroid with normal baseline FSH and prolactin levels, no identifiable cause of infertility and have had three or more failed IVF cycles. Women with history of chocolate cyst, fibroids, mild to severe endometriosis, polycystic ovary syndrome (PCOS), Chlamydia infections and any pelvic pathology including pelvic inflammatory disease (PID) and adhesions were excluded. All samples were collected after obtaining written consent from the couples participating in the study. Once ovulation was confirmed by ultrasonography, endometrial samples collected on the 7th day post-ovulation during implantation window (D21). The collected tissue were divided into two parts: the first part was used for PCR, BACTEC culture and ZN smear test, and the second part fixed in 4% formaldehyde and kept at 4 °C until used for immunohistochemistry (IHC) and scanning electron microscopy (SEM).

### 2.2 Immunohistochemistry

Tissue sections of 3-5 μm size were obtained from formaldehyde fixed, paraffin embedded tissue using Microtome (Microm HM325, ThermoFisher Scientific, Germany). Paraffin sections were deparaffinized using xylene and tissue sections hydrated using graded ethanol. Antigen retrieval was done using Tris-EDTA buffer (pH 9.0) and the endogenous peroxidase activity quenched using 0.3% hydrogen peroxide for 15 min. Slides were then blocked using power block for 10 min (Super Sensitive Detection system, Biogenex, USA). After washing with TBST, slides were incubated with monoclonal antibody produced in mouse specific to αvβ3 integrin and LIF (R&D Systems, Minneapolis, MN, USA), MECA-79, MUC-1 and E-Cadherin (Santa Cruz biotechnology, INC, Santa Cruz, California, USA) for 60 min. Excess primary antibody was washed with TBST and super enhancer used. Labeled cells were visualized with DAB following incubation with poly-HRP reagent (Super Sensitive Detection system, Biogenex, USA) for 30 min and sections counterstained with hematoxylin. Next, the sections were dehydrated using series of alcohol, mounted using DPX (Merck, India) and examined under bright-field microscope (Carl Zeiss, Germany).

#### 2.2.1 Scoring of IHC

Semi-quantitative scoring was done independently by two observers to assess the staining intensity which provides a measure of the expression of these molecules as suggested by Remmele and Stegner [10]. Immunostaining was classified on the basis of intensity of staining ((no staining-0; weak-1 point; moderate-2 points; strong-3 points) and percentage/extent of stained cells (0%-0 point; <10%-1 point; 11%-50%-2 points; 51%-80%-3 points; >80%-4 points). A final immunohistochemical score (Score 0-12) were obtained by multiplying intensity score and extent of stained cells.

### 2.3 Scanning Electron Microscopy

Formaldehyde-fixed tissues were washed in PBS and dehydrated in a series of alcohol gradient (50%, 70%, 90%, 95%, 100%), each for 10 min, dipped in HMDS (1,1,1,3,3,3- hexamethyl disilazane; Sigma, USA) and air dried. It was then mounted and coated with gold and the endometrial surface thoroughly examined under SEM (Jeol JSM-5800 Scanning Microscope, OXFORD). Expression of pinopodes on the surface of the endometrium was evaluated semi-quantitatively and scored as well developed, poorly developed and few/absent.

### 2.4 Statistical analysis

Chi-squared test or Fisher exact test was used to analyze immunostaining of endometrial receptivity markers and expression of pinopodes. Analysis, discrimination, and classification of data were performed with Graphpad prism and MedCalc software. Statistical significance of the test was defined as p<0.05 and p<0.001.

### 3. Results

A significant decrease in αvβ3 integrin, LIF, E-cadherin, MECA-79 and MUC-1 expression was observed in women with LGTB as compared to controls (Figs. 1, see also Table 1). Table 2 shows the sensitivity, specificity and overall accuracy values with 95% confidence of interval (CI) of all biochemical markers expression for distinguishing LGTB and controls.
Fig. 1 Immunohistochemical staining of endometrium during implantation window to assess the expression of Mucin, αvβ3 integrin, E-cadherin, LIF and MECA-79 (top-bottom) in fertile controls (A, C, E, G, I) and women with LGTB (B, D, F, H, J). Magnification X 200.

Table 1 Semi-quantitative scoring method of immunohistochemistry-based expression pattern of different endometrial receptivity markers in endometrium of women with LGTB and controls during implantation window.

<table>
<thead>
<tr>
<th>Biochemical Markers</th>
<th>Scoring Categories</th>
<th>LGTB (n=30)</th>
<th>Control (n=25)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC-1</td>
<td>Score 0-3</td>
<td>21 (70%)</td>
<td>6 (24%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Score 4-12</td>
<td>9 (30%)</td>
<td>19 (76%)</td>
<td></td>
</tr>
<tr>
<td>αvβ3 integrin</td>
<td>Score 0-3</td>
<td>23 (76.67%)</td>
<td>8 (32%)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
In contrast to controls, which showed well-formed pinopodes in most of the cases, poorly developed pinopodes were observed in women with LGTB (Fig. 2). A significant decrease in the expression of pinopodes was seen in LGTB as compared to controls (Table 3).

![Typical microscopic images showing the apical surface of endometrial epithelium during implantation window in women with LGTB. Pinopodes are graded as (A) well developed (B) poorly developed and (C) few/absent pinopodes.](image)

### Table 2 Sensitivity, specificity and overall accuracy of different endometrial receptivity markers in differentiating LGTB and controls.

<table>
<thead>
<tr>
<th>Biochemical Markers</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>Accuracy (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC-1</td>
<td>70% (50.6%-85.3%)</td>
<td>76% (54.9%-90.6%)</td>
<td>73% (61.27%-84.73%)</td>
</tr>
<tr>
<td>avß3 integrin</td>
<td>76.67% (57.7%-90.1%)</td>
<td>68% (46.5%-85.1%)</td>
<td>72.33% (60.51%-84.15%)</td>
</tr>
<tr>
<td>LIF</td>
<td>73.33% (54.1%-87.7%)</td>
<td>76% (54.9%-90.6%)</td>
<td>74.66% (63.16%-86.16%)</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>70% (50.6%-85.3%)</td>
<td>72% (50.6%-87.9%)</td>
<td>71% (59.01%-82.99%)</td>
</tr>
<tr>
<td>MECA-79</td>
<td>66.67% (47.2%-82.7%)</td>
<td>72% (50.6%-87.9%)</td>
<td>69.33% (57.14%-81.52%)</td>
</tr>
</tbody>
</table>

CI= Confidence of Interval

### Table 3 Scoring based on expression of apical protrusions in endometrium during implantation window in women with LGTB and controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>Well developed</th>
<th>Poorly developed</th>
<th>Few/Absent</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGTB</td>
<td>6</td>
<td>17</td>
<td>7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Controls</td>
<td>16</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

4. Discussion

Considering the fact that incidence of tuberculosis in the Indian subcontinent is extremely high, we felt the need to evaluate women partners of unexplained infertile couples for LGTB as a potential cause of repeated IVF failure. It is
well established that endometrial receptivity indicates the molecular status of the endometrium when it is favourable for blastocyst implantation and plays a critical role in determining the IVF outcome [3]. Various cell adhesion molecules, cytokines, and pinopodes, expressed during the implantation window, are being increasingly recognized as potential biomarkers of uterine receptivity [6, 11].

We observed a significant decrease in the expression of αvβ3 integrin, a transmembrane glycoprotein, in women with LGTB. αvβ3 integrin plays an important role in the adhesion of blastocyst to the endometrium during implantation. There is evidence of its reduced expression associated with unreceptive endometrium [6]. Though E-cadherin represents the most studied subclass of glycoproteins responsible for the calcium-dependent cell-to-cell adhesion mechanism, its expression in LGTB is not reported so far. We found a decrease in the expression of E-cadherin in women with LGTB. L-selectin molecules help the blastocyst to roll freely over the endometrium. It is expressed by the human trophoblast and bind with the ligand, MECA-79, aiding the initial steps of blastocyst adhesion to the uterine wall. Absence of MECA-79 is suggested to be the reason for repeated implantation failure in infertile patients [12, 13]. Expression of L-selectin ligand during implantation widow has not been investigated in women with LGTB so far. We observed a significantly reduced expression of MECA-79 in women with LGTB.

LIF, a glycoprotein of the interleukin-6 family, attracts the blastocyst to the optimal implantation site over the endometrium. MUC-1, an anti-adhesion molecule, holds off the blastocyst from implanting to an improper endometrial implantation region [6]. Pinopodes appear as apical cellular protrusions over the endometrium during implantation window and are believed to be progesterone-dependent morphological markers of receptivity. They are considered to be the site of blastocyst attachment to the endometrium [14]. Expression of LIF, MUC-1 and pinopodes in endometrium of women with LGTB were also observed to be significantly less during implantation window.

Based on our microscopic findings, it is suggested that dormant M. tuberculosis, present in the basal endometrial layer, possibly interferes with implantation in women with LGTB. This theory is evidenced by the poor expression of the biochemical mediators of receptivity. Expression of morphologically abnormal pinopodes in LGTB during implantation window further confirms the hypothesis. Assessment of implantation markers expression pattern may provide an improved understanding of the mechanism regulating embryo implantation in LGTB. This is expected to assist clinicians in the treatment of genital tuberculosis related infertility, prevent early pregnancy loss and also prevent frank tuberculosis in future.

References