ARTICLE

Possible role of natural killer and natural killer T-like cells in implantation failure after IVF

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Abstract During implantation, maternal immunoactivation and tolerance are not only limited to the decidua but are also observed in the periphery, predominantly affecting the innate immune system. Since unexplained female infertility, as well as recurrent spontaneous abortion and implantation failure, are thought to be associated with pathological maternal immunotolerance mechanisms, this study focused on immune profile analysis of IVF candidates. Previous studies on peripheral natural killer (NK) cell characteristics of IVF patients have been limited to the comparison of blood samples taken prior to the IVF procedure. This study performed a follow-up study and compared patient’s data obtained on the day of oocyte collection with the data 1 week after embryo transfer. The aim was to investigate phenotypic (subpopulations, CD69, T-cell immunoglobulin mucin 3 and NK-activating receptor expression) and functional (perforin and CD107a expression) changes in the peripheral NK and NK T (NKT)-like cell populations. During this short period of time around the IVF procedure, women with failed IVF reflected unfavourable Th1-oriented changes of NK and NKT-like cells. In comparison the follow-up data for women with successful conception remained principally constant. The observed peripheral changes during early pregnancy in the same individual may also have importance in successful embryo implantation.

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Introduction

At the time of implantation, recognition of the semiallogenic fetus by the maternal immune system is crucial for successful placentation and maintaining pregnancy. Locally, recruited immune cells of the decidua come into direct contact with fetal antigens presented by trophoblast cells leading to immunooactivation and inducing tolerance via type 2 immunity particularly in the innate immune system. Uterine natural killer (NK) cells, the dominant lymphocyte subpopulation found in the decidua, play a central role in efficient placentation (Santoni et al., 2008). Recognition of fetal non-polymorphic human leukocyte antigen (HLA)-G and HLA-E by uterine NK cells usually induces the secretion of Th2-type cytokines and contributes to the success of pregnancy (Carosella et al., 2008; Hunt and Langat, 2009; Hviid, 2006; Ishitani et al., 2006). On the other hand, recognition of paternal HLA-C molecules expressed on the trophoblast results in a local inflammatory response (Koopman et al., 2003; Lash et al., 2006; Li et al., 2001), which, by loosening the tissue, facilitates extravillous trophoblast invasion (Sargent et al., 2006). Additionally, interferon-γ, produced during inflammation, promotes uterine vascular remodelling (Croy et al., 2003).

Disturbed maternal recognition of fetal antigens may lead to pregnancy pathologies. Hiby et al. (2004) identified a specific genetic combination of maternal killer immunoglobulin-like receptors and fetal HLA-C genes that results in inhibition of trophoblast invasion and leads to pre-eclampsia. In the case of recurrent spontaneous abortion, women lacking appropriate inhibitory killer immunoglobulin-like receptors to fetal HLA-C molecules fail to deliver inhibitory signals to NK cells (Varla-Leftherioti et al., 2005).

In healthy pregnancy, the type 2 shift of the immune responses is not only limited to the implantation site but it can also be observed systemically (Marzi et al., 1996; Raghupathy et al., 2000; Saito et al., 1999). Besides the involvement of Th cells, changes in the innate lymphocyte population – NK and NK T (NKT)-like cells – are thought to be dominant (Borzyskowski et al., 2005). Human pregnancy is characterized by a low peripheral NK activity reflecting the local immune responses in the decidua (Aoki et al., 1995; Szeredny et al., 1999).

Several studies have focused on the role of NK cells in the pathogenesis of recurrent miscarriage or repeated IVF failure (King et al., 2009; Quenby and Farquharson, 2006). It has been shown that an increased percentage of peripheral blood NK cells, enhanced NK activity and cytotoxicity are associated with poorer pregnancy outcome (Ntrivalas et al., 2001). Furthermore, Coulam et al. (1995) suggested that systemic NK cell percentage could predict pregnancy outcome.

NK cells represent the predominant lymphocyte population of the innate immune system possessing both effector and immunoregulatory functions. Phenotypically, NK cells are characterized by the lack of CD3 and the expression of CD56 on their cell surface (Cooper et al., 2001). The CD56bright and CD56dim NK subpopulations stand for different functional activities. CD56dim NK cells account for approximately 90% of human peripheral blood NK lymphocytes and have a mainly cytotoxic action via perforin-dependent lysis or inducing apoptosis of the target cell through FasL-Fas interaction (Cooper et al., 2001). CD56dim NK cells also express high concentrations of FcγRIII (CD16), a molecule which is involved in antibody-dependent cellular cytotoxicity reactions (Cooper et al., 2001). Contrarily, the minor CD56bright peripheral NK cell subset indirectly modulates the immune response by secreting Th1/Th2 cytokines (Cooper et al., 2001) and this subpopulation is capable of decidual recruitment from the blood at ovulation and during early gestation (van den Heuvel et al., 2008).

Since NK cells do not require major histocompatibility complex (MHC)-restricted antigen presentation, antigens can be recognized directly by different activating and inhibitory NK receptors on NK cells (Yokoyama and Riley, 2008). The ‘missing self’ hypothesis declares the principal way of their function: NK cells recognize and eliminate cells that fail to express self-MHC class-I molecules: a common consequence of virus infection or malignant transformation (Raulet, 2006). On NK cells, the functional predominance of inhibitory receptors compared with activating ones ensures the protection of autologous tissues against NK cell-mediated lysis (Lanier, 2005; Szeredny-Bartho, 2008). Alter et al. (2004) demonstrated that the induction of CD107a expression on the surface of NK cells is in concert with cytokine secretion and target cell lysis, so it can be used as a general marker of NK functional activity. The use of CD107a as readout of NK activity permits the discrimination of multiple populations of NK cells based on their ability to respond to different stimuli.

NKT-like CD3+CD56+ cells are unique T cells co-expressing a T-cell receptor complex (CD3) and NK receptors (CD56) comprise a minor subpopulation of human peripheral blood lymphocytes (Kronenberg and Gapin, 2002). Similar to NK cells, the involvement of NKT-like cells in the immunological changes observed during implantation and later on during pregnancy has been previously described (Borzyskowski et al., 2005). Additionally, immunoglobulin IV therapy in women with repeated implantation failure could decrease the elevated blood NKT level, which strongly correlates with successful pregnancy (van den Heuvel et al., 2007).

Moreover, other novel mechanisms could play a role in the regulation of NK and NKT-like cells during maternal tolerance. The immunoglobulin superfamily member T-cell immunoglobulin mucin 3 (TIM-3) was first published by Monney et al. (2002) as a cell-surface marker preferentially expressed by Th1 cells, but the in-vivo functions of TIM-3 have remained unknown. Expression of TIM-3 on Th1 cells provides a key checkpoint that serves to dampen pro-inflammatory Th1-dependent T-cell responses and limit the associated tissue injury.

The aim of this study was to investigate phenotypic and functional changes in the peripheral NK and NKT-like cell populations in women undergoing IVF treatment during the time of IVF (from oocyte collection to embryo implantation).

Materials and methods

Study population

This study investigated 19 women undergoing IVF treatment cycles. All women in both groups received the same...
hormonal supplementation as described in the next section. IVF cycles were successful in eight patients, defined by positive blood β-human chorionic gonadotrophin (HCG) tests performed in the first and third week after embryo transfer followed by live birth at term. The failed IVF group was defined by negative blood HCG tests performed on both the first and the third week after embryo transfer (11 women). The patients’ obstetric data (birthweight of the babies and gestational age at birth) showed that the pregnancies progressed normally in the successful IVF patient group.

This study was approved by the local ethics committee and informed consent was obtained from each person. The patients’ demographic, gynaecological and treatment characteristics are summarized in Table 1.

Peripheral venous blood (10 ml) was taken from each donor on the day of oocyte collection; the procedure was repeated once 1 week after embryo transfer.

**IVF procedure**

Pituitary down-regulation was achieved with triptorelin (Decapeptyl; Ferring) at the mid-luteal phase. Ovarian stimulation was carried out with recombinant FSH (Gonal-F; Serono; Puregon; Organon) after 14 days of triptorelin treatment (0.05 mg/day). When follicles reached pre-ovulatory size (18–22 mm), 250 μg of recombinant HCG (Ovitrelle, Serono) was administered. Oocytes were aspirated (Repromed, IM Services) using transvaginal ultrasound guidance (SA 6000C; Medison) 34–36 h after HCG administration. All embryos were allowed to cleave and the best one, two or three embryos were selected for transfer (one in the case of top-quality embryo transfer). Embryo transfer was performed on day 3 or 5 using a soft catheter (Wallace). Vaginal progesterone supplementation for luteal support (Utrogestan; Besins International), 300 mg three times per day, was commenced 1 day after oocyte aspiration and continued until a vaginal ultrasound examination was performed 3 weeks after embryo transfer.

**Lymphocyte separation, cryopreservation and thawing**

Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood on Ficoll-Paque (GE Healthcare) gradient. After washing the cells in RPMI 1640 medium the cells were counted and centrifuged at 650g for 5 min. Resuspension was performed in human serum containing 10% DMSO for cryoprotection. Cells were aliquoted in cryovials and stored in a −80°C mechanical freezer.

Thawing was carried out on the day of fluorescent cell labelling. Cryovials were warmed up as quickly as possible in an incubator at 37°C and DMSO (Sigma–Aldrich) was washed out in RPMI 1640 medium (Gibco).

**Antibodies**

Freshly thawed PBMC were used for surface and intracellular staining and analysis for NK and NKT-like cell markers. The following monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 (BD-Pharmingen), allophycocyanin (APC)-conjugated anti-human CD56 (BD-Pharmingen) phycoerythrin (PE)-conjugated anti-human perforin (BD-Pharmingen), PE-conjugated anti-human TIM-3 (R and D Systems), PE-conjugated anti-human CD69 (BD-Pharmingen), PE-conjugated anti-human Natural killer group 2, member D (NKG2D) NKG2D (R and D Systems) and PE-conjugated anti-human CD160 (R and D Systems). Control antibodies included isotype-matched FITC-conjugated, PE-conjugated and APC-conjugated mouse antibodies (all from BD-Pharmingen).

**Table 1** Patients’ demographic, gynaecological and treatment characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Successful IVF</th>
<th>Failed IVF</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36.87</td>
<td>35.18</td>
</tr>
<tr>
<td>Cause of infertility (%)</td>
<td></td>
<td></td>
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<tr>
<td>Tubal factor</td>
<td>27.27</td>
<td>22.5</td>
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<tr>
<td>Male factor</td>
<td>45.45</td>
<td>62.5</td>
</tr>
<tr>
<td>Other</td>
<td>27.27</td>
<td>25</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>6.06</td>
<td>4.95</td>
</tr>
<tr>
<td>Basal FSH concentrations (IU/ml)</td>
<td>7.63</td>
<td>6.29</td>
</tr>
<tr>
<td>Previous failed IVF attempts</td>
<td>2.37</td>
<td>1.45</td>
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<tr>
<td>Previous miscarriages</td>
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<td>0</td>
</tr>
<tr>
<td>Oocytes collected</td>
<td>6.6</td>
<td>9.63</td>
</tr>
<tr>
<td>Available embryos for IVF</td>
<td>4.62</td>
<td>4.45</td>
</tr>
<tr>
<td>Available embryos for transfer</td>
<td>2.87</td>
<td>2.45</td>
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<tr>
<td>Gestational age at birth (weeks)</td>
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<tr>
<td>Birthweight (g)</td>
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<td>—</td>
</tr>
<tr>
<td>No. of live birth/no. of embryos transferred</td>
<td>8/23</td>
<td>0/24</td>
</tr>
<tr>
<td>No. of live births/no. of patients&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8/8</td>
<td>0/11</td>
</tr>
</tbody>
</table>

Values are mean unless otherwise stated. Statistical comparisons were made by using one-tailed Student’s t-tests.

<sup>a</sup>P < 0.05.
Labeling of lymphocytes and flow cytometric analysis

Thawed PBMC (10⁶ in 100 μl phosphate-buffered saline (PBS)/tube) was incubated for 30 min at room temperature with the fluorochrome-labelled monoclonal antibodies. After surface staining, the cells were incubated with 1:10 diluted FACS Lysing Solution (BD-Pharmingen) for 10 min and washed twice with PBS buffer. Finally, the cells were resuspended in 300 μl PBS containing 1% paraformaldehyde and stored at 4°C in the dark until fluorescence-activated cell sorting (FACS) analysis.

For detecting perforin-positive cells, after surface labeling the cells were incubated with 1:10 diluted FACS Permeabilizing Solution (BD-Pharmingen) for 10 min and then washed with PBS. The cells were then incubated with PE-conjugated mouse anti-human perforin for 30 min at room temperature, washed with PBS and fixed with PBS containing 1% paraformaldehyde.

Labeled cells were analysed with a FACSCalibur flow cytometer (BD Immunocytometry Systems) equipped with the CellQuest software program (BD Biosciences) for data acquisition and analysis.

NK cell functional assay

Cytotoxic activity was assayed by the detection CD107a expression, as previously described (Andzelm et al., 2007).

To determine CD107a surface expression on NK cells, PBMC were incubated for 4 h at 37°C in the presence of PE-conjugated anti-CD107a monoclonal antibody (mAb; BD-Pharmin-gen) in RPMI 1640 medium containing 10% fetal bovine serum, penicillin and streptomyycin, immunoglobulin (Sigma–Aldrich) and phorbol myristate acetate (Sigma–Aldrich), after which cells were washed and resuspended in PBS. Cells were incubated with FITC-conjugated CD3 mAb and APC-conjugated CD56 mAb (BD-Pharmingen) for 30 min, washed twice, fixed with 1% paraformaldehyde and evaluated by FACS.

Statistical analysis

Statistical comparisons were made using one- and two-tailed Student’s t-tests. The results were expressed as the mean ± SEM. Differences were considered significant for \( P = 0.05 \).

Results

Table 2 presents changes in the NK cell phenotype characteristics before and after IVF in women with successful IVF and in women with failed IVF (fIVF). Comparing the data from the two dates, significant differences were only seen in the failed IVF group. In the peripheral blood of failed IVF patients there was a significant increase in the NK cell ratio within the lymphogate (\( P = 0.002 \)) after 1 week of the procedure affecting both the CD56bright (\( P = 0.04 \)) and the CD56dim (\( P = 0.001 \)) subsets. After IVF, NK cells of failed

| Table 2 | Natural killer cell phenotype characteristics in women with successful and failed IVF. |
|------------------|----------------------------------------|------------------|------------------|------------------|
| No. of patients  | Successful IVF | Failed IVF | P-value | Successful IVF | Failed IVF | P-value |
|                  | Before IFV | After IFV |  | Before IFV | After IFV |  |
| 8                 |  |  |  | 5.28 ± 0.74 | 9.66 ± 1.11 | 0.002  |
| Cells in lymphogate |  |  |  | 0.47 ± 0.07 | 1.26 ± 0.39 | 0.04  |
| CD3−CD56−        | 11.09 ± 3.16 | 11.01 ± 2.8 | NS | 3.82 ± 3.38 | 50.47 ± 3.53 | NS |
| CD3−CD56bright   | 1.41 ± 0.42 | 1.67 ± 0.56 | NS | 41.93 ± 4.06 | 54.07 ± 5.73 | NS |
| CD3−CD56dim      | 9.94 ± 2.85 | 9.61 ± 2.38 | NS | 44.15 ± 3.47 | 49.52 ± 3.21 | NS |
| TIM-3 expression | 38.98 ± 3.38 | 40.22 ± 6.49 | NS | 2.84 ± 0.7 | 3.27 ± 0.91 | NS |
| CD3−CD56−        | 44.23 ± 5.61 | 43.51 ± 9.05 | NS | 1.38 ± 0.38 | 1.55 ± 0.82 | NS |
| CD3−CD56bright   | 38.26 ± 3.26 | 39.81 ± 6.49 | NS | 2.89 ± 0.69 | 3.31 ± 0.95 | NS |
| CD3−CD56dim      | 2.84 ± 0.7 | 3.27 ± 0.91 | NS | 3.51 ± 0.64 | 4.04 ± 0.82 | NS |
| CD69 expression  | 11.09 ± 3.16 | 11.01 ± 2.8 | NS | 2.89 ± 0.69 | 3.31 ± 0.95 | NS |
| CD3−CD56−        | 2.84 ± 0.7 | 3.27 ± 0.91 | NS | 3.51 ± 0.64 | 4.04 ± 0.82 | NS |
| CD3−CD56bright   | 1.38 ± 0.38 | 1.55 ± 0.82 | NS | 1.26 ± 0.38 | 2.24 ± 0.51 | NS |
| CD3−CD56dim      | 2.89 ± 0.69 | 3.31 ± 0.95 | NS | 3.7 ± 0.7 | 4.21 ± 0.87 | NS |
| CD160 expression | 26.31 ± 4.43 | 24.68 ± 5.69 | NS | 2.84 ± 0.69 | 3.31 ± 0.95 | NS |
| CD3−CD56−        | 26.31 ± 4.43 | 24.68 ± 5.69 | NS | 2.84 ± 0.69 | 3.31 ± 0.95 | NS |
| CD3−CD56bright   | 9.58 ± 1.64 | 10.22 ± 3.49 | NS | 23.51 ± 3.51 | 28.61 ± 2.84 | 0.05 |
| CD3−CD56dim      | 28.14 ± 4.69 | 26.04 ± 6.11 | NS | 21.25 ± 3.51 | 28.61 ± 2.84 | 0.05 |
| NKG2D expression | 73.7 ± 9.12 | 69.93 ± 8.6 | NS | 73.7 ± 9.12 | 69.93 ± 8.6 | NS |
| CD3−CD56−        | 73.7 ± 9.12 | 69.93 ± 8.6 | NS | 50.14 ± 8.35 | 71.64 ± 9.28 | 0.01 |
| CD3−CD56bright   | 84.66 ± 3.63 | 78.01 ± 6.81 | NS | 73.1 ± 7.51 | 79.91 ± 7.15 | 0.03 |
| CD3−CD56dim      | 71.77 ± 9.38 | 68.27 ± 9.07 | NS | 47.81 ± 8.16 | 70.71 ± 9.38 | 0.01 |

Values are mean percentage ± SEM.
In both groups, statistical comparisons were made using two-tailed Student’s t-tests between data obtained before and after IVF. Differences were considered statistically significant for \( P \)-values ≤0.05. NS = not statistically significant.
IVF women expressed significantly more CD160 ($P = 0.03$) and NKG2D receptors ($P = 0.01$) compared with their expression before IVF. Changes in these NK cell receptor expressions applied to both NK cell subsets (CD56$^{\text{bright}}$: $P = 0.01$ and $P = 0.03$; CD56$^{\text{dim}}$: $P = 0.05$ and $P = 0.01$, respectively).

**Table 3** represents the changes of NK cell functional characteristics before and after IVF in women with successful conception and in women with failed IVF. In the peripheral blood of women with failed IVF attempts, significantly more peripheral CD56$^{\text{bright}}$ NK cells contained cytotoxic perforin molecules ($P = 0.02$). In the case of women with failed IVF, the ratio of activated and degranulated NK cells significantly increased after IVF, shown by the increased CD107a expression ($P = 0.02$). These findings predominantly affect the CD56$^{\text{dim}}$ subpopulation ($P = 0.02$).

**Table 4** shows alterations in the NKT-like cell phenotype characteristics before and after IVF in successful IVF and failed IVF patients. In women with failed conception, the ratio of peripheral NKT-like cells significantly increased after the IVF procedure ($P = 0.01$). After IVF, there was a significant ($P = 0.02$) decrease in perforin-containing NKT-like cells compared with their percentage before fertilization in the successful IVF group. NKT-like cells of failed IVF women expressed significantly more CD160 ($P = 0.04$) and NKG2D receptors ($P = 0.01$) compared with their expression before IVF performance. CD160 expression of NKT-like cells in women with successful IVF significantly decreased after the embryo transfer ($P = 0.05$).

**Discussion**

Assisted reproductive techniques are now routine gynaecological procedures. Although their efficiency is undoubted, a notable proportion of IVF cycles result in failed conception resulting in emotional and financial strain for the couples. Much effort has been made to predict IVF treatment outcome and characterize IVF patients’ chances prior to the procedure. A major field of interest is immune profile analysis of IVF candidates since unexplained female infertility,
as well as recurrent spontaneous abortion and implantation failure, is thought to be associated with pathological maternal immunotolerance mechanisms. Therefore, NK cell characteristics have been intensively investigated. Exploring data of idiopathic infertile women with multiple IVF failures, Beer et al. (1996) suggested that elevation of CD56+ NK cells to over 18% in the peripheral blood of pregnant women is a negative prognostic factor for pregnancy maintenance. Coulam et al. (2003) revealed that systemic enumeration of CD56+ cells could predict pregnancy outcome. Contrarily, Thum et al. (2005) couldn’t find any significant differences of simple peripheral NK cell counts between patients with successful IVF treatment and IVF patients with failed conception, but another study (Thum et al., 2004) reported that in IVF patients, an increase in the peripheral cytotoxic CD56dimCD16+CD69+ NK cell subpopulation is associated with a poorer pregnancy outcome. In a prospective study, enhanced activation marker (CD69, CD161) expression and elevated cytotoxicity of peripheral NK cells were found to be risk factors for IVF implantation failure (Coulam and Roussev, 2003).

The current study analysed IVF patients’ data from a different point of view. Besides separating successful IVF patients from failed conception, it performed a follow-up study and compared patients’ data on the day of oocyte retrieval with the data 1 week after embryo transfer. Different changes of NK and NKT-like cell characteristics were found during this interval in pregnant and non-pregnant women. At 1 week after IVF, women with failed IVF showed elevated peripheral NK (both CD56 brightly and CD56 dim) and NKT-like cell ratios, increased perforin-containing CD56 brightly cells, more activated and degranulated NK dim cells and enhanced NK cell-activating receptor expression on both cell types as well as on both NK cell sub-sets (CD160, NKG2D). According to the hypothesis of van den Heuvel et al. (2008), elevated CD56 brightly NK cells in the failed IVF group may account for reduced decidual recruitment and could predict implantation failure. The current findings concerning the increased activating NK cell receptor expression during pregnancy failure are consistent with the data published by others (Ntrivalas et al., 2005; Varla-Leftherioti et al., 2003). Although the ratio of NK cells before IVF was significantly higher in the successful IVF compared with failed IVF group, on the one hand this value did not reach the abnormally increased level (18%) published by several papers (Beer et al., 1996; Kwak-Kim and Gilman-Sachs, 2008), but on the other hand it is assumed that one of the crucial points in failed IVF treatment is the significant increase in NK cells from the date of oocyte collection to the time 1 week after embryo transfer in the same individual. All these findings in the failed IVF group reflect unfavourable, Th1-oriented changes of NK and NKT-like cells during this short period of time around the IVF procedure. Comparing the follow-up data of pregnant women, they remain principally constant. There is a significant decrease in perforin-containing and CD160 expressing NKT-like cells in successful IVF patients, which is beneficial compared with the changes in failed IVF women.

Winger et al. (2009) demonstrated that treatment with tumour necrosis factor-α or intravenous immunoglobulin could be beneficial in young infertile women with Th1/Th2 cytokine elevation. The current findings also confirm the importance of immune therapy, in patients with previous pregnancy failure, during IVF procedures.

Previous studies on peripheral NK cell characteristics of IVF patients were limited to the comparison of pregnant and non-pregnant patients’ data from one blood sample prior to the IVF procedure. Here, the current study demonstrates that changes of NK and NKT-like cell phenotype during the implantation window and early pregnancy in the same individual may also be important for successful embryo implantation.

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