Intrauterine administration of autologous peripheral blood mononuclear cells promotes implantation rates in patients with repeated failure of IVF–embryo transfer

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BACKGROUND: There are few effective approaches to infertile patients with repeated failure in IVF–embryo transfer therapy. Since recent evidence suggests that some populations of maternal immune cells positively support embryo implantation, we have developed a new approach using peripheral blood mononuclear cells (PBMCs). METHODS: Patients who had not experienced successful pregnancy despite four or more IVF–embryo transfer sessions were enrolled in this study (n = 35, 35 cycles). PBMCs were obtained from patients on the day of oocyte retrieval and were cultured with HCG for 48 h. Two days later, PBMCs were freshly isolated from patients again, combined with cultured PBMC and then administered to the intrauterine cavity of the patients. Blastocyst transfer was performed on day 5, and the success of implantation in the PBMC-treated group was compared with that in the non-treated group. RESULTS: Clinical pregnancy rate, implantation rate and live birth rate in the PBMC-treated group (41.2, 23.4 and 35.3%; n = 17, 47 and 16, respectively) were significantly higher than those in the non-treated group (11.1, 4.1 and 5.5%; n = 18, 49 and 18, respectively). CONCLUSION: Intrauterine administration of autologous PBMC may be an effective approach to improve embryo implantation in patients with repeated IVF failures.

Key words: embryo implantation/PBMC/IVF outcome

Introduction
Embryo implantation in the uterus is an essential phenomenon for mammals. In general, the endocrine system regulates endometrial differentiation to prepare for embryo implantation. Estrogen-primed endometrium is further differentiated to secretory endometrium by progesterone stimulation. In mice, it is widely accepted that endometrium can receive embryo implantation during a limited period, that is, the implantation window (Psychoyos, 1993). Although human endometrium is also considered to have a limited period for embryo receptivity (Lessey, 2000), there have been few experiments investigating this concept. Using a primary monolayer culture of human endometrial epithelial cells and BeWo cell spheroids (John et al., 1993), a human choriocarcinoma-derived cell line (Pattillo and Gey, 1968), we observed that the adhesive ability of endometrial epithelial cells for BeWo cells was increased in those derived from women in the midsecretory phase (Kosaka et al., 2003), supporting the idea that the receptive property of endometrial epithelial cells changes according to menstrual cycle (Lessey, 2000). It has been widely accepted that poorly developed endometrium is associated with infertility (Fanchin, 2001). Consequently, sex steroid hormone supplementation has been clinically applied to infertile patients to induce adequate endometrial differentiation. However, when endometrial reactivity to sex steroid hormones is poor, this supplementation has little effect on endometrial function. Thus, alternative therapies other than endocrine approaches have been explored (Sher and Fisch, 2002).

Although the endocrine system plays a main role in preparing the endometrium for embryo implantation, there is evidence suggesting that the immune system also participates in supporting embryo implantation when the oocyte is fertilized. The presence of a developing embryo in the genital tract is one of the most important events for human females. More than two decades ago, early pregnancy factor was reported to be secreted from the developing embryo and to influence immune cell function and early events related to development and implantation of the embryo (Clarke, 1992; Morton et al., 1992; Morton, 1998). We report that immune cells derived from pregnant mice directly promote endometrial differentiation at the peri-implantation period in a manner independent of the endocrine system (Takahatake et al., 1997a,b). Later, CD4(+)CD8(-) lymphocytes in the thymus derived from non-pregnant mice were also shown to enhance endometrial differentiation to induce embryo implantation, suggesting that...
some populations of immune cells can affect endometrial differentiation and open the implantation window even when the female is not pregnant (Fujita et al., 1998). To support this, when endometrial epithelial cells were incubated with autologous peripheral blood mononuclear cells (PBMCs), attachment of BeWo cell spheroids was significantly promoted in endometrial epithelial cell culture derived from women in the late proliferative and early secretory phases (Kosaka et al., 2003). In addition, PBMCs obtained from women early in pregnancy were shown to promote murine blastocyst spreading and invasion and BeWo cell invasion in vitro, and these promoting effects were enhanced by HCG (Egawa et al., 2002; Nakayama et al., 2002). Furthermore, HCG is able to stimulate chemokine production by PBMC (Kosaka et al., 2002). Together, these findings suggest that the maternal immune system supports embryo implantation in the uterus as a complementary pathway and that HCG can induce functional changes in PBMC to facilitate embryo implantation (Fujiwara, 2006).

Based on the above evidence, we have developed a new approach for IVF-embryo transfer. PBMCs were isolated from the blood circulation of patients on the day of oocyte retrieval and incubated in the presence of HCG. Two days later, cultured PBMCs and freshly isolated PBMCs were combined and administered to the uterine cavity of patients 3 days before embryo transfer. In this study, we applied this approach to patients with repeated failure in IVF-embryo transfer therapy and evaluated the therapeutic effects of this approach.

Materials and methods

Subjects

A total of 35 cycles in 35 patients were included in this study. All patients had experienced four or more failures of IVF-embryo transfer therapy without poor ovarian reserve (FSH < 15 mIU/ml).

IVF procedure

Ovarian stimulation, oocyte collection and embryo culture were performed as described previously with minor modifications (Fujiwara et al., 2002). In brief, administration of a GnRH agonist (buserelin acetate; Aventis Pharma Co., Tokyo; Mochida Pharmaceutical Co., Osaka, Japan) was initiated in the midluteal phase or early follicular phase. All patients subsequently received pure FSH (Serono Japan, Co., Tokyo, Japan) or HMG (Organon Japan Co., Tokyo, Japan) from cycle day 3 for ovarian stimulation until the dominant follicle reached a diameter of >18 mm, followed by injection of HCG (5000 IU, Mochida Pharmaceutical Co., Osaka, Japan) 36 h before oocyte retrieval. After oocyte retrieval, HCG (3000 IU) was administrated on day 1 and day 5 to support luteal function.

After oocytes were retrieved, they were cultured in Quinn’s Advantage Fertilization Medium (Sage BioPharma, Inc., Bedminster, NJ, USA) with 10% serum protein substitute (SPS, Sage BioPharma, Inc) in 5% CO2, 5%O2 and 90% N2. Conventional insemination or ICSI was performed for IVF.

Embryo culture

After fertilization was confirmed the day after fertilization (day 1), the zygotes were cultured for another 2 days. For blastocyst transfer, embryos were further cultured in Blastocyst Medium (Irvine Scientific Sales Co., Inc., Santa Ana, CA, USA) with 10% SPS. The quality of blastocyst on day 5 was evaluated according to the scoring system by Gardner and Schoolcraft (1999) and types 2BB or better were assessed as good blastocysts. No more than three embryos showing the highest grade were transferred to the uterine cavity on day 5.

Preparation and intrauterine administration of PBMC

On the day of oocyte retrieval, blood samples were obtained from individual patients, and PBMCs (1 × 107 cells) were isolated by Ficoll–Hypaque centrifugation as described previously (Hashii et al., 1998). After centrifugation, PBMCs were collected from the interphase layer and washed four times with Roswell Park Memorial Institute 1640 (RPMI 1640). PBMCs (1 × 105 cells/ml) suspended in RPMI 1640 supplemented with 10% SPS were incubated in the presence of HCG (5 IU/ml, Mochida Pharmaceutical Co.) for 48 h. PBMCs (1 × 107 cells) were also obtained from the same patients 2 days after oocyte retrieval. After isolating PBMCs, these fresh PBMCs were immediately combined with 2-day cultured PBMC and suspended in PBS (2 × 107 cells/200 μl). This cell suspension was gently administrated to the uterine cavity using an embryo transfer catheter (Kitazato Supply Co., Shizuoka, Japan) on day 2.

Ethics

This study was performed according to the ethical guidelines regarding studies in which human gametes or embryos are used as materials; these guidelines are issued by the Ethics Committee of the Japan Society of Obstetrics and Gynecology. This study was approved by the Ethics Committee of Kyoto University Hospital, and informed consent for the use of spare embryos in this study was obtained from all donor couples attending the IVF unit of Kyoto University Hospital.

Data analysis

Differences between PBMC-treated and non-treated groups with regard to age, numbers of previous IVF–embryo transfer attempts, numbers of embryos transferred and numbers of good quality embryos were analysed by the two-tailed t-test. Chi-square test was used for comparisons of the clinical pregnancy rate, implantation rate and live birth rate between groups. A difference of <0.05 was considered significant.

Results

Patients who received blastocyst transfer after four or more failures of IVF-embryo transfer therapy were classified into PBMC-treated (n = 17, 17 cycles) and non-treated groups (n = 18, 18 cycles) based on the patient’s treatment preferences. Only first trials were included in this study. There were no significant differences in age, numbers of previous IVF–embryo transfer attempts, numbers of embryos transferred or numbers of good quality embryos were analysed by the two-tailed t-test. Chi-square test was used for comparisons of the clinical pregnancy rate, implantation rate and live birth rate between groups. A difference of <0.05 was considered significant.

The clinical pregnancy rate, implantation rate and live birth rate in the PBMC-treated group were significantly higher than those in the non-treated group (Table I). However, when the groups were further subdivided by patient age (<40 and ≥40), there was no significant difference between the PBMC-treated and non-treated groups in patients ≥40 years. In contrast, in patients <40 years old, the difference observed was more evident. The clinical outcomes of the PBMC-treated group were significantly better than that of the non-treated group (Table II). There were no significant differences in age, numbers of previous IVF–embryo transfer attempts, numbers of embryos transferred...
There was also no significant difference in endometrial thickness as assessed by ultrasonography on the day of oocyte retrieval between the two groups (Table II).

<table>
<thead>
<tr>
<th>Characteristics of the patients</th>
<th>PBMC treated</th>
<th>Non-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>37.5 ± 4.4</td>
<td>36.6 ± 4.4</td>
</tr>
<tr>
<td><strong>Number of IVF–embryo transfer previous attempts</strong></td>
<td>5.76 ± 2.5</td>
<td>5.2 ± 1.4</td>
</tr>
<tr>
<td><strong>Endometrial thickness (mm) on day of oocyte retrieval</strong></td>
<td>9.59 ± 1.47</td>
<td>10.7 ± 1.97</td>
</tr>
<tr>
<td><strong>Number of embryos transferred</strong></td>
<td>2.76 ± 0.56</td>
<td>2.72 ± 0.58</td>
</tr>
<tr>
<td><strong>Number of good quality embryos</strong></td>
<td>1.65 ± 0.70</td>
<td>1.50 ± 0.71</td>
</tr>
</tbody>
</table>

**Clinical outcome**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PBMC treated</th>
<th>Non-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical pregnancy rate</strong></td>
<td>41.2% (7/17)</td>
<td>11.1% (2/18) (P = 0.042)</td>
</tr>
<tr>
<td><strong>Implantation rate</strong></td>
<td>23.4% (11/47)</td>
<td>4.1% (2/49) (P = 0.011)</td>
</tr>
<tr>
<td><strong>Live birth rate</strong></td>
<td>35.3% (6/17)</td>
<td>5.5% (1/18) (P = 0.028)</td>
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PBMC, peripheral blood mononuclear cells; n.s., not significant.

**Table II. Characteristics and clinical outcome of the patients under 40 years**

<table>
<thead>
<tr>
<th>Characteristics of the patients</th>
<th>PBMC treated</th>
<th>Non-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>34.1 ± 3.06</td>
<td>34.4 ± 2.32</td>
</tr>
<tr>
<td><strong>Number of IVF–embryo transfer previous attempts</strong></td>
<td>5.67 ± 2.65</td>
<td>5.23 ± 1.36</td>
</tr>
<tr>
<td><strong>Endometrial thickness (mm) on day of oocyte retrieval</strong></td>
<td>9.56 ± 1.59</td>
<td>11.3 ± 2.17</td>
</tr>
<tr>
<td><strong>Number of embryos transferred</strong></td>
<td>3.0 ± 0.0</td>
<td>2.85 ± 0.60</td>
</tr>
<tr>
<td><strong>Number of good quality embryos</strong></td>
<td>1.78 ± 0.67</td>
<td>1.69 ± 0.79</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PBMC treated</th>
<th>Non-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical pregnancy rate</strong></td>
<td>66.7% (6/9)</td>
<td>15.4% (2/13) (P = 0.014)</td>
</tr>
<tr>
<td><strong>Implantation rate</strong></td>
<td>37.5% (9/25)</td>
<td>5.4% (2/37) (P = 0.0034)</td>
</tr>
<tr>
<td><strong>Live birth rate</strong></td>
<td>55.6% (5/9)</td>
<td>7.6% (1/13) (P = 0.013)</td>
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</table>

PBMC, peripheral blood mononuclear cells; n.s., not significant.

Discussion

This study showed that intrauterine administration of autologous PBMC promoted implantation and clinical pregnancy rates in patients <40 years old who underwent IVF–embryo transfer. Although there was no such significant difference observed among patients ≥40 years, this finding suggests that intrauterine administration of autologous PBMC is an effective approach to improve the clinical outcome of infertility therapy.

The protocol employed here was initially designed based on the concept that in the presence of an embryo, the maternal immune system positively influences the function of the reproductive organs including the corpus luteum and endometrium, facilitating embryo implantation in the uterus (Takabatake et al., 1997a; Hashii et al., 1998). However, the precise mechanism(s) of the effects of PBMC observed in this study is still unclear.

It has been proposed that inflammation of the endometrium can affect embryo implantation. In mice, mechanical stimulation also induces the decidual reaction in the endometrium (De Feo, 1967). Biologically active substances produced by inflammation can affect the microenvironment of the endometrium and contractility of the uterine muscle. In humans, infectious inflammation is speculated to cause implantation failure or spontaneous abortion (Romero et al., 2004). Intrauterine devices are also considered to cause inflammatory reactions which prevent embryo implantation (Rivera et al., 1999). In this study however, we cannot exclude the possibility that mechanical stimulation of intrauterine insertion of the catheter for PBMC administration contributed to a successful outcome. Because a sham procedure for intrauterine insertion was not performed in the PBMC non-treated group for ethical reasons, this issue remains undetermined.

Although PBMCs are autologous cells from the patient, we should note the possibility that PBMC by themselves evoke favourable inflammatory reactions in the uterine cavity. In general, immune cells become activated when they migrate from blood circulation to stromal tissues in vivo. It is well known that immune cells are activated by attachment on dishes in vitro, producing several cytokines. We have observed that adhesive PBMCs are increased in the presence of HCG (unpublished data). Several investigators have reported that the secretion of several cytokines is modified by HCG stimulation of PBMC in vitro (Schafer et al., 1992; Yousefi et al., 1993; Komorowski et al., 1997). In baboons, chorionic gonadotropin has been shown to regulate endometrial differentiation in vivo (Fazleabas et al., 1999). Recently, in vivo infusion of interleukin (IL)-1β and chorionic gonadotropin has been shown to induce endometrial changes that mimic early pregnancy events (Strakova et al., 2005). We have also demonstrated that HCG stimulates IL-8 production by PBMC through lectin–glycan interaction and that its basal production by PBMC is increased in women at 4–5 weeks of gestational age (Kosaka et al., 2002). Several studies have suggested important roles of chemokines including IL-8 in embryo implantation (Caballero-Cumpo et al., 2002; Selam et al., 2002; Jones et al., 2004). We have further demonstrated that chemokines regulate human extravilous trophoblast invasion (Sato et al., 2002, 2003, 2005; Fujivara et al., 2005). Thus, it is possible that PBMCs evoke some inflammation in the uterine cavity to facilitate embryo implantation.

In this study, to expect both in vivo and in vitro effects of HCG on PBMC, we combined PBMC that had been cultured with HCG for 2 days with PBMC that had been influenced by HCG injection in vivo and applied them for intrauterine administration. However, this study provides no definitive evidence showing the positive effect of HCG on PBMC function in embryo implantation in vivo. Therefore this issue should be further explored to develop more adequate protocols for PBMC activation.

It should also be noted that PBMC can secrete proteases. Various proteases and their inhibitors have been reported to be produced by human endometrium in the secretory phase and are proposed to regulate endometrial function (Salamonsen, 2003). These proteases may effectively change the function or structure of surface molecules expressed on the endometrial
REFERENCES


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