Histologic characteristics of the endometrium predicts success when utilizing autologous endometrial coculture in patients with IVF failure

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Purpose: To analyze the success of autologous endometrial coculture (AEC) in improving embryo quality and pregnancy outcome based on the histologic characteristic of the biopsy.

Methods: Prospective study of 86 consecutive patients undergoing IVF utilizing AEC.

Results: The patients were on average 37.4 ± 4.0 years with a history of 2.6 ± 1.8 failed previous attempts. An overall clinical pregnancy rate of 45.3% per ET was found. The embryos grown in AEC were of an improved quality in comparison to those grown in conventional media. 33.7% (29/86) of the biopsies were out of phase (>3 days). In-phase (IP) and OOP (out of phase) specimens both demonstrated an improvement in embryo quality. However, OOP endometrial biopsies that displayed significant retarded endometrial development ($<$ cycle day 19) did not demonstrate an improvement in embryos grown on AEC as compared to IP endometrial biopsies or OOP endometrial biopsies that demonstrated at least an endometrial development of cycle day 19.

Conclusions: We have demonstrated a significant improvement in embryo quality with AEC. We have also demonstrated that histologic dating of the endometrium is predictive of IVF outcome when utilizing AEC.

KEY WORDS: Autologous endometrial coculture; cytokines; endometrium; IVF.

INTRODUCTION

Autologous endometrial coculture is a useful technique for women with multiple failed IVF-ET cycles. We have previously demonstrated that utilizing autologous endometrial coculture is positively associated with embryo quality and pregnancy outcome (1,2).

We have shown that the success of autologous endometrial coculture is dependent upon the date of the endometrial biopsy (3). Our work has shown that autologous endometrial coculture improves embryo quality through the elaboration of cytokines by the stromal and epithelial cells of the endometrium. It appears that several cytokines (GM-CSF, LIF, and the IL-1 system) are associated with IVF outcome when utilizing autologous endometrial coculture (4–6). Therefore, the success of our autologous endometrial coculture system is dependent upon an appropriately developed endometrium. We undertook this study to analyze the correlation between histologic evaluation of the endometrium and success when utilizing autologous endometrial coculture (AEC).

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MATERIALS AND METHODS

Eighty-six consecutive patients enrolled in our AECC program were studied. Patients were selected to be included in the AECC study if they had poor embryo development in their previous cycle ($n = 25$), multiple failed IVF cycles ($n = 16$) or both ($n = 45$). The overall decision to utilize AECC was at the attending physicians' discretion. Patient outcomes were analyzed based on the histologic evaluation of the endometrium utilized in the AECC cycle. All histologic specimens were evaluated by a single gynecologic pathologist (RS) in a blinded fashion. The criteria for dating were the standardized methods of Noyes *et al.* (7).

Endometrial Coculture Techniques

Endometrium was obtained from each patient in a natural, nonmedicated cycle prior to her IVF attempt. Patients with anovulatory cycles were not utilized for this study. The endometrium was obtained by a luteal phase endometrial biopsy using a Pipelle Endometrial Suction Curette (Unimar, Wilton, CT). The biopsy was performed 5–11 days after a urinary ovulation predictor kit or a serum test detected the LH surge (3). The digestion, separation and preparation of the endometrium have been previously described (3).

In brief, the tissue was initially minced into small pieces (1–3 mm³) and washed with Hank's Balanced Salt Solution (HBSS; Gibco BRL, Grand Island, NY) supplemented with 5000 ug/100 mL Penicillin-Streptomycin (Gibco BRL, Grand Island, NY) to remove excess red blood cells and mucus.

Incubation of the tissue pieces for 5–6 min at 37°C in a shaking water bath in 10 mL of HBSS containing 0.2% collagenase type 2 (SIGMA, St Louis, MO) was then performed. Cell clumps were dispersed by brisk manipulation through a sterile transfer pipette. The digested tissue pieces were then allowed to settle by differential sedimentation at unit gravity for 5 min. After sedimentation, the supernatant, containing a mixture of single stromal cells and small intact glands, were transferred into a separate 15 mL polyethylene test tube and centrifuged at $400 \times g$ for 5 min. The pellet was resuspended in RPMI Medium 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% patient's serum (RPMI/10% serum) and 5000 ug/100 mL penicillin-streptomycin. The above steps were repeated four to five times resulting in a combined 4 mL of single stromal cells

mixed with small glands. This stroma and small gland sample underwent another differential sedimentation at unit gravity for 45 min to separate the majority of small glands from the single stromal cells remaining in solution. The supernatant, containing the stroma-enriched fraction, was centrifuged at $400 \times g$ for 5 min and the cell pellet was resuspended in RPMI/10% patient serum. A small aliquot of the sample was diluted 1:1 with Trypan Blue Stain 0.4% (Gibco BRL, Grand Island, NY) and cell yield and viability were determined quantitatively on a hemacytometer. Tissue culture flasks (25 cm²) were seeded with $\sim 5 \times 10^5$ cells.

The tissue pieces, which remained after the four digests, contained predominately intact glands mixed with small amounts of undigested connective tissue and stromal clumps. Concurrently, the glands were further purified by resuspension in 10 ml of HBBS. After approximately 30 s, the largest fragments (stromal clumps, undigested tissue) settled to the bottom of the 15 ml test tube and the top 8 mL, which had a "snow flake" appearance (glands and single stromal cells), were transferred to another 15 mL test tube and allowed to settle for 30 min at unit gravity. This sedimentation allowed the majority of glands to form a pellet at the bottom of the test tube while leaving the remaining single stromal cells in the supernatant that was removed. This glandular enriched pellet was then resuspended in RPMI/10% FCS and plated into one 25 cm² tissue-culture flask. After several hours of incubation for the glandular enriched pellet, the supernatant was removed and replated in a new tissue flask. This was done to improve the purity of the glandular component based on the fact that stromal cells plate quickly and glands are much slower to attach to the tissue flask.

The seeded tissue flasks were maintained at 37°C in 5% CO₂ air atmosphere and the culture medium were changed every 2 days. After approximately 6–10 days the cells reached confluence and were released with Trypsin-EDTA (Gibco BRL, Grand Island, NY). The cells were cryopreserved in a 15% glycerol solution and frozen at -70°C overnight then transferred to liquid-nitrogen storage.

Approximately equal mixtures of the glandular and stromal cells were thawed two to three days prior to placement of the human embryos on the coculture cells. Cell count and viability were determined and $\sim 4 \times 10^5$ cells were seeded into a four-well tissue culture plate containing 800 μL of Hams F-10 with 15% human serum. Usually, three wells were plated. In general, $\sim 90\%$ confluence was achieved when the

human embryos were placed into the coculture system. Conditioned media was changed every 1–2 days prior to exposure to human embryos.

Embryos were placed on AECC the day after oocyte retrieval when fertilization was determined. Fresh media consisting of Hams F-10 with 15% human serum was utilized prior to placement of the embryos on the coculture. Embryos were grouped and placed in one or two of the wells. The embryos remained on AECC until just prior to the embryo transfer. During this 2-day period, the media was not changed.

IVF Methods

Patients were treated with standard ovulation induction protocols and underwent IVF-ET as previously described (8). Patients underwent stimulation as determined by the attending physician. Human chorionic gonadotrophin (HCG) was administered (3300–10,000 IU) when at least two follicles reached or exceeded seventeen mm mean diameter as measured by transvaginal ultrasound. Oocytes were harvested by transvaginal ultrasound-guided follicular puncture 35 h after HCG administration.

Conventional oocyte insemination or ICSI was performed as indicated. The best morphologically appearing normal embryos were transferred into the uterine cavity approximately 72 h after retrieval. The number of embryos transferred was dependent on maternal age, according to our standard protocol. In general, two to three embryos were transferred to patients under 36 years of age, patients 36–39 years of age received four embryos, and patients over 40 underwent transfer of up to five embryos when available. Progesterone supplementation was initiated on the third day after HCG administration (25–50 mg i.m./day) and was continued until the sonographic assessment of the pregnancy and a fetal heart was visualized.

Statistical Analysis

Data are presented as mean ($\pm$ standard deviation). The data was not normally distributed, and therefore, continuous data were compared utilizing non-parametric tests. Categorical data was compared utilizing chi-square analysis. A p -value <0.05 was considered significant.

IRB approval for the study was obtained for the study.

RESULTS

A total of 86 patients participated in the study over a 48-month time period. The mean age was $36.8 (\pm 4.0)$ years. The patients had on average a history of $2.6 (\pm 1.8)$ failed prior attempts. Patients were selected to be included in the AECC study if they had poor embryo development in their previous cycle ($n = 25$), multiple failed IVF cycles ($n = 16$) or both ($n = 45$). In the prior cycle immediately preceding the treatment (AECC) cycle, there were no clinical pregnancies. The previous cycle did not differ significantly from the treatment cycle in respect to peak estradiol levels or number of oocytes obtained (1537 ± 656 pg/mL vs. 1499 ± 588 pg/mL, 8.6 ± 4.3 vs. 7.9 ± 4.9 oocytes, treatment vs. previous cycle, respectively). The embryos grown in AECC during the treatment cycle were of an improved quality in comparison to those grown in conventional media from the previous cycle (6.3 ± 0.2 vs. 5.3 ± 0.14 blastomeres, $p = 0.003$; $16.1 \pm 1.7\%$ vs. $21.2 \pm 1.3\%$ fragmentation, $p < 0.001$; paired t -test).

During the IVF treatment cycle, some embryos were grown on AECC and others were grown utilizing conventional media. The embryos grown in AECC were of an improved quality in comparison to those grown in conventional media (6.3 ± 0.2 vs. 5.4 ± 0.2 blastomeres, $p = 0.003$; $16.1 \pm 1.7\%$ vs. $21.4 \pm 1.9\%$ fragmentation, $p = 0.006$; Wilcoxon Rank-Sum). It is interesting to note that there was no difference in the embryo quality (cell number or fragmentation) grown in conventional media from the previous cycle or the treatment cycle. Only the embryos grown in AECC were of an improved quality.

An overall clinical pregnancy rate of 45.3% was found.

Just over one third [29/86 (33.7%)] of the endometrial biopsies were out of phase. In-phase and out of phase endometrial biopsies both demonstrated an improvement in embryo quality. No differences were found in clinical pregnancy outcome (47.6 vs. 36.4%, in phase vs. out of phase, $p = 0.38$, chi-square test).

Previous work had suggested that the success of coculture depended on the endometrium being at least five days post an LH surge. We therefore examined the success based on histologic characteristics of at least cycle day 19 in the OOP specimens. Out of phase endometrial biopsies that displayed significant retarded endometrial development ($<$ cycle day 19) did not demonstrate an improvement in embryos grown on AECC as compared to in phase endometrial biopsies or out of phase endometrial biopsies

that demonstrated at least an endometrial development of cycle day 19. Despite this lack of apparent embryo improvement, no differences in pregnancy outcome were noted.

DISCUSSION

We have demonstrated a clinically applicable, autologous endometrial coculture (AECC) system for patients with a history of multiple failed IVF attempts. We demonstrated an overall clinical pregnancy rate of approximately 40% when utilizing autologous endometrial coculture in patients with a history of multiple IVF failures. We have also found an improvement in embryo quality when utilizing this autologous endometrial coculture system.

We have previously demonstrated that the AECC system depends upon the date of the endometrial biopsy as well as the elaboration of various cytokines and growth factors. It therefore is logical to evaluate the histologic development of the endometrium as a factor predicting success when utilizing AECC. In previous work we have demonstrated that endometrial biopsies obtained before cycle day 19 (based on LH surge as day 14) were not helpful in AECC embryonic development (3). Thus the findings of this study demonstrating that out of phase endometrial biopsies with significant retardation of endometrial development were not helpful in improving embryo quality when utilizing AECC is consistent with our prior work. We speculate that with further study and more patients that if the lack of embryonic development improvement seen with significantly retarded endometrial development continues, we would find differences in pregnancy outcome.

Substantial embryonic-maternal cross-talk at the time of early embryonic development via cytokines and growth factors is well documented (9). Many cytokines and growth factors play important roles in the establishing and promoting early embryonic development and implantation (10). Previously, in earlier studies, we have established the importance of various cytokines (LIF, GM-CSF and the IL-1 system) in the autologous endometrial coculture system (4-6). Autologous endometrial coculture outcome was positively associated with the production of these cytokines. Because the endometrial function and cytokine production may be altered in the patient with out of phase endometrial biopsies, it can be hypothesized that there may be alterations in the

production of the growth factors and cytokines that help improve embryonic development when utilizing AECC.

Another consideration for the failed cycles when a significant OOP was detected that these patients may represent a subset of individuals with implantation abnormalities related to the development of the endometrium. During the IVF cycle the patients were exposed to supernormal levels of estradiol and progesterone; therefore, the abnormalities found during a natural cycle biopsy may not remain. Further work is aimed at determining the role of the endometrium and its development in these patients with multiple failed IVF cycles.

In summary, we have demonstrated a successful autologous endometrial coculture system. We have shown that when the endometrium displays significant retarded epithelial development, there appears to be a lack of improved embryo development. This work suggests that when utilizing AECC, it may be prudent to evaluate the endometrium histologically.

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