Autologous Endometrial Co-culture in Patients with Repeated Failures of Implantation After In Vitro Fertilization—Embryo Transfer

LARRY I. BARMAT,^{1,2} HUNG-CHING LIU,¹ STEPHEN D. SPANDORFER,¹ ANIA KOWALIK,¹ CAROL MELE,¹ KANGPU XU,¹ LUCINDA VEECK,¹ MARK DAMARIO,¹ and ZEV ROSENWAKS¹

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Purpose: Our purpose was to evaluate the effect of co-culture on preembryo development and clinical outcome.

Methods: Enrolled patients underwent a luteal-phase endometrial biopsy. The tissue was then enzymatically digested (collagenase) and the stromal and glandular cells were separated by differential sedimentation rates. These cells were cultured to confluence, released, and then cryopreserved until the patient's in vitro fertilization (IVF)–embryo transfer (ET) cycle. All normally fertilized oocytes were then placed on the co-cultured cells until transfer on day 3. Preembryo development on co-culture was compared to that in the patient's noncocultured previous cycle. Implantation and clinical pregnancy rates were compared to those in a control group of patients undergoing IVF during the study period who were matched for age, stimulation protocol, number of oocytes retrieved, and preembryos transferred.

Results: Twenty-nine women underwent 31 cycles of IVF-ET. On day 3 the overall mean number of blastomeres per preembryo on co-culture compared to that in the patient's previous cycle was 6.3 ± 1.8 vs. 5.6 ± 1.2 ($P = 0.04$). The average percentage of cytoplasmic fragments on co-culture compared to the previous cycle was $16 \pm 9\%$ vs. $19 \pm 9\%$ ($P = 0.32$). At transfer, after preembryo selection, the mean number of blastomeres per preembryo on co-culture compared to that in the patient's previous cycle was 6.8 ± 1.6 vs. 6.6 ± 1.3 ($P = 0.5$). The implantation and clinical pregnancy rates between co-culture and the matched control group were 15% (14/93) vs. 13% (16/124) ($P = 0.79$) and 29% (9/31) vs. 25% (10/40) ($P = 0.45$).

Conclusions: There was a significant improvement in the average number of blastomeres per preembryo on co-culture

compared to that in the patient's previous noncoculture cycle. The overall implantation and clinical pregnancy rates between co-culture and a matched control group were not significantly different.

KEY WORDS: co-culture; endometrium; in vitro fertilization; embryo; morphology.

INTRODUCTION

Since the landmark report in 1978 of the first human birth resulting from in vitro fertilization (IVF)–embryo transfer (ET), numerous advances and modifications have occurred in the field of assisted reproduction. The introduction of gonadotropin-releasing hormone agonists has allowed greater control of follicular stimulation, with an increased number of oocytes retrieved and improved pregnancy rates per stimulated cycle (1). Also, the development of intracytoplasmic sperm injection enables fertilization rates comparable to those with conventional IVF, revolutionizing the treatment of male infertility (2). Unfortunately, despite these advances in assisted reproductive technologies, the take-home baby rate remains stubbornly low.

One factor that may contribute to these poor success rates is the current in vitro culture conditions, which sustain only approximately 20–50% of preembryos to the blastocyst stage (3–5). Therefore, to minimize in vitro preembryo fragmentation and degeneration and to maximize the opportunity for embryo replacement, many IVF-ET programs transfer zygote to eight-cell-stage preembryos into the uterus. This uterine environment may not be optimal for cleavage of early preembryos to the blastocyst stage prior to implantation and may further contribute to lower pregnancy rates. Also, many mammalian embryos, including human, display

¹ The Center for Reproductive Medicine and Infertility, The New York Hospital–Cornell Medical Center, New York, New York.

² To whom correspondence should be addressed at Crozer Reproductive Endocrinology and Fertility Center, One Medical Center Boulevard, Upland, Pennsylvania 19013-3995.

specific blocks to development in vitro that are related to the transition from maternally derived components of genetic information to the activation of the embryonic genome (6). These developmental delays can be partially alleviated by culturing preembryos in the presence of somatic cells or with conditioned and/or growth factor-supplemented media (7). In fact, growth rates and morphology have been significantly improved for preembryos maintained in co-culture systems (7). Although controversial, there is also evidence that preembryo development on somatic cell lines may enhance implantation rates in human IVF (5). Therefore, we have developed a unique autologous endometrial co-culture system and successfully applied this to our clinical IVF-ET program. We report our findings on preembryo development and clinical outcome in a select group of patients with histories of failed implantations who subsequently underwent IVF-ET using their own endometrial cells for co-culture.

MATERIALS AND METHODS

Between January 1996 and June 1997, 29 patients with a history of at least one previous failed IVF attempt with poor preembryo quality were entered into this study. Poor preembryo quality was suggested by preembryos of less than six cells at transfer or morphology of less than grade 2 (8). Couples were treated with IVF for a variety of infertility disorders. All patients signed a consent form which was approved by the New York Hospital–Cornell Medical Center's Institutional Review Board.

Enrolled patients underwent a luteal-phase endometrial biopsy using a Pipelle Endometrial Suction Curette (Unimar, Wilton, CT) in a cycle prior to their IVF procedure. The sample was transferred to the laboratory in a sterile container filled with normal saline solution. The tissue was then minced into small pieces (1 to 2 mm³) and washed with Hank's balanced salt solution (HBSS) (Gibco BRL, Grand Island, NY) supplemented with 5000 µg per 100 ml penicillin–streptomycin (Gibco BRL) to remove excess red blood cells and mucus. A small portion of each endometrial biopsy was placed in 10% neutral buffered formalin solution for histologic assessment. All tissue samples revealed morphologic changes consistent with secretory endometrium ranging from cycle day 16 to cycle day 25. Tissue dissociation was then accomplished by four enzymatic digestion steps, with separation of glands and stromal cells by differential sedimentation at unit gravity.

The methodology involved modifications to previously published techniques to make them less time-consuming, while maintaining highly purified cell populations for nonclinical research purposes (9). Our technique involves incubation of the tissue pieces for 5 min at 37°C in a shaking water bath in 10 ml of HBSS containing 0.2% collagenase type 2 (Sigma, St. Louis, MO) and 5000 µg per 100 ml of penicillin–streptomycin. Cell clumps were dispersed by brisk aspiration 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, was transferred into a separate 15-ml polyethylene test tube and centrifuged at 400g for 5 min. The pellet was resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL) supplemented with 10% patient's serum (RPMI medium/10% serum) and 5000 µg per 100 ml of penicillin–streptomycin. The above steps were repeated four 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 400g for 5 min, and the cell pellet was resuspended in RPMI medium/10% patient serum. A small aliquot of the sample was diluted 1:1 with Trypan blue stain 0.4% (Gibco BRL), and the cell yield and viability were determined quantitatively on a hemacytometer. Tissue culture flasks (25 cm²) were seeded with approximately 5×10^5 cells.

The tissue pieces, which remained after the four digests, contained predominantly intact glands mixed with undigested connective tissue and stromal clumps. Concurrently, the glands were further purified by resuspension in 10 ml of HBSS. After approximately 30 sec 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 "snowflake" appearance (glands and single stromal cells), was 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, which was removed and discarded. This glandular enriched pellet was then resuspended in RPMI medium/10% patient serum and plated into one to three 25-cm² tissue culture flasks, depending on a gross estimate of the yield.

The seeded tissue flasks were maintained at 37°C in a 5% CO₂ air atmosphere and the culture medium was changed every 2–3 days. After approximately 1 week the cells reached confluence and were released with Trypsin–EDTA (Gibco BRL). The cells were cryopreserved in a 15% glycerol solution and frozen at –70°C overnight, then transferred to liquid nitrogen storage.

An approximately equal mixture of the glandular and stromal cells was thawed on the estimated day before the administration of human chorionic gonadotropin during the patient's IVF treatment cycle. This ratio was chosen, as histologic assessment of luteal-phase biopsies revealed a similar ratio of glandular and stromal cells in vivo. Cell count and viability were determined and approximately 3×10^5 cells were seeded into a four-well tissue culture plate containing 1 ml of Ham's F-10 medium (Gibco BRL), supplemented with 15% patient serum. In general, approximately 50–75% confluence was achieved when preembryos were placed into the co-culture system, and at transfer close to a monolayer existed. The medium was changed just prior to preembryo placement on the co-culture cells to allow an adequate reservoir of nutrients for both the preembryo and the helper cells.

The procedure for our assisted reproductive program has been described previously (2). Briefly, a variety of controlled ovarian stimulation protocols (long and short leuprolide acetate plus gonadotropins and clomiphene citrate plus gonadotropins) was implemented based on the patient's previous response to ovarian stimulation, age, and day 3 hormonal status. Daily estradiol levels and pelvic ultrasounds monitored follicular maturation. Human chorionic gonadotropin (5000–10,000 IU) was administered when at least two follicles were 17 mm or greater, followed by transvaginal oocyte retrieval 35 hr later. Conventional in vitro insemination or microsurgical insemination occurred based on appropriate indications (2). After 12–18 hr of incubation fertilization was confirmed by the identification of two pronuclei. Preembryos were then transferred to growth in the autologous endometrial co-culture system until transfer on day 3. All preembryos were maintained at 37°C in a 5% CO₂ air atmosphere. Cleavage rates and morphologic appearance were assessed daily. The morphologically best preembryos were transferred back to the patient 72 hr after retrieval. Selective assisted hatching was performed on all patients as previously described (2). After preembryo transfer the co-culture cells were immunostained using a monoclonal antipapillary keratin (Sigma), demon-

strating on average approximately 25–50% glandular epithelial cells.

The Wilcoxon matched-pairs test and the median test were used to compare differences between the patients' co-culture cycle and their previous cycle. All values are reported as mean $\pm$ standard deviation except the number of preembryos transferred, which is reported as median $\pm$ semiinterquartile range based on the distribution of values. The clinical outcomes of the co-culture patients were compared to those of a separate matched control group. This control group included all patients who underwent IVF-ET during the study period and was matched for age, ovarian stimulation protocol, number of oocytes retrieved, and number of preembryos replaced. These data were analyzed using a χ^2 test for independence. The implantation rate was defined as the number of intrauterine sacs with a fetal heartbeat per the number of preembryos transferred. Clinical pregnancies included only those pregnancies with a fetal heartbeat documented on transvaginal ultrasound by day 49.

RESULTS

Twenty-nine women aged 39.0 ± 3.7 years underwent 31 cycles of IVF-ET. Their ages were similar to their previous cycle mean age of 38.0 ± 3.5 (95% CI = 37.6–40.3). They had an average of 4.1 ± 3.1 previous IVF-ET attempts. The couples suffered from a variety of infertility disorders: 9 idiopathic, 9 tubo-peritoneal, and 11 male factor. The ovarian stimulation protocols used in the patients' previous cycle and co-culture cycle included long leuprolide acetate plus gonadotropins (24 vs. 15), short leuprolide acetate plus gonadotropins (5 vs. 11), and clomiphene citrate plus gonadotropins (2 vs. 5).

The estradiol level on the day of human chorionic gonadotropin in the previous cycle compared to the co-culture cycle was 1385 ± 490 vs. 1224 ± 440 pg/ml ($P = 0.1$). The number of oocytes retrieved and fertilizing normally in the patients' previous cycles compared to the co-culture cycles was 9.4 ± 4.0 vs. 7.4 ± 3.1 ($P = 0.003$) and 5.8 ± 2.5 vs. 3.9 ± 1.9 ($P = 0.0002$). There were significantly fewer preembryos transferred in the co-culture cycle compared to the patients' previous cycle (3 ± 1 vs. 4 ± 0.5 ; $P = 0.001$). These data are given in Table I.

A total of 170 cleaved preembryos in the previous cycle and 114 cleaved preembryos in the co-culture cycle was available for morphologic analysis. The average percentage of cytoplasmic fragments on the

Table I. Comparison of the Co-culture Cycle to the Previous Cycle

	Previous cycle	Co-culture cycle	<i>P</i> value
Age ^a	38.0 ± 3.5	39 ± 3.7	
Estradiol on day of hCG ^a	1385 ± 490	1224 ± 440	0.10
No. of oocytes retrieved ^a	9.4 ± 4.0	7.4 ± 3.1	0.003
No. of 2 pronuclei ^a	5.8 ± 2.5	3.9 ± 1.9	0.0002
Mean No. of blastomeres per preembryo on day 3 ^a	5.6 ± 1.2	6.3 ± 1.8	0.041
Percentage fragmentation prior to assisted hatching ^a	19 ± 9	16 ± 9	0.32
No. of preembryos at transfer ^b	4 ± 0.5	3 ± 1	0.0001
Mean No. of blastomeres per preembryo transfer	6.6 ± 1.3	6.8 ± 1.6	0.5
Implantation rate	0% (0/116)	15% (14/93)	
Clinical pregnancy rate	0% (0/31)	29% (9/31)	

^a Mean ± SD.^b Median ± semiinterquartile range.

day of transfer prior to assisted hatching was $19 \pm 9\%$ in the prior cycle in conventional medium, compared to $16 \pm 9\%$ ($P = 0.32$) for the preembryos in co-culture. The average number of blastomeres per preembryo on day 3 for the cohort was 5.6 ± 1.2 in the previous cycle versus 6.3 ± 1.8 ($P = 0.04$) in the subsequent cycle on co-culture. There was a total of 116 preembryos selected for transfer in the previous cycle, compared to 95 preembryos in the co-culture cycle. The average number of blastomeres per preembryo at transfer was 6.6 ± 1.3 in the previous cycle, compared to 6.8 ± 1.6 ($P = 0.5$) in the co-culture cycle. These data are also shown in Table I.

The clinical outcomes of the co-culture patients were compared to those of the matched control group. The age, stimulation protocol, number of oocytes retrieved, number of fertilized oocytes with two pronuclei, and preembryos transferred were the same in the co-culture and control groups (Table II). Overall, implantation and clinical pregnancy rates between the co-culture and the control groups were not significantly different [$14/93 = 0.15$ vs. $16/124 = 0.13$ ($P = 0.79$) and $9/31 = 0.29$ vs. $10/40 = 0.25$ ($P = 0.45$)]. The co-culture patients did undergo significantly more previous IVF-ET attempts than the matched control group

(4.1 ± 3.1 vs. 0.58 ± 0.08 ; $P = 0.001$). The patients and controls were then stratified to those younger than 40 and those 40 years of age or older. The average age of the patients younger than 40 was 36.2 ± 2.9 . The implantation rate and clinical pregnancy rate of this subgroup using co-culture compared to the control group were 25% (13/52) vs. 19% (14/73) ($P = 0.42$) and 50% (8/16) vs. 35% (8/23) ($P = 0.35$). The patients 40 years of age or older had an average age of 41.8 ± 1.7 years. The implantation rates and clinical pregnancy rates between the co-culture and the control patients 40 years of age or older were 2.4% (1/41) vs. 3.9% (2/51) ($P = 0.7$) and 6.6% (1/15) vs. 11.8% (2/17) ($P = 0.5$).

DISCUSSION

Various attempts at altering the in vitro culture conditions to mimic more closely the in vivo reproductive environment have involved modifications of medium electrolyte and energy sources, with limited success (10). Therefore, in an effort to increase the potential dynamic interactions between somatic cells and the developing preembryo, the application of co-culture

Table II. Comparison of Co-culture Versus Matched Control Patients

	Co-culture	Control	<i>P</i> value
Age ^a	39 ± 3.7	39 ± 3.7	
No. of previous IVF attempts ^a	4.1 ± 3.1	0.58 ± 0.08	0.001
No. of oocytes retrieved ^a	7.4 ± 3.1	7.4 ± 3.7	
No. of 2 pronuclei ^a	3.9 ± 1.9	4.0 ± 2.1	
No. of preembryos at transfer ^a	3.0 ± 1	3.0 ± 1	
Implantation rate	15% (14/93)	13% (16/124)	0.79
Clinical pregnancy rate	29% (9/31)	25% (10/40)	0.45

^a Mean ± SD.

has been used in human IVF-ET. The most commonly used cell lines include bovine reproductive tract cells (5), African green monkey kidney cells (Vero) (11), and human oviduct and granulosa cells lines (12,13). The inherent fear of using xenologous and heterologous cell lines is the risk of disease transmission to the exposed preembryos. Only recently have continuously subcultured autologous endometrial cells been used in human IVF (14).

We have developed a unique autologous endometrial co-culture system utilizing first passaged-cryopreserved stroma and glandular epithelial cells as confirmed by immunohistologic staining. Preembryo growth with monolayers of somatic cells has been shown to overcome developmental blocks in several mammalian species in vitro, and it has been proposed that this effect was mediated primarily via the production of growth factors by the feeder cells. It has been demonstrated by Northern analysis that co-culture cell lines used in human IVF express a number of growth factors (15). Although many cell types in the reproductive tract synthesize embryotrophic factors, Pollard (16) has suggested that the one of the most important sources may be the secretory epithelial cells lining the uterine lumen. Utilizing polarized in vitro systems, there is evidence that uterine epithelial cells secrete interleukin-1 α , and when uterine epithelial cells were co-cultured with uterine stromal cells there was a severalfold increase in stromal cell secretory products (17). It was proposed that the secretion of interleukin-1 α by epithelial cells might function in an autocrine or paracrine manner to regulate aspects of uterine function during implantation. Another group reported the presence of the interleukin-1 system in human preembryos by immunohistochemical experiments and failed to detect the secretion of these cytokines when preembryos were cultured in a simple defined medium (18). Further work noted the selective release of interleukin-1 only when preembryos were cocultured with human endometrial epithelial cells or endometrial epithelial conditioned medium compared to endometrial stromal cells (18). This suggested an obligate role of the endometrial epithelium in the regulation of the embryonic interleukin-1 system. These studies demonstrate the importance of intercellular interactions in regulating both feeder cell line and preembryo function. Therefore our co-culture system, containing stroma and glandular epithelial cells, will provide the conditions necessary for these autocrine and paracrine interactions to occur in an effort to enhance preembryo quality.

The effect of somatic cell lines on human preembryo growth has been evaluated in a number of studies.

Improvements in preembryo grade, an increase in the average number of blastomeres, and a decrease in the average percentage of fragments per preembryo have been demonstrated in controlled studies utilizing bovine oviduct and fibroblast, Vero, and human tubal co-culture cell lines (4,5,19,20). A recent prospective randomized trial found a higher percentage of fertilized oocytes developed to the eight-cell stage in three co-culture systems compared to serum-supplemented medium (21). Additional studies randomizing deselected "spare" preembryo growth between cumulus or human ampullary co-culture and chemically defined media have reported significantly more preembryos reaching the blastocyst stage, with more cells per blastocyst, when co-culture is used (13,22). There is also evidence that patients with multiple failures of IVF who subsequently undergo bovine oviduct co-culture have a significant improvement in both the average number of cells and the fragmentation rate per preembryo on co-culture compared to their previous noncoculture cycle (23). Although the majority of research suggests improved preembryo development on feeder cell lines, this is still controversial, as there are also studies which have not demonstrated differences in mean cell numbers per preembryo or blastulation rates on bovine oviduct or Vero co-culture compared to control medium (24,25).

In our study, after approximately 48 hr of preembryo growth on the autologous endometrial co-culture cells, there was a significant improvement in the average number of blastomeres per preembryo on co-culture compared to that in the patient's previous cycle. These findings are consistent with those of Wiemer *et al.* (23), who evaluated a similar deselected population and noted a subsequent therapeutic benefit of co-culture. We were not able to note any significant difference in fragmentation rates between the co-culture cycle and the control cycle prior to assisted hatching. Although at preembryo transfer there was no significant difference in mean cell number between the co-cultured and the noncocultured preembryos, this may be a function of the significantly larger cohort of preembryos available to choose from in the previous cycle compared to the co-culture cycle.

The impact of feeder cell lines on clinical outcome in human IVF-ET remains controversial. There are both randomized (5,19) and nonrandomized (16,12,23) studies utilizing a variety of co-culture cell lines that demonstrate the beneficial effects of helper cell lines on in vitro preembryo development and clinical outcome. Furthermore, a recent study suggests that co-cultured preembryos are more likely than their nonco-

cultured counterparts to implant following cryopreservation (25). However, other investigators have shown that, while preembryo growth is improved on helper cell lines, there are no significant effects of co-culture on pregnancy rates (4,24).

There was no difference in the overall clinical outcome for our co-culture patients compared to the matched control group. When patients were separated based on age, the co-culture patients younger than 40 had a trend toward an improved implantation rate and clinical pregnancy rate compared to the controls. Although this was not statistically different, due to the small sample size we cannot rule out a type II error. A power analysis showed that a sample size of 366 would be needed to detect a 15% difference in clinical pregnancy rates between the two groups (50% for co-culture and 35% for control) with a power of 80% and a significance level of 0.05.

In conclusion, we have developed a unique first passaged-cryopreserved autologous endometrial co-culture system utilizing both the stromal and the glandular cell populations. This differs from the only other reported use of the patient's own endometrial cells for co-culture, where a simple digestion technique resulted in a mixture of stromal and epithelial cells that were continuously subcultured until the patient underwent oocyte retrieval (14). The theoretical benefit of our system may allow for a greater degree of intercellular interactions than continuously subcultured co-culture systems, which tend to be overgrown by a single population of fibroblasts. There is evidence that our autologous endometrial co-culture system increases the average number of blastomeres per preembryo compared to the patient's previous noncocultured cycle. Although we did not detect any significant differences in the implantation and clinical pregnancy rates with the use of co-culture compared to the controls, we are encouraged that in this deselected population the clinical outcome was at least similar to that of the controls, who had significantly fewer IVF-ET attempts. Further larger prospective randomized trials comparing co-cultured versus noncocultured preembryos are necessary to determine the clinical impact on IVF-ET success rates.

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