

Human preembryo development on autologous endometrial coculture versus conventional medium

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Objective: To evaluate the effect of autologous endometrial coculture versus conventional medium on preembryo development.

Design: Controlled systematic clinical study.

Setting: University-based IVF center.

Patient(s): Women with a history of failed IVF-ET with poor preembryo quality.

Intervention(s): Patients underwent a luteal phase endometrial biopsy. The tissue then was digested enzymatically, 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 IVF-ET cycle. All normally fertilized oocytes then were allocated systematically to growth on autologous endometrial coculture or conventional medium until transfer on day 3.

Main Outcome Measure(s): Preembryo blastomere numbers and cytoplasmic fragmentation rates were measured.

Result(s): Forty-two women underwent 44 cycles of IVF-ET. In the morning on day 3, the mean (\pm SD) number of blastomeres and cytoplasmic fragments per preembryo on coculture compared with conventional medium was 5.9 ± 1.5 versus 5.5 ± 1.4 and $21\% \pm 13\%$ versus $24\% \pm 11\%$. At transfer the mean (\pm SD) number of blastomeres per preembryo on coculture was 7.4 ± 1.3 versus 6.7 ± 1.9 on conventional medium.

Conclusion(s): There was a significant improvement in the mean (\pm SD) number of blastomeres per preembryo and decrease in the fragmentation rate for preembryos on autologous endometrial coculture compared with noncultured preembryos from the same patient. (Fertil Steril® 1998;70:1109-13. ©1998 by American Society for Reproductive Medicine.)

Key Words: Endometrial, coculture, IVF-ET

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The quality of the in vitro culture conditions for human preembryos is one of the most critical aspects of successful IVF-ET. Studies in lower mammalian species have suggested that the in vitro preembryo rate of development (1), cell number (2), biochemical synthetic activity (3), and survival after ET (4) are inferior to in vivo developed preembryos. Specific developmental blocks related to the transition from maternal to zygotic genomic activation are displayed in preembryos grown in an in vitro environment (5).

Various attempts at improving in vitro culture conditions by modification of medium electrolyte and energy sources have met with

limited success (6, 7). The development of preembryo coculture systems that have used a variety of types of "helper cells" has provided a more efficient means to maintain preembryos in vitro, allowing improved developmental rates and possibly improved pregnancy rates (8).

Although the beneficial effects of coculture systems have been demonstrated by a number of researchers, the mechanism of action of these helper cells is not understood fully. Coculture cells have been demonstrated to both produce embryotrophic factors (9) and may also serve to detoxify the culture medium (10). The variability in success rates associated with coculture systems can be attributed to differ-

ences in types of cell lines, maintenance of the cells, and various environmental factors within each laboratory. We have developed a unique coculture system that uses the patient's own endometrial cells and successfully applied this to our clinical IVF-ET program. We report our findings of human preembryo development on autologous endometrial coculture cells versus conventional medium.

MATERIALS AND METHODS

Forty-two patients who were undergoing IVF-ET treatment between January 1996 and June 1997 at The Center for Reproductive Medicine and Infertility of the New York Hospital-Cornell Medical Center and who had a history of at least one previously failed IVF-ET attempt with poor preembryo quality were entered into this study. Poor preembryo quality was defined as preembryos of <6 cells or preembryos of <grade 2 morphology at transfer on day 3 (11). The couples were treated with IVF-ET for a variety of infertility disorders. The clinical application of coculture was approved by the New York Hospital-Cornell Medical Center's Institutional Review Board, and all patients gave informed consent to participate in this study.

A luteal phase endometrial biopsy was performed in a cycle before the patient's IVF procedure with use of a Pipelle Endometrial Suction Curette (Unimar, Wilton, CT). The sample was transferred to the laboratory in a sterile container filled with normal saline solution. The tissue then was minced into small pieces (1–2 mm³) and washed with Hank's balanced salt solution (HBSS) supplemented with 5,000 µg per 100 mL of penicillin-streptomycin (Gibco BRL, Grand Island, NY) 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.

Tissue then was dissociated by four enzymatic digestion steps with separation of the glandular epithelium and stromal cells by differential sedimentation at unit gravity. The methodology involved modifications to previously published reports, which are more time-consuming, and developed to achieve highly purified cell populations for nonclinical research purposes (12). Our technique involves incubation of the tissue pieces for 5 minutes at 37°C in a shaking water bath in 10 mL of HBSS containing 0.2% collagenase type 2 (Sigma, St. Louis, MO) and 5,000 µg per 100 mL of penicillin-streptomycin.

Cell clumps were dispersed by brisk aspiration through a sterile transfer pipette. The digested tissue pieces then were allowed to settle by differential sedimentation at unit gravity for 5 minutes. 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 × g for 5 minutes. The pellet was resuspended in RPMI medium 1640 (Gibco BRL) sup-

plemented with 10% patient's serum (RPMI-10% serum) and 5,000 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 minutes to separate most small glands from the single stromal cells remaining in solution. The supernatant, containing the stroma enriched fraction, was centrifuged at 400 × g for 5 minutes, 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), and cell yield and viability were determined quantitatively on a hemacytometer. Tissue culture flasks (25 cm²) were seeded with approximately 5 × 10⁵ cells.

The tissue pieces, which remained after the four digests, contained predominately intact glands mixed with undigested connective tissue and stromal clumps. Concurrently, the glands were purified further by resuspension in 10 mL of HBBS. After approximately 30 seconds, the largest fragments (stromal clumps and 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) were transferred to another 15-mL test tube and allowed to settle for 30 minutes at unit gravity. This sedimentation allowed most of the 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 and discarded. This glandular enriched pellet then was resuspended in RPMI-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 5% CO₂ air atmosphere, and the culture medium was changed every 2–3 days. After approximately 1 week, the cells reached to confluence and were released with trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco BRL). The cells were cryopreserved in a 15% glycerol solution and frozen at –70°C overnight and then transferred to liquid nitrogen storage.

Approximately an equal mixture of the glandular and stromal cells were thawed on the estimated day before the administration of hCG during the patient's IVF treatment cycle. Cell count and viability were determined, and approximately 3 × 10⁵ 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 75% confluence was achieved when preembryos were placed into the coculture system.

A detailed description of the methodologies used in our IVF program were previously published (13). Briefly, controlled ovarian stimulation protocols based on patient's previous response to ovarian stimulation, age, and day 3 FSH and E₂ hormonal status were implemented. Follicular matu-

TABLE 1

Characteristics of human preembryos developed on autologous endometrial coculture and conventional medium.

Preembryo characteristics	Coculture	Conventional medium	Wilcoxon's matched-pairs test
No. of preembryos	203	186	
Mean (\pm SD) no. of blastomeres per preembryo on day 3	5.9 \pm 1.5	5.5 \pm 1.4	0.19
Mean (\pm SD) percentage of fragmentation before assisted hatching	21 \pm 13	24 \pm 11	0.045
No. of preembryos transferred	90	83	
Mean (\pm SD) no. of blastomeres per preembryo transfer	7.4 \pm 1.3	6.7 \pm 1.9	0.032

ration was monitored by daily E₂ levels and transvaginal ultrasounds. Human chorionic gonadotropin (5,000–10,000 IU) was administered when at least two follicles reached a mean diameter of \geq 17 mm, and transvaginal oocyte retrieval was performed 35 hours later. Conventional in vitro insemination or intracytoplasmic sperm injection was performed on the basis of appropriate indications (13).

After 12–18 hours of incubation, fertilization was confirmed by the identification of two pronuclei. The zygotes then were removed from the insemination droplet and alternately allocated to growth in conventional medium (human tubal fluid plus 15% maternal serum) or autologous endometrial coculture with Ham's F-10 medium (Gibco BRL) supplemented with 15% maternal serum. All preembryos were maintained at 37°C in 5% CO₂ air atmosphere. Cleavage rates and morphological appearance were assessed daily. Embryologists were not blinded to the culture system used because this would require greater manipulation of the cocultured preembryos by transfer to a control system with prolonged exposure outside the incubator. Selective assisted hatching was performed as previously described (14).

The morphologically best preembryos were transferred back to the patient 72 hours after retrieval irrespective of culture system. After preembryo transfer, the coculture cells were fixed in 4% paraformaldehyde. Immunostaining of the coculture cells using a monoclonal antipancytokeratin antibody (Sigma, St. Louis, MO) demonstrated approximately 25%–50% glandular epithelial cells per coculture well.

Wilcoxon's matched-pairs test was used to compare differences between the cocultured and conventionally cultured preembryos. All values are reported as means \pm SD. A *P* value of $<.05$ was considered statistically significant. The implantation rate was defined as the number of intrauterine sacs with fetal cardiac activity per number of preembryos transferred. Clinical pregnancies included only those pregnancies with a fetal heart beat documented on transvaginal ultrasound by day 49.

RESULTS

Forty-two women with a mean (\pm SD) age of 36.7 \pm 4.3 years underwent 44 cycles with an ET. They had a mean

(\pm SD) of 3.0 \pm 2.5 previous unsuccessful IVF attempts. The ovarian stimulation protocols used included long leuprolide acetate (*n* = 32), short leuprolide acetate (*n* = 7), and clomiphene citrate plus gonadotropins (*n* = 5). The couples had a variety of infertility disorders: idiopathic (*n* = 6), tuboperitoneal (*n* = 14), anovulation (*n* = 2), male factor (*n* = 20).

The mean (\pm SD) E₂ level on the day of hCG was 1,554 \pm 621 pg/mL. There was a mean (\pm SD) of 13.6 \pm 6.3 oocytes retrieved, and 9.0 \pm 4.7 demonstrated two pronuclei after insemination or intracytoplasmic sperm injection. In all cycles the cohort of zygotes was alternated systematically between growth in conventional medium or on autologous endometrial coculture. A total of 203 preembryos were placed on coculture, and 186 preembryos were placed in conventional medium. There were 90 cocultured and 83 noncocultured preembryos that were transferred back to the patients.

Table 1 summarizes the preembryo characteristics. The third morning after oocyte retrieval there was a mean (\pm SD) of 5.9 \pm 1.5 blastomeres per preembryo on autologous endometrial coculture compared with 5.5 \pm 1.4 (*P* = .19) blastomeres per preembryo on conventional medium. The fragmentation rate before assisted hatching was 21% \pm 13% on coculture versus 24% \pm 11% (*P* = .045) for the noncocultured preembryos. Six hours later, at transfer, the mean (\pm SD) number of cells per preembryo in the cocultured compared with noncocultured preembryos was 7.4 \pm 1.3 versus 6.7 \pm 1.9 (*P* = .032).

Endometrial biopsy samples were available for 32 of the patients for histologic assessment. Five of the patients demonstrated proliferative endometrium, whereas 27 had cellular changes consistent with secretory endometrium ranging from cycle days 15–22.

The patients who had late proliferative phase biopsies had a total of 42 preembryos of which 21 were grown on coculture and 21 were grown on conventional media. The mean (\pm SD) number of blastomeres on day 3 in coculture versus conventional media was 6.1 \pm 1.7 versus 6.3 \pm 1.6 (*P* = .5) with 22% \pm 18% versus 23% \pm 18% (*P* = .8) fragmentation rate. There was a total of 18 preembryos transferred with a mean (\pm SD) of 8 \pm 0.6 cells for the cocultured preembryos and 7.4 \pm 1.1 cells for conventional

media ($P = .3$). The implantation rate was 17% (3 of 18), and clinical pregnancy rate of 60% (3 of 5). There did not appear to be a significant effect with the use of proliferative phase cells, although the numbers are small.

The overall implantation rate was 15% (26 of 173), and the clinical pregnancy rate was 50% (22 of 44). Two patients underwent preembryo transfer with all cocultured preembryos. Both patients failed to conceive. There were three patients whose preembryo transfer included only conventionally cultured preembryos. One patient conceived with triplets (3 preembryos transferred), and the other two failed to conceive.

DISCUSSION

We have developed a unique autologous endometrial coculture system using first passaged-cryopreserved stroma and glandular epithelial cells. In this study, patients consented to alternately grow their cohort of preembryos on conventional medium or coculture. The preembryos placed on the patient's own endometrial cells had fewer cytoplasmic fragments and a greater number of blastomeres at uterine transfer. Therefore, it appears that autologous endometrial coculture cells may provide a more suitable environment for preembryo growth than conventional medium.

The optimal culture conditions for preembryo development currently are unknown. Efforts to formulate a medium based on the composition of human tubal fluid have suggested that the ratio of Na^+ to K^+ ions in the culture medium are important for determining the quality of the preembryos and subsequent pregnancy rates (6). Others have demonstrated that the addition of glutamine, lack of glucose in the initial stages of cleavage, and the addition of EDTA may be beneficial in improving preembryo development (7). Unfortunately, these media are devoid of growth factors secreted by reproductive tract cells and potential paracrine signals, which could enhance preembryo growth and development.

It is becoming increasingly apparent that the preembryo is a dynamic organism that is able to respond to both autocrine as well as paracrine signals from the surrounding environment. When human preembryos are cultured in groups, morphological characteristics appear to improve, suggesting that factors released by the preembryos may enhance their quality (15). Embryonic signals also have been shown to stimulate endometrial stromal cell synthesis and secretion of insulin-like binding proteins that, in turn, may modify insulin growth factor action on mouse embryo development (16).

A recent study demonstrated enhanced gene expression of activin-receptors from deselected human preimplantation embryos when cocultured on human endometrial stromal cells. Because the activin ligand was expressed only in the cocultured cells while the activin receptor was detected only in the developing preembryo, a paracrine mechanism for activin in early preimplantation development was postulated

(17). Therefore, the autocrine and paracrine interactions between the preembryo and its culture environment may play a role, enhancing preembryo quality and possibly improving pregnancy rates in IVF-ET.

Because reports in the mid-1960s demonstrated that a higher percentage of mouse preembryos developed and hatched in vitro when cultured on a feeder cell line, the use of coculture has been applied successfully to both animal and human IVF programs (8). These helper cell lines appear to enhance in vitro growth conditions and provide the necessary factors that may allow preembryos of several species to overcome their specific in vitro developmental blocks. In fact, growth rates and morphology have been improved significantly for preembryos maintained in coculture systems (8).

The most commonly used cell lines in human IVF-ET include bovine reproductive tract cells (18), African Green Monkey kidney cells (Vero) (19), and human oviduct and granulosa cells lines (20, 21). The inherent fear of using xenologous and heterologous cell lines is the risk of disease transmission to the exposed preembryos. Only recently has autologous endometrial cells been used in human IVF (22, 23).

A number of studies have evaluated the effect of various somatic cell lines on human preembryo development. When surplus human preembryos were cocultured on fallopian tube epithelium, significantly higher rates of blastocyst formation with more nuclei per blastocyst were demonstrated compared with conventionally cultured preembryos (20).

Weimer et al. (18) found that cocultured preembryos grown on bovine oviductal cells had significantly more blastomeres and fewer cytoplasmic fragments than conventionally grown preembryos in randomly allocated patients. Other researchers, with the aid of videocinematography, noted a number of morphological features were improved significantly for cocultured compared to noncocultured preembryos on bovine oviductal cells (24). In addition, a recent prospective randomized trial found a significantly higher percentage of fertilized oocytes developed to the eight-cell stage on three different coculture systems compared with serum-supplemented media (25).

Although the literature predominantly suggests improved preembryo development on feeder cell lines, there are some studies that have not demonstrated differences in mean (\pm SD) cell numbers or blastulation rates in cocultured preembryos compared with those grown in control medium (26, 27).

In this controlled study, fertilized oocytes from the same patients were allocated systematically to growth on autologous endometrial coculture or conventional medium. The fragmentation rate was significantly less for the preembryos in the coculture system compared with noncocultured preembryos. Also, at the time of preembryo replacement, the mean (\pm SD) number of blastomeres per preembryo was greater in the cocultured preembryos compared with the noncocultured preembryos. These results are consistent with

previous reports demonstrating reduced fragmentation rates (22) and improved development rates (21) for patients whose preembryos were allocated randomly between growth in coculture versus conventional media. Because preembryo transfers in this study contained both cocultured and non-cocultured preembryos, we are not able to draw conclusions on clinical outcome.

A confounding variable in this study is the different medium used to culture the control and experimental embryos. The standard medium used in our IVF-ET laboratory is human tubal fluid plus 15% maternal serum. Unfortunately, the nutrient requirements of preembryos and coculture cells are different, and a more complex medium was necessary to support the coculture cells. Therefore, it is possible that the Ham's F-10 medium may have contributed to the improved preembryo quality on coculture. Also, because the embryologists were not blinded to the culture systems, we cannot rule out selection bias in the assessment of embryo number and fragmentation rates.

This study suggests that in vitro culture conditions may be enhanced with the use of autologous endometrial coculture as reflected in improved preembryo quality. There is evidence of a correlation between preembryo morphology and clinical pregnancy rates, which would suggest that this coculture system ultimately may have a beneficial effect on IVF-ET success (28, 29). In addition, by using the patient's own reproductive tract cells, the potential infectious risks of heterologous and xenologous coculture systems will be avoided.

Further research is necessary to determine if this coculture system is better able to support preembryo growth to the blastocyst stage, as has been suggested by others using different cell lines (19, 21). It remains to be demonstrated whether this system will improve implantation rates, enabling the transfer of fewer better quality preembryos with the potential reduction of the multiple gestation rate. A randomized controlled trial is currently underway at our institution comparing the growth and transfer of preembryos on coculture versus conventional media.

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