

# Importance of the biopsy date in autologous endometrial cocultures for patients with multiple implantation failures

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**Objective:** To analyze the effectiveness of autologous endometrial coculture by the cycle day of the endometrial biopsy.

**Design:** Retrospective study.

**Setting:** University-based IVF center.

**Patient(s):** Two hundred eight patients with multiple IVF failures.

**Intervention(s):** Embryos were split and randomly allocated to growth on autologous endometrial coculture or conventional media.

**Main Outcome Measure(s):** Embryo quality and pregnancy outcome.

**Result(s):** The overall clinical pregnancy rate was 41.8%. Embryos grown on autologous endometrial coculture were of higher quality (more blastomeres and less fragmentation) than embryos grown with conventional media. Early luteal biopsies (<5 days after LH surge) for autologous endometrial coculture did not demonstrate an improvement in embryo quality as compared to the significant improvement demonstrated with later luteal endometrial biopsies (≥5 days after LH surge). The date of the biopsy was predictive of pregnancy outcome when using autologous endometrial coculture (44.7% [≥5 days after LH surge] vs. 18.8% [<5 days after LH surge],  $P=.012$ ).

**Conclusion(s):** We have demonstrated an improvement in embryo quality when using autologous endometrial coculture. The improvement in embryo quality and higher pregnancy rates were limited to biopsies ≥5 days after the LH surge. This suggests that mid/late luteal phase endometrium contains factors that enhanced embryo growth and subsequent implantation. (*Fertil Steril*® 2002;77:1209–13. ©2002 by American Society for Reproductive Medicine.)

**Key Words:** Embryo, autologous endometrial coculture, IVF

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Success after IVF-ET is dependent on many factors. Maternal age and oocyte quality are the most important predictors associated with outcome (1, 2). However, many other factors are involved, and enhancing the development of human embryos in vitro has remained a major concern. Many strategies have been used including assisted hatching, improved culture media, and coculture techniques (3–14). Coculture techniques provide helper cell lines that appear to enhance the in vitro conditions and provide necessary factors to allow embryos to demonstrate improved in vitro development (12–14).

Many studies have evaluated the effect of various somatic cell lines on human embryo development. Most of these studies have found significantly decreased fragmentation and improved blastomere numbers in embryos grown in coculture (8–14). Why coculture works in enhancing embryonic development in the in vitro environment is not fully known. Theories include cell-to-cell interactions, cytokine and growth factor elaboration, and detoxification of the culture media.

A dominant theory of how autologous endometrial coculture (AECC) improves embryo quality is through the release of cytokines and

growth factors. We have previously demonstrated the role of granulocyte-macrophage colony-stimulating factor, interleukin-1, and leukemia-inhibiting factor in improving embryo quality (12, 13, 15). Furthermore, factors like insulin-like growth factor-I, transforming growth factor, and epidermal growth factor are released by various coculture cells, and these factors are known to have a direct enhancement effect on embryonic development (11). The endometrium is dynamic, secreting many cytokines, and the elaboration of these cytokines vary according to the date of the endometrium. Therefore, we undertook this study to evaluate the influence of the cycle date of the luteal phase endometrial biopsy, which was performed for use in our AECC program.

## MATERIALS AND METHODS

Two hundred eight patients with a history of multiple implantation failures (at least two) after IVF-ET were enrolled in our AECC study.

### Endometrial Coculture

Endometrium was obtained from each patient in a non-medicated cycle before her IVF attempt. The endometrium was obtained by a luteal phase endometrial biopsy using a Pipelle Endometrial Suction Curette (Unimar, Wilton, CT). The biopsy was performed 1–12 days after a urinary ovulation predictor kit detected the LH surge. The digestion and separation of the endometrium has been previously described (12).

In brief, the tissue was then minced into small pieces (1–2 mm<sup>3</sup>) and washed with Hank's balanced salt solution (GIBCO-BRL, Grand Island, NY) supplemented with 5,000 µg/100 mL penicillin-streptomycin (GIBCO-BRL) to remove excess red blood cells and mucus.

Incubation of the tissue pieces for 5 minutes at 37°C in a shaking water bath in 10 mL of Hank's balanced salt solution containing 0.2% collagenase type 2 (Sigma, St. Louis, MO) was then performed. 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 minutes. 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 400 × *g* for 5 minutes. The pellet was resuspended in RPMI medium 1640 (GIBCO-BRL) supplemented with 10% patient's serum (RPMI/10% serum) and 5,000 µg/100 mL penicillin-streptomycin. The above steps were repeated four times, resulting in 4 mL of single stromal cells mixed with small glands. Tissue culture flasks (25 cm<sup>2</sup>) were seeded with ~5 × 10<sup>5</sup> cells.

The tissue pieces, which remained after the four digests, contained predominately intact glands mixed with undigested connective tissue and stromal clumps. Another 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/10% patient serum and plated into one 25-cm<sup>2</sup> tissue culture flask.

The seeded tissue flasks were maintained at 37°C in 5% CO<sub>2</sub> air atmosphere and the culture medium were 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.

Approximately equal mixtures of the glandular and stromal cells were thawed 3 days before placement of the human embryos on the coculture cells. Cell count and viability were determined and ~4 × 10<sup>5</sup> 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, ~90% confluence was achieved when the human embryos were placed into the coculture system. Conditioned media was changed every 1–2 days before 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 used before 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 before the embryo transfer. During this 2-day period, the media was not changed. Patients with six or fewer embryos had all of their embryos placed on the coculture. Patients with more than six embryos had their embryo randomly split by the embryologist to growth on AECC or conventional media. This was determined by putting the first half on AECC and the second half on conventional media from numbering assigned to the oocytes before fertilization occurred. The fertilized embryos were then split in numerical order without preferences to the appearance of the fertilized embryos.

### IVF Methods

Patients were treated with standard ovulation induction protocols and underwent IVF-ET as previously described (1). In brief, most women were treated with luteal phase leuprolide acetate (Lupron; Tap Pharmaceuticals, Deerfield, IL), 1 mg SC daily until ovarian suppression was achieved. Women not treated with luteal leuprolide acetate began stimulation on day 2 of their treatment cycle. Ovarian stimulation was then effected with a combination of gonadotrophins (hMG) or pure FSH (Pergonal or Metrodin; Serono, Waltham, MA), using a step-down protocol (5). Human chorionic gonadotrophin was administered (3,300–10,000 IU) when at least two follicles reached or exceeded 16–17 mm mean diameter as measured by transvaginal ultrasound. Oocytes were harvested by transvaginal ultrasound-guided follicular puncture 35–36 hours after hCG administration.

Conventional oocyte insemination or micromanipulation was performed as indicated. Morphologically normal embryos were transferred into the uterine cavity approximately 72 hours after retrieval. The number of embryos transferred was dependent on maternal age, according to our standard protocol. In general, patients <35 years of age received three embryos, patients 35–40 years of age received four embryos, and patients >40 years underwent transfer of up to five embryos when available. Methylprednisolone (16 mg/d) and tetracycline (250 mg every 6 hours) were administered for 4 days to all patients commencing on the day of oocyte retrieval. Progesterone supplementation was initiated on the third day after hCG administration (25–50 mg IM/d) and was continued until the sonographic assessment of the pregnancy.

Embryos were evaluated each day after fertilization under the direction of our senior embryologist (LV). Fragmentation was assessed as a percentage by the embryologist. The embryo grade was determined by a unified system developed by our embryology team. The number of blastomeres was determined by a count by the embryologist.

Clinical pregnancy was defined as the presence of a fetal heart beat. All pregnancy rates are reported based on retrieval as the coculture was not used until after fertilization occurred.

The study was approved by the internal review board and all patients signed informed consent according to our internal review board policies.

### Statistical Analysis

Data is presented as mean ( $\pm$  SD). The data were not normally distributed, and therefore, continuous data were compared using nonparametric tests. Categorical data were compared using  $\chi^2$  analysis. A *P* value < .05 was considered statistically significant.

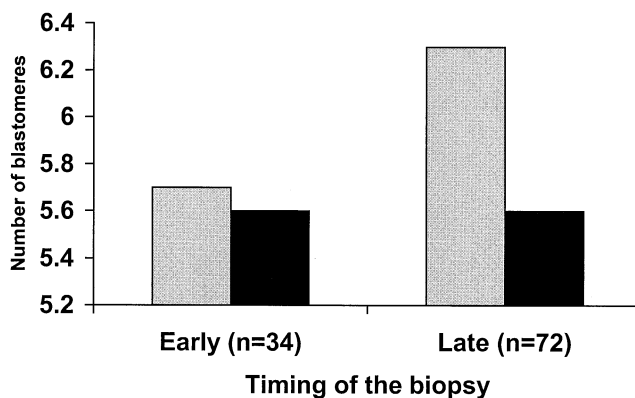
## RESULTS

A total of 208 patients underwent IVF using AECC. The average age of the patients was 37.6 years ( $\pm$ 3.9 years). The patients had had an average of 3.3 ( $\pm$ 2.4) previous attempts at IVF-ET. Seventy-nine of the 208 patients (37.9%) had an ongoing pregnancy after IVF-ET when using AECC.

To more accurately assess the embryonic improvement that the coculture system provided, we analyzed the group of patients (*n* = 106) that had enough embryos to allow for splitting and random allocation of embryos to AECC or conventional media. The average age of these patients was 36.7 years ( $\pm$ 4.1 years). The patients had had an average of 3.0 ( $\pm$ 2.1) previous attempts at IVF-ET. An improvement in number of blastomeres and a decrease in the amount of fragmentation in embryos grown on AECC was found compared to the randomly split embryos that were grown in conventional media (6.12  $\pm$  1.3 vs. 5.6  $\pm$  1.3 blastomeres,

**FIGURE 1**

Improvement in embryo development when using autologous endometrial coculture is restricted to biopsies performed at least 5 days after the LH surge. No significant difference was found when comparing the number of blastomeres between embryos grown in autologous endometrial coculture (light gray bars) from early biopsies as compared to conventional media (solid bars). On the other hand, embryos grown on autologous endometrial coculture from later biopsies demonstrated a significant improvement in number of blastomeres (*P* = .002) as compared to the split embryos randomly assigned to growth in conventional media.



Spandorfer. Autologous endometrial coculture and IVF. *Fertil Steril* 2002.

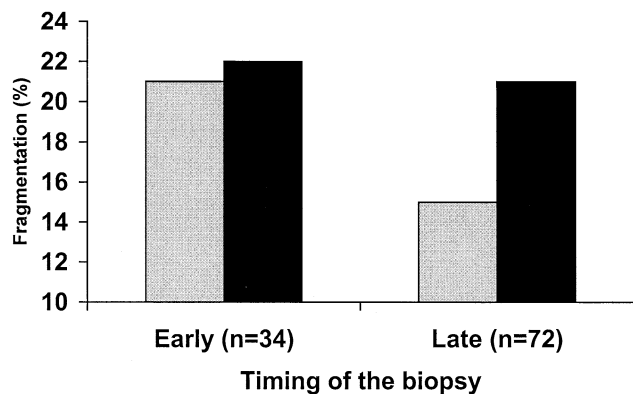
*P* = .0016; 17.9%  $\pm$  11.4% vs. 26.0%  $\pm$  38% fragmentation, *P* = .0003; AECC vs. conventional media, respectively).

We then compared the improvement of embryos grown on AECC as compared to conventional media based on the date of the endometrial biopsy. We compared early biopsies (biopsy performed <5 days after LH surge) to later endometrial biopsies ( $\geq$ 5 days after LH surge). Figures 1 and 2 illustrate that the improvement in AECC is only found in the biopsies performed at least 5 days after the LH surge. Later biopsies demonstrated an improvement in blastomere number as well as diminished fragmentation in contrast to the lack of improvement with earlier biopsies.

We then analyzed pregnancy outcome as determined by the date of the endometrial coculture biopsy. Because some patients had embryos grown on both coculture and conventional media, we restricted this analysis to the group of patients that received only embryos grown on AECC for their embryo transfer. Of the original 208 patients, 135 had only embryos grown on AECC replaced. Thirty-two patients (23.7%) had biopsies performed in the early luteal phase (<5 days after the LH surge) and 103 (76.3%) had biopsies performed in the later luteal phase ( $\geq$ 5 days after the LH surge). When using AECC from biopsies obtained at least 5 days after the LH surge, there was a significantly higher clinical pregnancy rate than when using coculture cells from earlier biopsies (Fig. 3).

**FIGURE 2**

Improvement in embryo development when using autologous endometrial coculture is restricted to biopsies performed at least 5 days after the LH surge. No significant difference was found when comparing the percent of fragmentation between embryos grown in autologous endometrial coculture (*light gray bars*) from early biopsies as compared to conventional media (*solid bars*). On the other hand, embryos grown on autologous endometrial coculture from later biopsies demonstrated a significant improvement with decreased fragmentation ( $P=.001$ ) as compared to the split embryos randomly assigned to growth in conventional media.



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## DISCUSSION

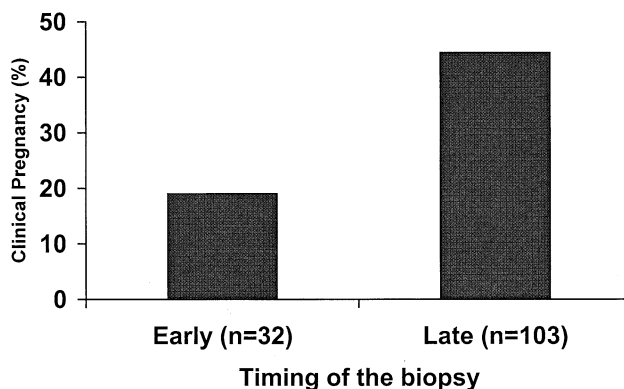
We demonstrated an overall clinical pregnancy rate of >40% when using AECC in patients with a history of multiple IVF failures. This work is in agreement with previous studies in demonstrating that AECC appears to be helpful in patients with multiple failed cycles of IVF-ET (5–12). Although many investigators have shown beneficial results when using AECC techniques, the mechanism of this action remains largely unknown.

We have previously postulated that AECC appears to improve embryonic development by the elaboration of growth factors (13–15). Because the endometrium is dynamic in its secretion of cytokines, we sought to analyze the results of our AECC program by the cycle date of the biopsy taken. All biopsies were timed by a LH surge; accurate dating was possible. We have clearly demonstrated that the benefits of AECC appear to be associated with mid to late luteal phase biopsies. In fact, early biopsies demonstrated no improvement in embryonic development; whereas biopsies performed at least 5 days after a LH surge were highly associated with improved embryonic quality. Furthermore, pregnancy rates were significantly better in the biopsies performed at least 5 days after the LH surge.

Because we were able to demonstrate an improvement in

**FIGURE 3**

A significantly higher pregnancy rate is seen when autologous endometrial coculture uses a biopsy at least 5 days after the LH surge in comparison to an earlier biopsy (<5 days after the LH surge;  $P=.012$ ).



Spandorfer. Autologous endometrial coculture and IVF. *Fertil Steril* 2002.

embryo quality in only the mid to late luteal phase biopsies, we can postulate that some factors from this part of the cycle is responsible for the improvement yielded by AECC. On the basis of this study, we subsequently limited biopsies to at least 5 days after the LH surge. In addition, we have expanded our investigations of possible cytokines that play a role in AECC, focusing on those elaborated in the mid to late luteal phase of the menstrual cycle.

These cultured cells have lost the normal three-dimensional architecture of *in vivo* endometrial cells and are possibly transformed by the digestion process. We acknowledge that these cells are different from *in vivo*, but we believe that they still possess the ability to secrete various growth factors and cytokines that may be important to the AECC process. Furthermore, because they are first-pass cell lines, transformation may not be as great as cell lines that have undergone multiple passages of growth.

Furthermore, because embryos grown on coculture were not improved when using earlier biopsies as compared to embryos grown in conventional medium, this allows us to make two important observations. First, this demonstrates that the coculture system works by more than merely allowing the embryos to undergo cell-to-cell contact. Second, we have demonstrated that the process of clustering a few embryos together in a single well does not account for the beneficial effect of coculture.

## References

1. Rosenwaks Z, Davis OK, Damario MA. The role of maternal age in assisted reproduction. *Hum Reprod* 1995;10 Suppl 1:165–73.
2. Spandorfer SD, Avrech OM, Colombero LT, Palermo GD, Rosenwaks Z. Effect of parental age on fertilization and pregnancy characteristics in couples treated by intracytoplasmic sperm injection. *Hum Reprod* 1998;13:334–8.

3. Liu H-C, Cohen J, Alikani M, Noyes N, Rosenwaks Z. Assisted hatching facilitates earlier implantation. *Fertil Steril* 1993;60:871-5.
4. Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Hum Reprod Update* 1997;3:367-82.
5. Thibodeaux J, Godke R. In vitro enhancement of early stage embryos with coculture. *Arch Pathol Lab Med* 1992;116:364-72.
6. Nietro FS, Watkins WB, Lopata A, Baker HWG, Edgar DH. The effects of coculture with autologous cryopreserved endometrial cells on human in vitro fertilization and early embryo morphology: a randomized study. *J Assist Reprod Genetics* 1996;13:386-9.
7. Vald M, Walker D, Kennedy RC. Nuclei number in human embryos cocultured with human ampullary cells. *Hum Reprod* 1996;11:1678-86.
8. Wiemer KE, Hoffman DI, Maxson WS, Eager S, Muhlenberg B, Fiore I, Cuervo M. Embryonic morphology and rate of implantation of human embryos following coculture on bovine oviductal epithelial cells. *Hum Reprod* 1993;8:97-101.
9. Feng HL, Wen XH, Amet T, Pesser SC. Effect of different coculture systems in early human embryo development. *Hum Reprod* 1996;11:1525-8.
10. Bongso A, Ng SC, Fong C-Y, Ratnam S. Cocultures: a new lead in embryo quality improvement for assisted reproduction. *Fertil Steril* 1991;56:179-91.
11. Dirnfeld M, Goldman S, Gonene Y, Koifman M, Calderon I, Abramovici H. A simplified coculture system with luteinized granulosa cells improves embryo quality and implantation rates: a controlled study. *Fertil Steril* 1997;67:120-2.
12. Barmat LI, Liu HC, Spandorfer SD, Xu K, Veeck L, Damario M, et al. Human preembryo development on autologous endometrial coculture versus conventional medium: a randomized trial. *Fertil Steril* 1998;70:1109-13.
13. Spandorfer SD, Barmat LI, Liu H-C, Mele C, Veeck L, Rosenwaks Z. Granulocyte macrophage-colony stimulating factor production by autologous endometrial co-culture is associated with outcome for IVF patients with a history of multiple implantation failures. *Am J Reprod Immunol* 1998;40:377-81.
14. Spandorfer SD, Clarke R, Bovis L, Liu H-C, Neuer A, Witkin SS, et al. Interleukin-1 levels in the supernatant of conditioned media of embryos grown in autologous endometrial coculture: correlation with embryonic development and outcome for patients with a history of multiple implantation failures after IVF. *Am J Reprod Immunol* 2000;43:6-11.
15. Spandorfer SD, Clark R, Liu H-C, Veeck L, Rosenwaks Z. Leukemia inhibiting factor (LIF) production by autologous endometrial co-culture (AECC) is associated with outcome for patients with a history of multiple implantation failure. *Am J Reprod Immunol* 2001;46:375-80.