Improved Development of Very-Poor-Quality Human Preembryos by Coculture with Human Fallopian Ampullary Cells

Amnon Weichselbaum,¹ Yoav Paltieli,² Ralph Philosoph,³ Benjamin Rivnay,⁴ Raymond Coleman,⁵ Machel M. Seibel,¹ and Shalom Bar-Ami^{1,6,7}

Submitted March 22, 2001; accepted August 23, 2001

Purpose: To determine whether a confluent culture of fallopian ampullary epithelial cells, taken from women at the end of their reproductive life, is capable of rescuing very-poor-quality preembryos from cleavage arrest and/or degeneration.

Methods: Human preembryos, rejected for transfer or freezing because of very poor quality, and arrested within 24 h of cleavage, were cultured for 5 days in medium alone or over a confluent culture of fallopian ampullary epithelial cells. Morphological criteria were utilized to assess preembryo degeneration and stage of development.

Results: The described coculture rescued preembryos from degeneration, enhancing development to the blastocyst stage 2.2-fold, compared with cultures in medium alone. Furthermore, fully expanded and hatching blastocysts were observed only under coculture conditions.

Conclusions: Very-poor-quality human preembryos may be rescued from degeneration, and their growth and development dramatically improved, when cocultured with a confluent culture of fallopian ampullary epithelial cells.

KEY WORDS: Blastocyst; coculture; fallopian ampullary cells; IVF; preembryos.

INTRODUCTION

Accumulated evidence indicates that a beneficial environment for growth and development of human preembryos can be created when they are cocultured with

various cell types derived from the genital organs and tracts. The more established cell types in this regard include cumulus cells (1), luteinized granulosa cells (2,3), fallopian ampullary epithelial cells (FAEC) (4), and endometrial epithelial cells (5). Growth and development of human preembryos is also enhanced when cocultured with various cells of nonhuman origin. Such systems include fetal bovine uterine fibroblast cells (6), green monkey kidney epithelial cells (Vero cells) (7,8), and buffalo rat liver cells (9). Thus, it appears that various cell types, from both genital and nongenital organs, may provide a supportive environment for in vitro development of human preembryos.

In vitro growth and development of human preembryos is determined by many factors, such as the type of culture vessel, incubator parameters, medium formulation, oil overlay, duration of culture, and technician and physician handling. It has been suggested

¹ Faulkner Institute for Reproductive Medicine, Boston, Massachusetts.

² Department of Obstetrics and Gynecology, Bnai Zion Medical Center, Haifa, Israel.

³ Caritas-Norwood Hospital, Norwood, Massachusetts.

⁴ Repromedix Corporation, Woburn, Massachusetts.

⁵ Department of Anatomy and Cell Biology, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel.

⁶ Department of Obstetrics and Gynecology, Boston City Hospital and Boston University School of Medicine, Boston, Massachusetts.

⁷ To whom correspondence should be addressed at Department of Obstetrics and Gynecology, Poriya Government Hospital, 15208 Hagalil Hatachton, Israel; e-mail: sgys@zahav.net.il.

that coculture assists the in vitro growth and development of preembryos when the in vitro parameters are suboptimal (10). Other studies have indicated that the beneficial effect of coculture may involve necessary cross-talk signals between the preembryo and the supporting cells. This cross-talk may assist in both preembryo growth and development (11–13), as well as in preembryo competence for implantation (9,14).

Although it is widely accepted that coculture of human preembryos with various cell types improves growth and development of preembryos, it should be noted that in any coculture system not all the preembryos develop into blastocysts. They may be blocked at the morula or early blastocyst stages or may be doomed to degenerate. Thus, it is conceivable that coculture improves the growth and development of only a limited fraction of preembryos, presumably those that are of higher quality.

In this study we deliberately selected the poorest available preembryos (unsuitable for transfer or freezing). Following this, these preembryos were randomly divided into two groups, one for culture in medium alone and the other for coculture with FAEC. The purpose of the study was to assess whether coculture conditions could rescue such preembryos and redirect them into a normal, healthy course of development. This is particularly important since morphological criteria have routinely been utilized to select preembryos for transfer following IVF.

MATERIALS AND METHODS

Fallopian Ampullary Cell Culture

Healthy fallopian tubes were collected from women at the end of reproductive life (40–45 years old) or not having a menstrual cycle (usually, older than 45 years) who underwent hysterectomy. Tissue samples were used for this study after patients signed their consent. Tubes were collected into Dulbecco's modified Eagle's medium/nutrient mixture F12 Ham (DMEM/F12 medium) (Sigma, St. Louis, MO) containing 10% fetal calf serum, 4 mM L-glutamine buffered with 15 mM HEPES, and 50 µg/mL gentamicin (all from GIBCO-BRL, Rockville, MD). Fallopian tubes were taken to the laboratory at room temperature within 30 min of removal. The ampullary region was dissected out and opened longitudinally, and the inner lining was gently scraped to dislodge epithelial cells.

The FAEC in the DMEM/F12 medium were then centrifuged at 100g for 5 min, and the cell pellet was

suspended in fresh DMEM/F12 medium. The cells were immediately plated in a Primaria 24-well tissue culture plate (Falcon/BD Franklin Lakes, NJ) and incubated at 37°C in 5% CO₂ in air. The plates were left undisturbed for 4–5 days to allow cell attachment to the bottom of the wells. Following this initial culture period, the medium was replaced every 48 h with fresh medium, including the period of coculture.

Coculture of Preembryos with FAEC

In the Faulkner Institute for Reproductive Medicine, the quality of preembryos for transfer or freezing is assessed about 48 h after the retrieval of cumulus-oocyte complexes (COC); in cases with repeated IVF failures, a longer culture period is attempted to assess preembryo developmental competence toward the blastocyte stage.

In the present study, assessment of preembryo quality took place 48 h after COC retrieval. Preembryos defined as poor-quality, as judged by morphological parameters, and therefore rejected for transfer or freezing were allocated to the present study. Poor-quality parameters included at least the following three factors: blastomeres of unequal size, cells with grainy to dark cytoplasm, and 50% or more of the preembryo volume consisting of fragments.

These poor-quality preembryos were donated for the present study by women with an age range of 35–41. Initially, these preembryos were incubated for another 24 h and then put in subgroups according to their stage of growth, defined according to the number of blastomeres per preembryo. Subsequently, the preembryos in each subgroup were randomly distributed between the two study groups, control and coculture with FAEC. Twentyfive preembryos were allocated to coculture, and 21 were cultured in medium alone. Coculture was initiated 72 h after COC retrieval. One day before coculture, the DMEM/F12 medium in the FAEC culture was replaced with human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA), which was the medium for the rest of the coculture or noncoculture (control) period. The HTF culture medium was replaced every 48 h with fresh culture medium for both the control and the coculture. Preembryo growth and development was recorded in the two study groups both before the start of study (72 h after COC retrieval) and during the next 5 days of culture.

Morphological criteria were utilized to assess the developmental stage of preembryos (morula, cavitation, well-defined blastocyst, expanding blastocyst,

and hatching blastocyst). Assessment of the proportional volume between blastomeres and fragments was used to define the quality of the preembryos. The preembryo was defined as in a process of degeneration either when the preembryo stopped cleaving, blastomere cell cytoplasm became dark and grainy, or the preembryo's blastomeres became totally degenerated or fragmented.

No attempt was made to transfer the rescued preembryos into the mother. The request submitted to the Institutional Review Board [IRB] was limited only to testing the beneficial effects of confluent cultures of FAEC upon growth and development of very-poor-quality preembryos. The small numbers of preembryos donated to this study did not allow a phase B for this study, i.e. the implantation competence of the rescued preembryos. Extension of this research direction will be needed in future studies to evaluate the clinical implications of this technology.

Light Microscopy

The morphology of viable FAEC cultures and preembryos was examined using an IMT3 inverted light microscope with $\times 40$ and Hoffman interference optics (Olympus, Tokyo Japan). FAEC were examined at $\times 400$ total magnification to view ciliary beating. When the plated FAEC became a confluent culture, the density of the beating cilia was utilized to determine that the majority of the cells were ampullary and functionally active. Preembryos were examined at $\times 100$ – $\times 400$ total magnification to determine their health, growth, and development, as described above.

Transmission Electron Microscopy

Specimens were dissected from the fresh fallopian tubes shortly after arrival from the operating room. One-millimeter blocks of material were immersion-fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.01% calcium chloride, pH 7.4, at room temperature. The samples were kept in the fixative at room temperature overnight, then rinsed and stored in 0.1 M sodium cacodylate buffer containing 7.5% (w/v) sucrose prior to treatment with an unbuffered 1% aqueous solution of osmium tetroxide for 2 h. This was followed by a cacodylate rinse and dehydration in ascending ethanol concentrations, prior to propylene oxide treatment and embedding in epoxy resin (Eponate 12; Pelco International, Redding, CA). After heat polymerization for 18 h at 60°C, the blocks were trimmed and 60- to 90-nm

sections cut with glass or diamond knives on an LKB Nova ultramicrotome (LKB, Bromma, Sweden). The sections were collected on uncoated copper grids and contrast-stained with uranyl acetate and lead citrate prior to examination in a JEOL 100SX transmission electron microscope at 60 kV.

Scanning Electron Microscopy

Confluent cultures of FAEC, cocultured with preembryos, were rinsed twice in phosphate-buffered saline (PBS) and fixed in 2.5% glutaraldehyde in 375 mOsmol PBS (final pH, 7.4) at room temperature for 3–5 h, followed by overnight fixation at 4°C. Specimens were rinsed thoroughly in PBS and postfixed in 1% osmium tetroxide in PBS for 1 h at room temperature and then dehydrated in ascending ethanol concentrations, dipped in hexamethyldisilazane, air-dried, and visualized under the dissecting microscope. Specimens were mounted on copper stubs with their epithelial side upwards, coated with gold 18-nm thick (Polaron coating unit E-5000), and viewed in a JEOL T-300 scanning electron microscope operated at 25 kV.

Statistical Analysis

The experimental data were subjected to χ^2 analysis. The results are expressed as mean $\pm$ SEM.

RESULTS

Light microscopic examination of fresh, unfixed preparations of tubal sections using an inverted microscope showed ciliated cells that maintained vigorous waves of ciliary beating. Examination of the ampullary samples with transmission electron microscopy revealed that the epithelium consisted of two distinct cell types: ciliated cells and secretory cells (Fig. 1). Within 4–5 days of culture, the majority of the ampullary cells became attached to the culture well surface (Fig. 2(A)). Scanning microscopy confirmed the presence of the two cell types: ciliated and secretory (Fig. 2(B)). The structure of the ciliated and secretory cells did not change markedly during the culture period.

The preembryos selected for the present study consisted mainly of fragments; however, in most cases about two to four blastomeres could be identified (Fig. 3). The preembryos were distributed almost equally according to their developmental stage

Fig. 1. Transmission electron micrograph showing details of human ampullary epithelial cells in section from human fallopian tube. The two cell types (ciliated and secretory) are easily distinguished, and the organelles are well-preserved.

between the coculture (with FAEC) or control (medium alone) treatments (Table I). During the following 5 days of culture, 76 and 44% of the preembryos in control and coculture treatments, respectively, failed to proceed to cavitation, and most of them demonstrated signs of degeneration (Table II). Only 24% of the 21 preembryos in the control group compared with 56% of the cocultured preembryos ($P < 0.05$) proceeded to cavitation (Table II). A significantly higher number of these preembryos in the coculture group (40%), as compared with the control group (5%) ($P < 0.05$), were defined as blastocysts between early to hatching stage. Fully expanded and hatching blastocysts (24%) were observed only in the cocultured preembryos (Table II). The very-poor-quality preembryos failed to develop normally to expanded blastocysts. Figure 3(B) shows that even when preembryos attained cavitation in 5 days' culture in medium alone (control group), the inner cell mass was small and the trophoctoderm cells were distributed in a disorderly fashion as compared with preembryos cocultured with FAEC (Fig. 3(C)–(F)).

Fig. 2. Micrographs of human ampullary epithelial cells after 5–7 days in culture in which full confluent cell culture was attained. (A) Light photomicrograph, the secretory cells are marked by arrowheads. (B) Scanning electron micrograph, the secretory cells are covered with small microvilli.

DISCUSSION

Research in different animal species indicates that when preembryos are arrested at a two- or four-cell stage, coculture of these preembryos with various cell types may prevent and overcome this cleavage block. In conventional human IVF, about 30% of fertilized eggs develop into cavitated preembryos or blastocysts within 6–7 days of culture. In most IVF units attempting coculture, about 60% of the fertilized eggs will develop into blastocysts. Nevertheless, there are still concerns regarding various aspects of the beneficial contribution of coculture in increasing growth and development of preembryos (10,15,16) and improving implantation and clinical pregnancy rates (9,17).

Table I. Developmental Stage of Very-Poor-Quality Preembryos Before the Start of Coculture^a

Group	Total no. of preembryos	Preembryo developmental stage		
		2–3 cells	4 cells	5–6 cells
Control	21	9 (43%)	10 (47%)	2 (10%)
Coculture	25	13 (52%)	10 (40%)	2 (8%)

^aBetween one and three preembryos were collected from (donated by) each patient. The preembryos were randomly allocated into the two treatment protocols.

Fig. 3. Morphology of very-poor-quality preembryos during 5 days' culture in the absence (A, B) and presence (C–F) of FAEC. (A) A five-blastomere preembryo with many fragments, 72 h after egg retrieval. (B) Arrested preembryo at the early blastocyst stage, with many degenerative cells and fragments, cultured in medium alone for 5 days, corresponding to the coculture period (=120 h). (C) Eight- to 12-cell preembryo after 24 h in coculture. (D) Compacted preembryo after 48–72 h in coculture. (E) Expanded blastocyst after 72–96 h in coculture. (F) Expanded preembryo at hatching state, after 96–120 h in coculture. (C–F) Note ampullary cells in background.

It is not clear from these studies whether coculture improves poor culture conditions and competency of human preembryos to develop *in vitro*, or if it selectively increases the ability of poor-quality preembryos to grow and develop under *in vitro* conditions. The present study indicates that many very-poor-quality preembryos can be rescued from cleavage arrest and degeneration if cocultured with FAEC.

Coculture of poor-quality preembryos has been attempted previously. Preembryos defined as poor-quality were cocultured on Vero cells and yielded 61% blastocysts (7). In addition, abnormal (tripronuclear) preembryos were cocultured with fetal bovine uterine fibroblasts and yielded seven of nine blastocysts (6). Interestingly, 55–60% blastulation was observed

when transferable preembryos were cocultured on various cell types such as Vero cells (18). Even when utilizing a more sophisticated system that attempted to mimic the *in vivo* journey of the preembryos, namely, by primary coculture of preembryos with ampullary epithelial cells followed by coculture with endometrial epithelial cells, only 63% of preembryos developed to blastocyst stage (19).

In the present study, coculture of very-poor-quality preembryos with FAEC increased the rate of blastocyst formation to 56%. This value is more than twofold greater than in the corresponding preembryos that were cultured in medium alone. Therefore, this study indicates that long-term coculture of even very-poor-quality preembryos yields about the

Table II. Development of Very-Poor-Quality Preembryos in Conventional IVF and Coculture

Group	Total no. of preembryos	Blocked or degenerated	Stage of blastocyst development				
			Cavity ^a	Early blastocyst	Expanding blastocyst	Hatching/hatched	Total blastocysts
Control	21	16 (76%)*	4 (19%)	1 (5%)	0	0	5 (24%) [†]
Coculture	25	11 (44%)*	4 (16%)	4 (16%)	3 (12%)	3 (12%)	14 (56%) [†]

^aInitial appearance of cavity in the morulae.

* $P < 0.05$.

[†] $P < 0.05$ by frequency comparison.

same rate (50–60%) of blastocyst formation as in the transferable-grade preembryos. This interesting similarity may indicate that poor preembryo morphology is not necessarily indicative of the preembryo potential for growth and development, if cocultured with a suitable cell type.

The increase in the percentage and quality of blastocysts obtained when fertilized eggs, preembryos, or morulas are cocultured with FAEC has been documented by many studies. The present study, however, indicates that coculture with FAEC has the capacity to prevent further cleavage arrest and degenerative processes in very-poor-quality preembryos. In addition, coculture with FAEC turns the very-poor-quality preembryos onto the track of growth and development to the blastocyst stage. Moreover, this study implies that the beneficial effects of FAEC are manifested in cells that have been collected from fallopian tubes of older women both at the end of reproductive life and postreproductive life.

Higher pregnancy rates were observed in patients with increasing numbers of high-quality blastocysts that were obtained within 5–6 days of culture (20,21). Actually, preembryos achieving blastocyst stage passed through various selection processes and therefore had a greater chance to produce clinical pregnancy. Therefore, it could be deduced that the increase in rate of blastocyst formation in coculture may help achieve this critical step toward clinical pregnancy by overcoming possible developmental incompetence up to the blastocyst stage. Indeed, patients with repeated incompetence for blastocyst formation and pregnancy failure achieved pregnancy once their preembryos were cocultured with supporting cell layers (22,23).

Blastocyst hatching appears to be the rate-limiting step for the outcome of pregnancy (24). Several studies have reported that preembryos that fail to proceed to the stage of expanded and/or hatched blastocyst stage have a thick zona pellucida (25,26). Blastocyst hatching has been induced following thinning of the zona pellucida by enzymatic approaches (27). It appears that certain growth factors that are usually produced by the supporting cultured cells can induce blastocyst hatching without a major change in the percentage of preembryos attaining the blastocyst stage (28). This specific action may provide an explanation for the increased hatching of blastocysts in coculture. The present study clearly demonstrated that in very-poor-quality preembryos, those that did not become expanded blastocysts manifested degenerative signs, in both coculture and noncoculture conditions.

Only in the coculture system did preembryos attain the stage of expanded and hatching blastocysts. Thus, our data are in agreement with the assumption that preembryos will degenerate once arrested at morula or even after cavitation, as well as with the assumption that coculture can support the development of expanded and hatching preembryos.

The present study implies that FAEC culture creates a beneficial environment for the development of very-poor-quality preembryos mostly arrested from cleavage between Days 2 and 3 of culture. It also suggests that coculture can rescue preembryos that, on the basis of current morphological criteria, are doomed for degeneration, even if they become cavitated. These beneficial effects were observed in FAEC collected from women at the late stage of reproductive life or even postreproductive life.

ACKNOWLEDGMENTS

The editorial assistance of Miss Ruth Singer and the technical assistance of Mrs. Pessiah Shenzer are gratefully acknowledged.

REFERENCES

1. Quinn P: Use of co-culture with cumulus cells in insemination medium in human in vitro fertilization (IVF). *J Assist Reprod Genet* 1994;11:270–277
2. Plachot M, Antoine JM, Alvarez S, Firmin C, Pfister A, Mandelbaum J, Junca AM, Salat-Baroux J: Granulosa cells improve human embryo development in vitro. *Hum Reprod* 1993;8:2133–2140
3. Dirnfeld M, Goldman S, Gonen Y, Koifman M, Calderon I, Abramovici H: A simplified co-culture system with luteinized granulosa cells improves embryo quality and implantation rates: A controlled study. *Fertil Steril* 1997;67:120–122
4. Bongso A, Soon-Chye N, Sathananthan H, Lian NP, Rauff M, Ratnam S: Improved quality of human embryos when co-cultured with human ampullary cells. *Hum Reprod* 1989;4:706–713
5. Jayot S, Parneix I, Verdaguer S, Discamps G, Audebert A, Empereire JC: Co-culture of embryos on homologous endometrial cells in patients with repeated failures of implantation. *Fertil Steril* 1995;63:109–114
6. Wiemer KE, Cohen J, Amborski GF, Wright G, Wiker S, Munyakazi L, Godke RA: In vitro development and implantation of human embryos following culture on fetal bovine uterine fibroblast cells. *Hum Reprod* 1989;4:595–600
7. Menezo YJ, Guerin JF, Czyba JC: Improvement of human early embryo development in vitro by co-culture on monolayers of Vero cells. *Biol Reprod* 1990;42:301–306
8. Schillaci R, Ciriminna R, Cefalu E: Vero cell effect on in vitro human blastocyst development: Preliminary results. *Hum Reprod* 1994;9:1131–1135

9. Hu Y, Maxson W, Hoffman D, Ory S, Eager S, Dupre J, Worrihow K: Co-culture with assisted hatching of human embryos using Buffalo rat liver cells. *Hum Reprod* 1998;13:165–168
10. Van Blerkom J: Development of human embryos to the hatched blastocyst stage in the presence or absence of a monolayer of Vero cells. *Hum Reprod* 1993;8:1525–1539
11. Lai YM, Wang HS, Lee CL, Lee JD, Huang HY, Chang FH, Lee JF, Soong YK: Insulin-like growth factor-binding proteins produced by Vero cells, human oviductal cells and human endometrial cells, and the role of insulin-like growth factor-binding protein-3 in mouse embryo co-culture systems. *Hum Reprod* 1996;11:1281–1286
12. Yeung WS, Lau EY, Chan AY, Ho PC: The production of interleukin-1 alpha immunoreactivity by human oviductal cells in a co-culture system. *J Assist Reprod Genet* 1996;13:772–775
13. Fukaya T, Chida S, Murakami T, Yajima A: Is direct cell-to-cell contact needed to improve embryonic development in co-culture? *Tohoku J Exp Med*, 1996;180:225–232
14. Simón C, Gimeno MJ, Mercader A, O'Connor JE, Remohí J, Polan ML, Pellicer A: Embryonic regulation of integrins β_3 , α_4 , and α_1 in human endometrial epithelial cells in vitro. *J Clin Endocrinol Metab* 1997;82:2607–2616
15. Bavister BD: Co-culture for embryo development: Is it really necessary? [editorial]. *Hum Reprod* 1992;7:1339–1341
16. Watson AJ, Watson PH, Warnes D, Walker SK, Armstrong DT, Seamark RF: Preimplantation development of in vitro-matured and in vitro-fertilized ovine zygotes: Comparison between co-culture on oviduct epithelial cell monolayers and culture under low oxygen atmosphere. *Biol Reprod* 1994;50:715–724
17. Wiemer KE, Garrisi J, Steuerwald N, Alikani M, Reing AM, Ferrara TA, Noyes N, Cohen J: Beneficial aspects of co-culture with assisted hatching when applied to multiple-failure in vitro fertilization patients. *Hum Reprod* 1996;11:2429–2433
18. Menezo Y, Hazout A, Dumont M, Herbaut N, Nicolle B: Co-culture of embryos on Vero cells and transfer of blastocysts in humans. *Hum Reprod* 1992;7(Suppl 1):101–106
19. Bongso, A, Fong C-Y, Ng S-C, Ratnam S: Human embryonic behavior in a sequential human oviduct-endometrial co-culture system. *Fertil Steril* 1994;61:976–978
20. Janny L, Menezo YJR: Maternal age effect on early human embryonic development and blastocyst formation. *Mol Reprod Dev* 1996;45:31–37
21. Balaban B, Urman B, Sertac A, Alatas C, Aksoy S, Nuhoglu A: Progression of excess embryos to the blastocyst stage predicts pregnancy and implantation rates after intracytoplasmic sperm injection. *Hum Reprod* 1998;13:2564–2567
22. Olivennes F, Hazout A, Lelaïdier C, Freitas S, Fanchin R, de Ziegler D, Frydman R: Four indications for embryo transfer at the blastocyst stage. *Hum Reprod* 1994;9:2367–2373
23. Shoukir Y, Chardonnens D, Campana A, Bischof P, Sakkas D: The rate of development and time of transfer play different roles in influencing the viability of human blastocysts. *Hum Reprod* 1998;13:676–681
24. Wun WS, Wun CC, Valdes CT, Dunn RC, Grunert GM: Blastocyst formation is a good indicator for attainment of assisted reproduction. *Chin J Physiol* 1997;40:237–242
25. Parikh FR, Kamat SA, Nadkarni S, Arawandekar D, Parikh RM: Assisted hatching in an in vitro fertilization programme. *J Reprod Fertil Suppl* 1996;50:121–125
26. Magli MC, Gianaroli L, Ferraretti AP, Fortini D, Aicardi G, Montanaro N: Rescue of implantation potential in embryos with poor prognosis by assisted zona hatching. *Hum Reprod* 1998;13:1331–1335
27. Lee DR, Lee JE, Yoon HS, Lee HJ, Kim MK, Roh SI: The supplementation of culture medium with protease improves the hatching rate of mouse embryos. *Hum Reprod* 1997;12:2493–2498
28. Martin KL, Barlow DH, Sargent IL: Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. *Hum Reprod* 1998;13:1645–1652