CLINICAL ASSISTED REPRODUCTION

Beneficial Effects of Coculture with Cumulus Cells on Blastocyst Formation in a Prospective Trial with Supernumerary Human Embryos*

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Purpose: *We reported previously on the use of coculture with cumulus cells in insemination medium for the development of human embryos in vitro. Here we describe a prospective trial to determine if this procedure has a significant beneficial effect.*

Methods: *On the day after insemination, zygotes were randomized for culture in either a fresh drop of medium without (−cum) or were left in their insemination drop with (+ cum) cumulus cells. Embryos with the best morphological quality were replaced on the third day of development at the eight-cell stage. The remaining embryos were cultured for a further 3 days and cryopreserved if they reached the fully expanded blastocyst (FEB) stage. Three different culture media were used over the period of this study.*

Results: *In 11 patients, supernumerary embryos were available only for continued culture in +cum and three patients had embryos cultured in only −cum. Thirty-nine other patients had embryos assigned to both +cum and −cum treatments. In the +cum group, 98 blastocysts developed from 216 embryos cultured for 6 days (45%), and this was significantly greater ($P < 0.01$) than the 48 blastocysts from 156 embryos (31%) developing in the absence of cumulus cells. In basal HTF medium (HTF medium with EDTA and glutamine) and basal XI HTF medium (similar to basal HTF but devoid of glucose and phosphate), culture of embryos with cumulus cells produced significantly more FEBs than in the absence of cumulus cells. There was no significant difference between the two culture treatments when regular HTF medium was used. Preliminary results indicate that pronectin-coated dishes provide a good substratum for cumulus cell attachment and embryo development.*

Conclusions: *The culture of human embryos with their cumulus cells in insemination drops of medium produces a significantly greater proportion of FEBs than when the zygotes are transferred to fresh culture drops devoid of cumulus cells. This is the first report of a significantly higher blastocyst rate with coculture in which a real comparison has been made between two culture treatments which differ only in the presence or absence of homologous cumulus cells in insemination drops.*

KEY WORDS: coculture; cumulus cell; blastocyst; extended culture.

INTRODUCTION

There are a number of benefits of extending the culture of preimplantation mammalian embryos, especially in the situation of human embryos in an assisted reproductive technology (ART) program. These benefits have been summarized on several occasions (e.g., see Ref. 1) and, briefly, include the following: (a) it allows the selection of embryos of better morphological quality for transfer; (b) it provides better chronological synchrony between the embryonic stage (e.g., eight-cell through early blastocyst) and the site of placement at transfer

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(uterus); (c) it gives a greater window of time for embryo biopsy and genetic diagnosis; and (d) more cells are available for biopsy where between-blastomere differences in genotype may occur (2,3). Coculture has been proposed as an effective way to maintain embryo quality during extended culture (4–6), although this supposition has been contradicted by some (7).

Several methods have been used for coculture of mammalian embryos, including bovine uterine and oviductal cells (4,8), human reproductive tract cells (9), cell lines (10), granulosa cells (11,12), and cumulus cells (1,13–16). The benefits and disadvantages and proposed mechanism of action of coculture with these various methods have recently been reviewed (1). It is becoming more evident that a simple, practical way to utilize coculture in a routine ART setting is by the use of homologous cumulus cells (17). The present study was undertaken to see if prospective allocation of supernumerary human embryos to continued culture in their insemination medium with cumulus cells and spermatozoa gave a superior blastocyst rate over 6 days of culture compared with transfer of the embryos at the zygote stage to fresh culture medium devoid of cumulus cells and inseminating spermatozoa. The use of development to the fully expanded blastocyst stage as an end point may obviate possible problems associated with the interpretation of data if earlier stages of development are utilized to appraise coculture because 35–45% of cleaving human embryos arrest between the eight-cell and the morula stage (18). Preliminary data are also presented on the use of pronectin-coated culture dishes used to enhance the attachment of cumulus cells to act as a feeder layer for human embryos in culture.

MATERIALS AND METHODS

The methodology used for human IVF and cumulus cell coculture has been described previously (1). Briefly, standard protocols were used for ovarian stimulation and monitoring of follicle growth. Oocytes were collected 36–37 hr after hCG injection and IVF was carried out in 30-μl drops of medium under equilibrated paraffin oil (BDH, Poole, England; Catalog No. 29436). The oocytes were inseminated with 25,000 to 40,000 motile spermatozoa prepared by Percoll gradient centrifugation. Three media were used in this study. They were HTF, HTF plus 0.1 m*M* EDTA and 1.0 m*M* glutamine (referred to as basal HTF), and HTF plus EDTA and glutamine but devoid of glucose and phosphate (referred to as basal XI HTF). On the day after insemination, zygotes either were randomized for culture in a fresh drop of medium (−cum) or were left in their insemination drop of medium (+cum). At the time oocytes were checked for fertilization, cumulus cells had plated onto the dish and covered 30 to 50% of the area in the culture drop. Zygotes were grouped together in a single insemination drop in which the cumulus cells were well spread, with minimal clumping and with few or no blood cells. Embryos with the best morphological quality were replaced in the patient on the third day of development at the eight-cell stage. The embryos selected for replacement were drawn from both the −cum and the +cum treatments, and this led to disparity in the numbers of supernumerary embryos remaining in each treatment. In three patients, supernumerary embryos remained only in the −cum treatment. In another group of patients ($n = 11$), all the drops of culture medium were used for insemination of oocytes and therefore there were no drops available for use in the −cum treatment. Supernumerary embryos were cultured for a further 3 days and cryopreserved if they reached the expanded blastocyst stage. In a preliminary trial to test the effect of culture on pronectin-coated dishes, the inside of 35-mm culture dishes (Corning Glass Works, Corning, NY; Catalog No. 25000) were coated with a solution of ProNectin F (Protein Polymer Technologies, San Diego, CA) at a concentration of 10 μg/ml in phosphate-buffered saline (PBS). This solution was left in place for 2 hr at room temperature, then removed, and the dishes were immediately rinsed twice with PBS and air-dried. The dishes were stored at room temperature and used within 4 months of preparation. Control dishes were not treated with pronectin. All patients gave informed consent to the procedures undertaken. The mean age of the patients was 33 years (range, 19–41 years). The etiology of the infertility of the 53 patients in this study was as follows: tubal, $n = 24$; unexplained, $n = 11$; egg donor, $n = 9$; endometriosis, $n = 4$; donor sperm used for male factor, $n = 1$; and other causes, $n = 4$.

Differences between treatments were analyzed using chi-square with Yates' correction for continuity. A probability level of $\leq 5\%$ was considered significant. All analyses were carried out using StatXact-Turbo software (Cytel Software Corporation, Cambridge, MA).

RESULTS

A total of 53 patients had supernumerary embryos remaining after embryo transfer or cryopreservation of pronuclear or cleavage-stage embryos. The development of these supernumerary embryos was first analyzed on a per-patient basis. The data obtained with the three culture media used showed homogeneity of their common odds ratio (Turbo StatXact, Cytel Software Corporation) so the results could be pooled over all media. A summary of these data is given in Table I. In 11 patients, supernumerary embryos were cultured only in the +cum treatment. Nine of these patients (82%) had at least one embryo develop to the blastocyst stage; 29 fully expanded blastocysts (FEBs) (52%) developed from 56 cultured embryos. Three other patients had their embryos cultured only in −cum; one FEB developed from a total of five embryos. The 39 remaining patients had embryos assigned to both +cum and −cum treatments. Twenty-eight (72%) of these patients had at least one embryo develop to the FEB stage. In this group of patients, the percentage of embryos reaching the FEB stage in +cum (43%) was significantly ($P < 0.05$) greater than the percentage (31%) in −cum. Overall, when the results for both the +cum and the −cum treatments were combined for all patients, the percentage of FEBs obtained in +cum (45%) was significantly ($P < 0.01$) higher than the percentage (31%) obtained in the −cum group. In some studies of coculture, only cleaved embryos with minimal fragmentation have been utilized (18). When such embryos were selected in the present study (<10% fragmentation on Day 2 of culture at the two- to four-cell stage), 71% of them developed into FEBs in +cum and 59% developed into FEBs in −cum (difference not significant). The overall number of embryos judged to be of good quality on Day 2 of development at the two- to four-cell stage following 24 hr of culture with cumulus cells (139 of 216 = 64%) was significantly ($P < 0.025$) greater than the proportion developing in the absence of cumulus cells in fresh medium (81 of 156 = 52%). There was no significant association between whether a patient became pregnant after transfer of sibling embryos and whether a FEB developed from her supernumerary embryos left in culture (data not shown).

A summary of the data analyzed on the basis of the type of culture medium used is given in Table II. With both basal and basal XI HTF medium, significantly more supernumerary embryos developed into FEBs after 6 days of culture when cocultured with cumulus cells than when the embryos were transferred to fresh medium lacking cumulus cells. There was no significant difference between the two culture treatments when regular HTF medium was used. In the presence of cumulus cells, the percentage of FEBs developing among the three media ranged from 36 to 51% but none of the differences were significant. When the differences in results among the three media in the absence of cumulus cells were compared, however, the percentage of FEBs in basal HTF medium (17%) was significantly lower ($P < 0.01$) than that in HTF medium (44%); no other comparisons were significant.

A preliminary trial was initiated in which IVF was performed using plastic dishes coated with pronectin. The cumulus cell plated down sooner and more uniformly on these dishes compared with the control, non-pronectin-coated dishes. From seven embryos cultured with cumulus cells on the pronectin-

Table I. Development of Supernumerary Human Embryos to Fully Expanded Blastocysts (FEBs) with (+cum) and Without (−cum) Cumulus Cell Coculture

Number of patients	Total number of embryos		Total number of good-quality Day 2 embryos[a]		Number of FEBs		Number of patients with FEBs (%)
	+cum	−cum	+cum	−cum	+cum	−cum	
11	56		40		29		9 (82)
3		5		3		1	1 (33)
39	160	151	99	78	69	47	28 (72)
53	216	156	139* (64%)	81* (52%)	98** (45%)	48** (31%)	38 (72)

[a] Less than 10% cytoplasmic fragmentation.
* Proportion of good-quality embryos in +cum significantly ($P < 0.025$) greater than in −cum.
** Proportion of FEBs from total embryos in +cum significantly ($P < 0.01$) greater than in −cum. Proportion from good-quality Day 2 embryos not significantly different.

Table II. Development of Supernumerary Human Embryos to Fully Expanded Blastocysts (FEBs) with and Without Cumulus Cell Coculture in Different Culture Media

Medium	+Cumulus	−Cumulus	*P*
Basal HTF	28/78* (36%)	9/53 (17%)	<0.04
Basal XI HTF	42/82 (51%)	18/55 (33%)	<0.05
HTF	28/56 (50%)	21/48 (44%)	>0.5
Total	98/216 (45%)	48/156 (31%)	<0.01

* Proportion of FEBs developing from total number of supernumerary embryos cultured.

coated dishes, four FEBs were obtained compared, with two FEBs from seven embryos in the control dishes.

A photograph of typical embryos after 6 days of culture is shown in Fig. 1. Blastocysts classified as grade A contained a fully expanded, single blastocoel cavity with well-defined polygonal cells visible in the trophectoderm and a single inner cell mass. Grade B blastocysts had a smaller blastocoel cavity, occupying 50% or less of the embryonic volume; on occasion in these blastocysts, several blastocoel cavities were visible and polygonal-shaped blastomeres were rarely visible in the trophectoderm.

DISCUSSION

The results from this study show that culture of human embryos with their cumulus cell in insemination drops of medium produced a significantly greater proportion of FEBs than when zygotes were transferred to fresh medium devoid of cumulus cells. This was a direct comparison between the presence and the absence of cumulus cells and inseminated spermatozoa. There were no other differences between the treatments and the study was prospective in design. The necessity for coculture to improve the development of human embryos in vitro has been criticized due to the use of inappropriate culture media and other unsuitable methodologies (7). Indeed, in a recent report by Wiemer *et al.* (8), the control treatment consisted of culture in microdroplets under oil and the coculture treatment was in 1 ml of medium with no oil overlay. This makes comparison of the effects of the two treatments more speculative than necessary. There are several reports that co-culture using Vero cells produces no significant difference in the in vitro development of embryos or implantation rates than controls (18,19). Some criticism of the use of inappro-

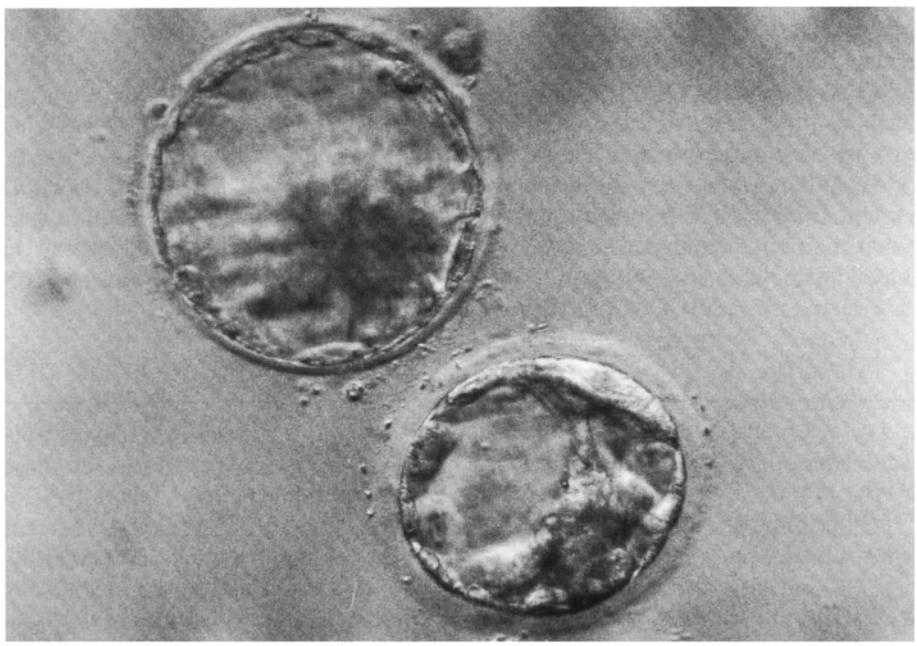

Fig. 1. Supernumerary human embryos on the sixth day of coculture. The embryos have been removed from their insemination drop with cumulus cells and are being prepared for cryopreservation. The blastocyst on the left was graded as Grade A and the one on the right was scored as Grade B. A Grade B blastocyst would not be cryopreserved unless at least one Grade A blastocyst was also available. Hoffman modulation contrast. Original magnification, ×260.

priate cell lines for coculture can be avoided by using human oviductal and endometrial cell lines (9), but this methodology is labor-intensive and requires microbial screening of the cell lines. A more easily obtained and perhaps more natural situation would be to use homologous cumulus cells for coculture of embryos (1,13,14). The methodology used by Saito *et al.* (14) whereby cumulus cells are cultured separately before the embryos are added appears unnecessary (1,13). It may also be unnecessary to change the medium in the insemination drop after checking the oocytes for fertilization, as is done by Mansour *et al.* (13). The presence of any dead and degenerating spermatozoa in the culture drops apparently had a minimal deleterious effect on the embryos in the present study. When higher concentrations of spermatozoa are used for male-factor cases, however, changing the medium the day after insemination, as described by Mansour *et al.* (13), may be warranted. This is because the higher numbers of spermatozoa may produce excessive amounts of free radicals and other metabolic wastes which could be deleterious to the embryos. The use of development to the FEB stage as described here gives a greater measure of the effectiveness of the methodology compared with development to the eight-cell stage, as described by Mansour *et al.* (13), because a substantial proportion (35–45%) of cleaving embryos have been reported to arrest between the eight-cell and the morula stage in vitro (18). The rate of development of supernumerary embryos into FEBs when cocultured with their cumulus cells in our laboratory, whether considering development of all cultured embryos or only those judged to have a good morphological appearance on Day 2 of development, approaches the 60% level proposed by Lopata (20) to indicate an optimized culture system for human preimplantation embryos. The viability of such blastocysts after cryopreservation, thawing, and replacement has been demonstrated (1).

A proportion (70%) of patients similar to that reported previously (1) had supernumerary embryos develop to the blastocyst stage in vitro. Van Blerkom (18) reported that an average of 56% of his patients had selected supernumerary embryos which reached the FEB stage with or without coculture on Vero cells. This high proportion of complete development in vitro makes this culture system very suitable to use for obtaining FEBs for preimplantation genetic diagnosis (PGD). It has been proposed that PGD at the blastocyst stage has unique advantages over the use of cleavage-stage embryos (3). Our study also showed the advantage of coculture with cumulus cells from the zygote stage because a higher proportion of embryos of good morphological quality was obtained on Day 2 after coculture than without it. This effect could explain the inability of others (18) to obtain any beneficial effect of coculture with Vero cells after the embryos were transferred to the monolayers after 12–20 hr of culture in fresh medium from the zygote stage to the two-cell stage. Even with three culture media being used in the present study, the beneficial effect of cumulus cell coculture was evident in all treatments, albeit not significant with regular HTF medium. This may have been because of the small number of patients and embryos utilized in this medium. When the differences in results among the media were analyzed, only the comparison between basal HTF and regular HTF medium in the absence of cumulus cells was significantly different. Again, this may well have been due to variation between patients and a prospective trial in which differences between media treatments within patients are compared would be needed before any meaningful discussion of these differences could be undertaken. The encouraging preliminary results obtained with pronectin-coated dishes warrant further investigation of this methodology to optimize the procedure and produce the best possible clinical outcome.

In summary, it has been shown that the culture of human embryos with their cumulus cells in insemination drops of medium produces a significantly greater proportion of FEBs than when zygotes are transferred to fresh culture drops devoid of cumulus cells. This is the first report of a significantly higher blastocyst rate with coculture in which a real comparison has been made between two culture treatments which differ only in the presence or absence of cumulus cells in insemination drops.

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