CLINICAL ASSISTED REPRODUCTION

Human Embryo Development and Pregnancies in an Homologous Granulosa Cell Coculture System

RAFFAELLA FABBRI,^{1,4} ELEONORA PORCU,¹ TIZIANA MARSELLA,¹ MARIA RITA PRIMAVERA,¹ SANDRA CECCONI,² STEFANIA A. NOTTOLA,³ PIETRO M. MOTTA,³ STEFANO VENTUROLI,¹ and CARLO FLAMIGNI¹

Submitted: January 6, 1999

Accepted: June 28, 1999

Purpose: Our purpose was to determine the effects of the coculture of embryos on human granulosa cells (GCs) in patients in the first cycle of IVF-ET treatment and in patients with repeated implantation failures and to investigate the presence of specific proteins in a 48-hr GC conditioned medium and the GC ultrastructural characteristics.

Methods: Eighteen patients with tubal or idiopathic infertility were enrolled in this study: 7 patients (Trial 1) were in the first cycle of IVF-ET treatment and 11 patients (Trial 2) had repeated implantation failures (one to five). Embryos from each patient were cocultured randomly either on homologous granulosa cells or on a conventional culture medium.

Results: At the end of the coculture period (day 5 or 6), 50% of the embryos (Trial 1) reached the blastocyst stage, with respect to 35% in Trial 2. The pregnancy rate per retrieval was 14.2 and 9%, respectively, in Trial 1 and in Trial 2. Many conditioned media showed proteins of 24–29 kDa, and some of them showed additional proteins of 90 kDa. The ultrastructural analysis of GCs showed healthy, metabolically active, protein-synthesizing, and mostly steroidogenic cells.

Conclusions: GC cultures improve embryo development but not pregnancy rates both in Trial 1 and in Trial 2.

KEY WORDS: blastocyst; coculture; human granulosa cells; human embryos; IVF implantation failures.

INTRODUCTION

The coculture of human embryos with cell types such as human ampullary cells, human endometrial cells, Vero cells, fetal bovine uterine fibroblast cells, bovine oviductal epithelial cells, and ovarian cancer cells was found to be successful in improving embryo morphology, in vitro development, and the subsequent implantation rate (1–5), suggesting that embryo–somatic cell interaction plays an important role in early embryogenesis and that a common mediator may be responsible for this beneficial effect (5).

There are a number of benefits to be had from extending the culture of preimplantation human embryos: (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 replacement at transfer (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 (6).

Human pregnancies have been reported after the transfer of cocultured blastocysts but an enhanced pregnancy rate on a large scale has been demonstrated in only a few cases (7).

The results of clinical trials using cocultured embryos are also conflicting, some suggesting that

¹ Infertility and IVF Centre, Human Reproductive Medicine Unit, Institute of Obstetrics and Gynaecology, University of Bologna, via Massarenti 13, 40138 Bologna, Italy.

² Department of Sciences and Biological Technologies, L'Aquila, Italy.

³ Department of Anatomy, University of Rome La Sapienza, Rome, Italy.

⁴ To whom correspondence should be addressed.

pregnancy rates are universally improved and others being unable to confirm this. Coculture in association with blastocyst transfer may be particularly useful for those patients who have had repeated IVF failure (8).

Surprisingly, the beneficial effects of feeder cells on embryo development and on pregnancy rate do not seem to be dependent on the cell type or tissue origin of the feeder cells (9). A study involving mouse embryos showed equally enhanced developmental rates and decreased fragmentation when cocultured with human granulosa-lutein cells, cumulus cells, and epithelial or fibroblast cells from the oviduct and endometrium (9,10). The use of homologous human and animal cells raises the question of the potential risk of transmitting viral or bacterial particles from the culture into the embryo or into the maternal genital tract. The zona pellucida has been found to block some bacteria, fungi, and large viruses (7) and could act as a contamination barrier, but these experiments were made on animal species and no experiments have been carried out on human embryos.

The use of homologous culture systems overcomes these problems; in particular, cumulus (6) and granulosa cells (GCs) (9–11) are usually those most utilized, as they are easy to recover and this procedure is simple to perform and does not require specialized equipment.

The aim of our study was to evaluate the effect of human granulosa cell cocultures on embryo development and pregnancy rate in a group of patients undergoing the first cycle of IVF treatment and in a selected group of patients with repeated implantation failures to whom blastocysts or morulae were transferred.

Basic observations on human GCs and cumulus corona cells (CCs) made at the time of zygote formation and early embryo cleavage revealed, *in vitro* as well as *in vivo*, that these cells show active signs of healthy metabolic functions such as steroid synthesis (12,13) and typical structural features of protein secreting cells by a paracrine model which activates a positive response with concurrent physiological activities including fluid secretions from the tubal mucosa. As a consequence, under *in vivo* conditions, the oocyte, CCs, and tubal fluids, under the control of general ovarian hormones and local paracrine secretion, are in the best condition to achieve successful fertilization and progress through early embryogenesis to implantation.

In the light of these basic relevant observations, a parallel study was performed to evaluate protein release by the GCs in culture (electrophoresis analysis) and to describe the fine structure of human GCs (trans-

mission electron microscopy) cultured for 48 hr in a serum-free medium.

MATERIALS AND METHODS

Patients

A total of 18 patients was selected and divided into two trials: Trial 1 was comprised of 7 patients of mean age 30 years (range, 27–34 years) with tubal or idiopathic infertility in their first cycle of IVF treatment and selected because they had not had previous implantation failures; Trial 2 included 11 patients of mean age 33 years (range, 26–39 years) with tubal or idiopathic infertility, having had from one to five previous implantation failures. No more than three morulae or blastocysts cultured in GCs were transferred on days 5–6.

The patients selected for Trial 1 and 2 were compared with two other groups of patients (controls) ($n = 12$ and $n = 19$, respectively) of the same age and having the same reasons for infertility (tubal or idiopathic) who had been treated with standard IVF in the same period and in whom the (two- to four-cell) embryos were transferred on day 2.

All the patients gave their informed written consent to participate in this experimental protocol.

Clinical pregnancies were confirmed by β hCG serum levels followed by ultrasound visualization of the intrauterine gestational sac; ongoing pregnancies had fetal heart activity confirmed by ultrasound.

Stimulation Protocol and IVF Procedure

The patients received follicle-stimulating hormone (FSH; Metrodin HP, 75 IU; Serono, Milan, Italy) following down-regulation with a gonadotropin releasing hormone analogue, leuprolide acetate, or decapeptil depot. Follicular growth was monitored by serum estradiol concentrations and ovarian ultrasonography. Human chorionic gonadotropin (hCG; Profasi; Serono) was administered when adequate follicular maturation (≥ 16 mm in mean diameter) had been obtained. Transvaginal ultrasound (US)-guided oocyte pickup was performed 34 hr later.

All oocytes were incubated on an HTF medium (Irvine Scientific, Santa Ana, CA) supplemented with 20% maternal serum (MS) at 37°C in a 5% CO₂ modified atmosphere for 3–4 hr. Spermatozoa from normospermic samples were prepared by the conventional Percoll method and the insemination was performed

using about 100,000 sperm/oocyte. Eighteen to twenty hours after insemination, oocytes were mechanically denuded of corona and cumulus cells to determine fertilization by the presence of two pronuclei and two polar bodies and reincubated on HTF medium supplemented with 20% MS at 37°C in 5% CO₂ until the next day when the embryonic development was observed.

Granulosa Cell Culture and Embryo Coculture

GCs, collected during oocyte pickup, were cultured according to the method previously described by Fab-bri *et al.* (14).

GCs in their follicular fluid were pelleted by centrifugation at 650g for 10 min. The pure follicular fluid was collected, filtered, and stored as incubating medium, while the pellet was resuspended in Tyrode's salt solution (Gibco BRL, Life Technologies LTD, Paisley, Scotland) at a volume one-half that of the original follicular fluid. The cells were dissociated mechanically with a transfusional set (Emoflo; Miramed, Mirandola, Italy).

Separation of the GCs from the red blood cells was achieved on a Percoll gradient: the cells suspended in Tyrode's salt solution were carefully layered on the 50% Percoll solution (Pharmacia Biotech AB, Uppsala, Sweden) at a 2:1 ratio (v/v) and centrifuged at 1000g for 13 min. The GCs formed a band at the interface, while the red blood cells and debris sedimented to the bottom of the tube. The cells were collected by aspiration, washed the first time with Tyrode's salt solution, and then with Ham's F10 medium (Gibco BRL) supplemented with 20% MS.

The viability of the GCs was assessed utilizing Trypan blue solution with a 1-to-10 ratio in a Neubauer counting chamber (Newbauer Improved; Assistant, Germany) and a value greater than >80% was always observed. About 250,000 cells/well were incubated on Nunclon Delta (Nalge Nunc International, Roskilde, Denmark) plates at 37°C in 5% CO₂ in Ham's F10 medium supplemented with 20% MS.

On day 2, after confluence, the cells were washed twice with Tyrode's solution to remove any residual erythrocytes and dead cells and they were reincubated with fresh Ham's F10 medium supplemented with MS. At this moment they were ready to receive the embryos.

When for each patient, more than six (two- to four-cell) embryos were obtained, they were equally and randomly divided into cocultures with confluent GCs and into HTF supplemented with 20% MS (control). When for each patient fewer than six embryos were

obtained, they were only put in culture in the presence of confluent GCs. The medium was replaced every 2 days until the end of the culture.

The embryo morphology was examined daily and the embryos were scored from the best to the worst as grade A, B, C, and D.

The morphology was graded both on the proportion of fragments and on blastomere symmetry as follows: grade A—uniform blastomeres, no anucleate fragments, and nongranular cytoplasm; grade B—slightly irregular blastomeres or less than 10% of anucleate fragments occupying the embryo volume and nongranular cytoplasm; grade C—irregular blastomeres, anucleate fragments less than 50%, and slightly granular cytoplasm; and grade D—more than 50% of fragmentation, nondistinct blastomeres, and cytoplasm very granular.

The embryos that failed to cleave for 2 consecutive days were discarded. On day 5 or 6, only morulae or blastocysts developed in coculture were transferred into the uterus: spare cocultured blastocysts and well-expanded blastocysts grown in the control medium were cryopreserved according to the Menezo method (3).

Blastocyst morphology was examined under an invertoscope (Olympus Optical, Co. LTD, Tokyo) and the blastocysts were graded on the basis of the expansion of the blastocoele cavity and the trophoectoderm diameter as follows: *early cavitated blastocysts*—compacted embryos with the beginnings of a blastocoele cavity or with a cavity less than two-thirds of the embryo volume; and *completely expanded blastocysts*—embryos with a blastocoele cavity occupying the entire volume of the embryo in which initiated herniation of the zona pellucida by the trophoectoderm may have begun.

Electrophoretic Analysis of GC Released Proteins

A parallel study was performed to evaluate the secretion of proteins (putative embryotrophic factors) in 48-hr serum-free conditioned media of human GC cultures obtained from 14 patients who underwent an IVF-ET clinical trial.

Briefly, the confluent GCs of each patient were divided into two groups: in the first group (defined as 2nd → 4th), on day 2, the cells were washed twice with Tyrode's salt solution (to eliminate the excess of serum proteins that could interfere with electrophoresis) and Ham's F10 (without MS) was replaced in each well and maintained for 48 hr. Thereafter, the

supernatant was recovered for electrophoretic analysis and Ham's F10 (with MS added) was replaced until days 10–12. In the second group (defined as 4th → 6th), on day 2, the cells were washed twice with Tyrode's salt solution, and Ham's F10 (without MS) was replaced in each well. On day 4, the GCs were washed and Ham's F10 (without MS) was replaced in each well and maintained for 48 hr. Thereafter, the supernatant was recovered for electrophoretic analysis and Ham's F10 (with MS added) was replaced until days 10–12. This protocol was applied to verify some differences in protein production after 4 or 6 days of culture.

A protease inhibitor, 0.2 M PMSF (phenylmethylsulfonyl fluoride, α -toluenesulfonyl fluoride; Sigma-Aldrich, Milan, Italy), was added to the supernatants. All the supernatants recovered were stored at -20°C .

Confluent GCs were cultured until days 10–12 to verify the cell culture quality and the formation of the complete monolayer.

Proteins released into conditioned media by the 48-hr cultured cells were precipitated with 10% TCA (trichloroacetic acid; Sigma-Aldrich Srl, Milan, Italy) (w/v, final concentration) for 30 min at 4°C . The samples were then centrifuged in a refrigerated Beckman ultracentrifuge at 15,000g for 60 min. After removing the supernatants, 1 N NaOH was added to each pellet to restore a pH of around 7. Protein concentration was determined using Bio-Rad assay. Similar protein aliquots (5–10 μg) were loaded on each lane and samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Miniprotean System, Bio-Rad Laboratories, Hercules, CA) according to Laemmli (15).

Prestained molecular weight standards (Bio-Rad Laboratories) in the range 7–200 kDa were loaded on each gel. Protein detection was performed using a standard silver staining procedure (16). Densitometric analysis of protein separation profiles was performed utilizing an LKB 2202 Ultrascan Laser Densitometer (LKB-Pharmacia, Bromma, Sweden).

Transmission Electron Microscopy (TEM) Procedures

In order to describe the fine structure of human GCs some wells containing confluent granulosa cells were washed twice, after 4 days of culture, with Tyrode's salt solution, and Ham's F10 without MS was replaced in each well and maintained for 48 hr.

Cultured GCs were fixed in glutaraldehyde 3% in 0.1 M phosphate buffer, pH 7.4, diluted with the culture medium (50/50). Subsequently, the cells were scraped

from the culture dish and pelleted in the same fixative by centrifugation at 1400 rpm for 5 min. After fixation for 2–5 days or longer at 4°C , the samples were washed in phosphate buffer, postfixed in 1% osmium tetroxide in distilled water, dehydrated in an ascending series of ethanol, and then immersed in propylene oxide (solvent substitution). Granulosa pellets were finally embedded in Epon 812 and sectioned by a Reichert–Jung Ultracut E ultramicrotome. Randomly oriented semithin sections (0.5–1 μm thick) were prepared for light microscopy (LM) examination and stained with toluidine blue. Ultrathin sections were cut with a diamond knife, contrasted by lead citrate and uranyl acetate, and observed by a Zeiss EM 10 transmission electron microscope.

Statistics

Differences between groups were analyzed by Student's *t* test and chi-square analysis. Values were considered significantly different if *P* was <0.05 with the use of a two-tailed test.

RESULTS

In Trial 1, on day 2 after insemination, a total of 103 embryos of variable quality at the two- to four-cell stage was obtained. These embryos were randomly allocated to cocultures on confluent GCs ($n = 65$; mean, 9.1 ± 2.7 per patient) or to control medium ($n = 38$; mean, 5.4 ± 3.1 per patient) (Table I).

At the end of the coculture period (Day 5 or 6), the percentage of embryos developed to the blastocyst stage on GCs was significantly higher for cocultured embryos compared to the control embryos (50.7 vs 7.8%; $P < 0.001$). The mean was 4.7 ± 1.7 and 0.4 ± 0.1 blastocysts per patient, respectively ($P < 0.001$) (Table I).

Early cavitated blastocysts constituted 45.5 and 33.3% in coculture and in the control, respectively, while completely expanded blastocysts constituted 54.5 and 66.6%, respectively: the differences were not statistically significant. Among cocultured embryos, 4 of 65 were compacted morulae (6.1%), while no morulae were found in the control group (Table I).

Eighteen embryos coming from GC cocultures were transferred (2.6 ± 0.3 per patient) (Table I): 14 (78%) were early or expanded blastocysts and 4 (22%) were morulae.

One pregnancy occurred and a normal baby was delivered. The pregnancy rate was 14.2% per retrieval (Table I).

Table I. Embryo Development and Pregnancy Rates in Patients in the First Cycle of IVF Treatment (Trial 1) and in Patients with Repeated Implantation Failures (Trial 2)*

	Trial 1, N (%)		Trial 2, N (%)	
	Coculture	Control	Coculture	Control
Embryos, total	65	38	77	31
(mean/patient $\pm$ SD)	9.1 $\pm$ 2.7	5.4 $\pm$ 3.1	7.0 $\pm$ 3.7	2.8 $\pm$ 1.3
Blastocysts	33 (50.7) ^a	3 (7.8) ^b	27 (35.0) ^c	2 (6.5) ^d
(mean/patient $\pm$ SD)	4.7 $\pm$ 1.7 ^e	0.4 $\pm$ 0.1 ^f	2.4 $\pm$ 1.7 ^g	0.2 $\pm$ 0.1 ^h
Early	15 (45.5)	1 (33.3)	13 (48.2)	1 (50.0)
Expanded	18 (54.5)	2 (66.6)	14 (51.8)	1 (50.0)
Morulae	4 (6.1)	0	9 (11.6) ⁱ	0 ^l
Transferred embryos	18 (27.7)	—	27 (35.0)	—
(mean/patient $\pm$ SD)	2.6 $\pm$ 0.3	—	2.4 $\pm$ 0.7	—
Pregnancies/retrieval	1 (14.2)	—	1 (9.1)	—
Frozen blastocysts	16 (48.5) ^m	—	4 (14.8) ⁿ	—

* a vs b, e vs f, and g vs h, $P < 0.001$; c vs d, $P < 0.005$; i vs l, $P < 0.05$; m vs n, $P < 0.01$.

All the untransferred blastocysts were cryopreserved (48.5%) (Table I).

The results of Trial 1 (7 patients) were compared with the data obtained in a slightly larger group ($n = 12$) of patients of the same age and having the same reasons for infertility (tubal or idiopathic), who had been treated with standard IVF in the same period, and in whom the embryos were transferred on day 2. A mean of 2.6 ± 0.3 and 2.7 ± 0.6 embryos was transferred per patient, respectively, and the pregnancy rates per retrieval were 14.2 and 16.6%, respectively. The differences were not statistically significant (Table II).

In Trial 2 (patients with previous implantation failures), on day 2 after insemination, a total of 108 embryos of variable quality was obtained at the two-to four-cell stage. These embryos were randomly allocated to cocultures on confluent GCs ($n = 77$; mean, 7.0 ± 3.7 per patient) or to control medium ($n = 31$; mean, 2.8 ± 1.3 per patient) (Table I).

On day 5 or 6 the incidence of blastocyst formation was significantly higher for the cocultured embryos compared with the control embryos (35 vs 6.5%; $P < 0.005$), with a mean of 2.4 ± 1.7 and 0.2 ± 0.1

blastocysts obtained per patient, respectively ($P < 0.001$) (Table I).

No differences were found in the percentage of early cavitated (48.2 vs 50.0%) and completely expanded (51.8 vs 50.0%) blastocysts in both groups. Eleven and six-tenths percent of the cocultured embryos reached the morula stage, while no morulae were found in the control-group embryos ($P < 0.05$).

Twenty-seven embryos coming from GC cocultures were transferred (2.4 ± 0.7 per patient) (Table 1): 20 (74%) were early or expanded blastocysts and 7 (26%) were morulae.

One pregnancy occurred and a normal baby was delivered. The pregnancy rate was 9.1% per retrieval (Table I).

All the untransferred blastocysts were cryopreserved (14.8%) (Table I).

The results of Trial 2 (11 patients) were compared with the data obtained in a slightly larger group ($n = 19$) of patients of the same age and having the same reasons for infertility (tubal or idiopathic), who had been treated with standard IVF in the same period, and in whom the embryos were transferred on day 2. A mean of 2.4 ± 0.7 and 2.8 ± 0.9 embryos was

Table II. Pregnancy Rates in Coculture Trials; Comparison with Standard In Vitro Fertilization (IVF) for the Same Types of Patients

	Trial 1 (coculture)	Standard IVF	Trial 2 (coculture)	Standard IVF
Day of transfer	5–6	2	5–6	2
Patients (N)	7	12	11	19
Transferred embryos, mean/patient $\pm$ SD	2.6 $\pm$ 0.3	2.7 $\pm$ 0.6	2.4 $\pm$ 0.7	2.8 $\pm$ 0.9
Pregnancies/retrieval, N (%)	1 (14.2)	2 (16.6)	1 (9.1)	2 (10.5)

transferred per patient, respectively, and the pregnancy rates per retrieval were 9.1 and 10.5%, respectively. The differences were not statistically significant (Table II).

Comparing the results obtained from Trial 1 and Trial 2, it was observed that a higher percentage of cocultured embryos developed to the blastocyst stage in Trial 1 in comparison with Trial 2 (50.7 vs 35.0%) and the differences were not statistically different (Table I).

Only a small percentage of the cocultured embryos (18% in Trial 1, 26% in Trial 2) reached the blastocyst stage on day 5, while the largest part were completely cavitated blastocysts on day 6 (82% in Trial 1, 74% in Trial 2).

A total of 16 (48.5%) spare blastocysts developed in GC coculture in Trial 1 and 4 (14.8%) in Trial 2 was frozen ($P < 0.01$) (Table I). Only expanded blastocysts with a well-developed inner cell mass or cavitating ones with a single well-defined blastocoele cavity were cryopreserved.

Embryo Morphology

A retrospective analysis of the embryo morphology showed that 24.2% of the embryos cocultured on confluent GCs were of grade A, 33.7% were of grade B, and 40.2 and 11.7% were of grades C and D, respectively.

On days 5–6 of the GC coculture, 54.5 and 39.5% of the embryos of grades A and B, respectively, reached the blastocyst stage, while 32.2% of the embryos of grade C and 25% of the very poor-quality embryos (grade D) were capable of rescuing and giving rise to blastocysts.

Electrophoretic Analysis of Secreted Proteins

The analysis of the proteins released by human GCs showed that the most representative proteic bands correspond to the range 24–29 and 45 kDa. There were no apparent differences between the 2nd → 4th group and the 4th → 6th group of GCs in terms of proteic release.

Five of 14 patients became pregnant. Their GC cultures were of high quality and reached the complete monolayer by the 12th day and the presence of an additional band of about 90 kDa was evident.

This group of patients showing the presence of the 90-kDa band was compared with the other group of patients showing the absence of the 90-kDa band. There was no relationship between the presence or

absence of the 90-kDa band and the mean age of the patients (31.40 ± 2.30 vs 33.13 ± 3.22), the E_2 levels on the day of hCG injection (1385.40 ± 961.93 vs 1246.57 ± 864.80), and the number of oocytes retrieved at pickup (13.20 ± 5.89 vs 18.43 ± 10.25).

On the contrary, the mean number of follicle stimulating hormone (FSH) ampoules administered was significantly lower in the group with the 90 kDa band with respect to the group without the 90-kDa band (23.20 ± 5.89 vs 36.78 ± 16.94 ; $P < 0.001$), while the percentage of good-quality embryos was significantly higher (64.89 ± 26.82 vs 39.44 ± 30.20 ; $P < 0.05$).

Transmission Electron Microscopy (TEM)

When observed in LM semithin sections, the GC pellets appeared to be formed by clusters of loosely packed cells. They appeared as irregularly rounded or polyhedral elements, varying in diameter from 18 to 25 μm . The GCs were generally well preserved and showed large, eccentrically located, rounded nuclei, provided with one or more nucleoli. Numerous GCs were rich in densely staining lipid droplets (Fig. 1a).

When observed by TEM, the GCs associated in small groups appeared to be joined together in some points by small linear gap junctions. Annular nexuses were also occasionally seen in the cytoplasm of some GCs. In other areas, the GCs plasma membrane created a network of intercellular spaces, irregularly shaped due to the protrusion of polymorphous evaginations (microvilli or blebs of varying size) from the cell surface. GC nuclei, surrounded by an occasionally indented nuclear membrane, were large, round, often eccentric, depicting in the nucleoplasm at least one (often two) prominent nucleoli and, sometimes, patches of heterochromatin. The cytoplasm appeared densely populated by a large variety of well-preserved organelles (Fig. 1b). They were rod-shaped or pleomorphic mitochondria, possessing cristae usually of the tubulovesicular type: membranes of smooth endoplasmic reticulum (SER), Golgi stacks and vesicles, isolated cisternae of rough endoplasmic reticulum (RER), and free ribosomes and polysomes (Figs. 1b, 2a and b). A number of dense bodies, probably lysosomal in nature, were also frequently seen in the cytoplasm (Fig. 2b). The presence of numerous highly electron-dense lipid droplets was a constant feature of most of the GCs. However, the lipid droplet quantity of cells varied among cells. In fact, whereas in some cells the lipid inclusions were randomly dispersed throughout the cytoplasm (Fig. 2a), in other cells they

Fig. 1. (a) Panoramic view of human granulosa cells in a 1- μ m thin section; toluidine blue. Note the presence of a cluster of loosely packed cells. LM; original magnification, 400X. (b) Human granulosa cell. Numerous well-preserved organelles are seen in the cytoplasm. N, nucleus; n, nucleolus; m, mitochondria; L, clusters of lipid droplets. TEM; original magnification, 4000X.

Fig. 2. (a) Human granulosa cell. Lipid droplets (L) scattered in the cytoplasm are observable. TEM; original magnification, 6300X. (b) Human granulosa cell. N, nucleus; L, lipid droplets; arrow, SER; m, mitochondria; db, dense bodies. TEM; original magnification, 12,500X.

usually grouped together and almost completely filled the cytoplasm (Fig. 1b).

DISCUSSION

Coculture systems have recently been introduced to enable the culturing of embryos to the blastocyst stage. The use of feeder cell monolayers provides the possibility of selecting embryos with a good developmental potential and of obtaining better synchrony between the embryo stage and the time of transfer compared to physiological cycles in natural pregnancies.

This study describes a simple coculture system with human autologous GCs, showing an increase in blastocyst formation with respect to conventional media both in patients at the first IVF treatment and in patients with previous implantation failures.

Data from the literature show that the percentage of blastocysts obtained with coculture systems or with culture media alone varied tremendously among studies (3–77%). Comparing the percentage of blastocysts obtained with culture media alone and coculture in the same study, Bongso *et al.* (17) found that the proportion was 33% with T6 medium versus 69% with ampullary cells. Menezo *et al.* (18) reported 3% with B2 medium versus 61% with Vero cells and Turner *et al.* (8) found 46% with HTF versus 77% with Vero cells. Plachot (11) had 9% with B2 versus 40% with GCs. The results obtained in our study showed a great improvement in the percentage of blastocysts developed in GC coculture (50%) with respect to 7% in a conventional medium.

Notwithstanding the increased rate of blastocyst formation, the pregnancy rate of 14.2% (obtained in patients at the first attempt of IVF-ET, in which a mean of 2.6 ± 0.3 blastocyst per patient was transferred) did not improve with respect to the overall pregnancy rate (16.6%) obtained during the same period in our standard IVF program when we transferred two- to four-cell-stage embryos on day 2.

Our results are confirmed by Guerin (19), who demonstrated that in routine IVF, day 5 embryo transfer resulted in the same pregnancy rate as conventional day 2 embryo transfer.

On the contrary, Wiemer *et al.* (4), using bovine oviductal epithelial cells, observed an improvement in both embryo morphology and pregnancy rate in a group of unselected patients in which two- to four-cell-stage embryos were transferred after a brief coculture period or from a control medium (43 vs 29%). The untransferred embryos left in coculture gave rise to a

significantly higher percentage of blastocysts compared with conventionally cultured embryos (58.46 vs 29.27%; $P < 0.05$).

Similarly, Freeman *et al.* (9) obtained a pregnancy rate of 54.2% by transferring a mean of 3.7 ± 0.1 embryos cocultured on GCs for 3 days after oocyte collection since they were two pronuclear zygotes. The incidence of blastocyst formation of untransferred embryos was 67.7%.

The selection of the “best embryos” by the evaluation of embryo development performance after 5, 6, or 7 days in prolonged coculture and the ability of the embryo to reach the expanded blastocyst stage would probably be more effective than normal day 2 assessment of morphological criteria (number of cells, fragments, and blastomere regularity) (7) in giving the patients a greater possibility of becoming pregnant in the first attempt of an IVF-ET trial and, particularly, to those with repeated implantation failures.

Similarly to the results obtained in the group of patients at the first attempt of IVF-ET in patients with multiple implantation failures, we achieved an encouraging blastocyst development rate (35%), but a lower pregnancy rate (9%) after the transfer of GC cocultured embryos with respect to the other coculture systems. In addition, the pregnancy rate was not increased with respect to that obtained in patients with similar characteristics during the same period in our standard IVF program when we transferred two- to four-cell-stage embryos on day 2.

Our findings are in agreement with those obtained by Plachot *et al.* (11), who observed that 30% of embryos in GC coculture system reached the blastocyst stage, showing a good embryo development in vitro. However, the pregnancy rate of the patients with at least three previous implantation failures was only 5.9%, which is similar to the control group without coculture (6.3%). In addition, Plachot observed that embryo development could depend on the clinical and biological history of the couples since a higher percentage of blastocyst formation (38%) but no pregnancy was found in patients older than 40 years, with a mean of 4.7 previous failures with respect to 33% of blastocysts and an 8.3% pregnancy rate in patients less than 38 years and a mean of 6 previous implantation failures. However, in the same study, it was observed that when implantation failures increased from seven to eight, only 17% of embryos developed to the blastocyst stage but no pregnancies were obtained ($P = 0.05$).

On the contrary, Olivennes *et al.* (7), transferring a mean of 1.5 ± 1.1 blastocysts cocultured with Vero cells into a group of patients selected for having had

at least four implantation failures in previous IVF attempts, obtained a pregnancy rate per transfer of 37.2%. Similarly Menezo *et al.* (3), transferring blastocysts developed on Vero cell cultures, obtained a pregnancy rate per transfer of 42% in patients with at least four previous failures and observed that 6% of patients had no embryo developed to the blastocyst stage, suggesting that a maternal effect may explain the repeated previous failures.

Bongso *et al.* (1), transferring blastocysts grown on human ampullary cells, in patients with at least two failed cycles, obtained a pregnancy rate of 30%. Our pregnancy rate of 9% is the lowest obtained if compared with all these studies, even if it is difficult to compare results found in the literature with those reported by Bongso because he replaced both two- to six-cell embryos and blastocysts in fallopian tubes, in utero and by sequential transfer; therefore, the author himself is not able to conclude whether the two- to six-cell or blastocyst-stage embryos implanted.

However, it is difficult to quantify the magnitude of the improvement in the quality of human cocultured embryos because of lack of information about the in vivo developing embryos and the lack of a standard objective method for assessment of embryo quality. High blastulation rates have often been reported in most coculture studies, but some authors have shown that blastocyst formation is a poor indicator of embryo quality and therefore not a reliable measurement for the efficacy of coculture system in order to improve the pregnancy rate (7,20).

Apparently normal blastocysts assessed at the level of light microscopy can, in fact, have a reduced cell number or a reduced incidence of hatching (7). hCG production by blastocysts in vitro has been used as an objective means of assessing development even if the hCG production does not necessarily correlate with the morphology and cleavage rate of an embryo (8). Nevertheless, in an experimental study, it has been shown that hCG secretion was significantly higher from embryos cocultured on Vero cells between day 9 and day 12 than from embryos in routine culture (8).

We found that GC coculture allows some embryos that were not suitable for freezing at the early cleavage stage to reach the blastocyst stage and to be frozen. We observed that 32% of the poor quality embryos developed into healthy blastocysts when cocultured on GCs until day 6. These observations are in agreement with the results reported by Dirnfeld *et al.* (10), who observed a significant decrease in poor-quality embryos (14 vs 51%) and an increase in the proportion of good-quality embryos (56.6 vs 4%) by comparing

the embryo quality of the embryos developed until the four- to eight-cell stage in GC coculture systems with the quality of those developed in HTF medium.

Moreover, they are supported by a recent study performed by Freeman (9), who compared the development and the fragmentation of human embryos cocultured with autologous GCs with embryos cocultured conventionally (Ham's F-10 plus 15% of maternal serum).

After examination for blastomere number and degree of fragmentation, it was found that coculture had no effect on the average number of blastomeres per embryo, but fragmentation was significantly decreased compared with the controls. In this study, the best three or four embryos were selected for transfer after 2 days of coculture, resulting in a pregnancy rate of 54.2%. The remaining embryos were cultured for 2–3 more days and 68% of these reached the blastocyst stage and were cryopreserved.

Notwithstanding the fact that the author selected the best embryos for transfer and only the nontransferred, with a poorer morphology, were cocultured until days 5–6, the rate of blastocyst formation was higher with respect to that found in our study.

Slight differences in GC culture methods could determine different culture conditions that could reflect on the embryo development to the blastocyst stage or on the pregnancy rate. Unlike our GC culture method, Freeman isolated GCs by sedimentation at unit gravity in culture medium without Percoll or centrifugation; in addition, 100,000 cells were plated in each dish, compared to about 250,000 cells plated/dish in our study. Probably both the more delicate cell isolation method and the lower cell number plated could create a better environment in which embryos develop well.

An autologous GC coculture system has several advantages over other coculture systems. First, screening of the GCs for infectious diseases is unnecessary in an autologous system. Second, the cells are easy to recover during oocyte pickup and ready for coculture 24 hr later. Other coculture systems, such as ampullary or endometrial cells, are limited by their finite life spans in culture: their mitotic activity declines through the sixth passage, with cell death following shortly thereafter (17). Our GC coculture system utilizes a primary cell culture. The monolayer remains epithelioid for the entire culture period up to 7 days. The culture medium is changed 24 hr after initiation and then every 2 days thereafter during the coculture period. The pH remains stable and there is very little debris present in the culture medium after the first change of medium.

The Vero cell coculture system is an exception, as Vero cells have an infinite life span *in vitro* and are well screened for infectious diseases, but it does not represent an autologous system. However, coculture results from these cells have been contradictory. Menzo *et al.* (18) suggested that coculture of "third choice" (nontransferred or frozen) embryos on a monolayer of Vero cells resulted in the "rescue" of many of these fragmented, early-degenerating embryos. However, he did not observe an increase in clinical pregnancy rate compared with conventional culture. In contrast, van Blerkom *et al.* (20) found no statistically significant improvement of any developmental parameter measured in embryos cocultured with Vero cells compared with conventional culture.

It seems that the improvement in embryo quality in both autologous and heterologous coculture systems is linked to the developmental stage in which the embryos are put into culture. In fact, van Blerkom *et al.* (20) observed that fragmented embryos that were put in Vero cell monolayers 30–34 hr postinsemination did not improve in morphology or continue development. These findings are not in agreement with our results since we observed a real improvement of the embryo quality in embryos put in GC coculture 40 hr postinsemination. These observations were confirmed by Plachot (11), who obtained a net improvement in embryo development 5 or 6 days after fertilization when the embryos were put into GC coculture at the pronuclear stage. However, we also obtained a good developmental rate even if we put the embryos in coculture at the two- to four-cell stage. On the other hand, the developmental stage at which the embryo is transferred in coculture did not seem to affect the pregnancy rate: the percentage of patients who became pregnant in our study compared well with that obtained by Plachot even if the embryonic stages at the time of transfer in coculture were different.

Vlad *et al.* (21) confirmed a significant increase in blastulation rate and a significantly higher average of nuclei per blastocyst using an ampullary cell coculture system with respect to the control medium.

The mechanisms of action of coculture are not entirely known, but it has been postulated that two mechanisms may exist which, in combination, may produce positive coculture effects. Somatic cells may stabilize the culture medium by detoxifying and removing undesirable factors from the medium (negative conditioning) and/or may release embryotrophic factors, such as proteins and glycoproteins (positive conditioning), which may have beneficial effects on the embryo.

The negative conditioning role can be achieved through the reduction of hypoxanthine, which is commonly found in most good culture media formulations and has been shown to induce the two-cell block in mouse embryos. The cells may stabilize the culture medium by means of the antioxidant activity and pH stabilization; furthermore, the serum proteins decrease embryotoxicity (17). Additional indirect support for the negative conditioning role was provided by Hartshorne *et al.* (22), who demonstrated that GCs actively migrate and exert phagocytotic activity on latex beads in culture.

The positive conditioning role is achieved by the production of growth factors, such as IGF binding protein (BP)-1 (22) and steroids (12). In particular, embryotrophic factors such as lactate, pyruvate, peptides, and glycoproteins seem to affect embryo development positively. Helper cells of different origin utilized during coculture procedures produce embryotrophic factors of high molecular weight such as proteins and glycoproteins (17,23). These factors seem to bind to the zona pellucida, to associate with the blastomere membranes, and to stimulate embryonic development *in vitro*.

In our study, we identified the presence of specific proteins with a possible putative embryotrophic role in a 48-hr conditioned medium from human GC cultures.

We found that patients having the 90-kDa band showed a better response to gonadotropin therapy and a better embryo quality.

GCs, after 48 hr of culture, are able to produce *in vitro* these high molecular factors, which are associated with better embryo quality and a superior pregnancy rate. It could be postulated that the presence of these proteins *in vivo* could improve the follicular microenvironment in which the oocytes develop: good-quality oocytes presumably could give rise to good-quality embryos with an increased chance of implantation.

However, since the patients studied (whose GCs released proteic factors) are not the same as those enrolled for the coculture protocol, we could not directly correlate the true embryotrophic activity of these peptides with the high quality of embryos maintained in coculture with GCs. However, even if the actual role of these proteins is unknown, their identification will help us to understand some of the mechanisms of coculture systems.

In addition, the ultrastructural characteristics of the GCs shown in this study were typical of healthy, metabolically active, protein-synthesizing, and mostly steroidogenic cells (12,13). These results agree with those described in *in vitro* spontaneously luteinizing GCs

and with those shown in GCs deriving from human follicles containing fertilizable oocytes (12).

The presence of linear gap junctions joining the cells and the presence of cytoplasmic annular nexuses reflect the same conditions in human GCs provided with morphological correlates of high steroid synthetic activity. These junctional elements could represent the remnants of a well-developed junctional complex, formed by a tandem of gap and adherence junctions, which link neighboring GCs during differentiation inside the follicle (12).

Development of polymorphous evaginations (microvilli or blebs of varying size) on the GC surface has been generally associated with GC luteinization, both in vitro and in vivo. Furthermore, their presence could represent a nonspecific cellular adaptation to the culture conditions in order to develop a greater surface of exchange with the medium (12).

The presence of large, rounded, and often eccentric nuclei provided with nucleoli and rare heterochromatin patches is typical of cells which are actively synthesizing steroid cells (12).

The abundant and diversified organelles such as pleomorphic mitochondria, SER membranes, Golgi vesicles, RER cisternae, ribosomes, polysomes, lysosomal bodies, and numerous electrodense lipid droplets are the parallel morphological expression in the cytoplasm of the steroid-synthesizing capability in GCs (24).

In particular, the presence of a rich network of SER tubuli and of a well-developed Golgi apparatus in the GCs confirms that luteinization and progesterone secretion are in progress (12,25).

The most representative organelles contained in the GCs observed are undoubtedly the lipids. The accumulation of lipid droplets is one of the early signs of luteinization (12,13). Lipid droplets appeared as isolated structures scattered in the cytoplasm of some cells, whereas in others they tended to be very numerous and clustered in some areas of the cytoplasm. This difference should be explained considering the presence of different subpopulations of GCs (mural, antral, cumulus–corona cells) in the follicular aspirate (24). Presumably, GCs containing isolated, randomly distributed lipid droplets do not seem to be completely mature (luteinized), whereas the accumulation of lipid droplets in the GC cytoplasm seems to be correlated with a profitable steroid synthesis in highly differentiated cells.

On the basis of this morphofunctional information, together with the biochemical and clinical data, we believe that luteinizing human GCs may exert a posi-

tive influence on oocyte fertilizability and early embryo development during coculture.

To conclude, granulosa cells allow embryo development but not the pregnancy rate to improve after transfer of cocultured blastocysts both in patients in the first cycle of IVF-ET treatment and in patients with multiple previous implantation failures. Of course, to draw a definite conclusion, it would be necessary to analyze the results obtained from a larger series of patients.

REFERENCES

1. Bongso A, Ng SC, Fong CY, Anandakumar C, Marshall B, Edirisinghe R, Ratnam S: Improved pregnancy rate after transfer of embryos grown in human fallopian tubal cell coculture. *Fertil Steril* 1992;58(3):569–574
2. Jayot S, Parneix I, Verdaguer S, Discamps G, Audebert A, Emperaire JC: Coculture of embryos on homologous endometrial cells in patients with repeated failures of implantation. *Fertil Steril* 1995;63(1):109–114
3. Menezo YJR, Sakkas D, Janny L: Co-culture of the early human embryo: Factors affecting human blastocyst formation in vitro. *Microsc Res Technique* 1995;32:50–56
4. Wiemer KE, Hoffman DI, Maxson WS, Eager S, Muhlberger B, Fiore I, Cuervo M: Embryonic morphology and rate of implantation of human embryos following co-culture on bovine oviductal epithelial cells. *Hum Reprod* 1993;8(1):97–101
5. Ben-Chetrit A, Jurisicova A, Casper RF: Coculture with ovarian cancer cell enhances human blastocyst formation in vitro. *Fertil Steril* 1996;65(3):664–666
6. Quinn P, Margalit R: Beneficial effects of coculture with cumulus cells on blastocyst formation in a prospective trial with supernumerary human embryos. *J Assist Reprod Genet* 1996;13(1):9–14
7. Olivennes F, Hazout A, Lelaidier C, Freitas S, Fanchin R, de Ziegler D, Frydman R: Four indications for embryo transfer at the blastocyst stage. *Hum Reprod* 1994;9(12):2367–2373
8. Turner K, Lenton EA: The influence of Vero cell culture on human embryo development and chorionic gonadotrophin production in vitro. *Hum Reprod* 1996;11(9):1966–1974
9. Freeman MR, Whitworth CM, Hill GA: Granulosa cell coculture enhances human embryo development and pregnancy rate following in-vitro fertilization. *Hum Reprod* 1995;10(2):408–414
10. Dirnfeld M, Goldman S, Gonen 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(1):120–122
11. 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(12):2133–2140
12. Nottola SA, Familiari G, Micara G, Aragona C, Motta PM: The ultrastructure of cumulus-corona cells at the time of fertilization and early embryogenesis. A scanning and transmission electron microscopic study in an in vitro fertilization program. *Arch Histol Cytol* 1991;54:145–161

13. Motta PM, Nottola SA, Pereda J, Croxatto HB, Familiari G: Ultrastructure of human cumulus oophorus: a transmission electron microscopic study on oviductal oocytes and fertilized eggs. *Hum Reprod* 1995;10:2361–2367
14. Fabbri R, Porcu E, Lenzi A, Gandini L, Marsella T, Flamigni C: Follicular fluid and human granulosa cell cultures: influence on sperm kinetic parameters, hyperactivation and acrosome reaction. *Fertil Steril* 1998;69(1):112–117
15. Laemmly UK: Cleavage of structural proteins during the assembly of head bacteriophage T4. *Nature* 1970;227:680–685
16. Merrill CR, Goldman D, Sedman SA, Ebert MH: *Science* 1981;211:1437–38
17. Bongso A, Fong CY, Ng SC, Ratnam S: The search for improved in-vitro systems should not be ignored: Embryo co-culture may be one of them. *Hum Reprod* 1993(8):1155–1162
18. Menezo Y, Guerin JF, Czyba JC: Improvement of human early embryo development in vitro by coculture on monolayers of Vero cells. *Biol Reprod* 1990;42:301–306
19. Guerin JF, Mathieu C, Pinatel MC, Regnier-Vigoroux G, Lornage J, Bouliou D, *et al.*: Coculture d'embryons humains avec des cellules epitheliales de rein de singe: donnees cliniques concernant les transferts a J3 et J5. *Contracept Fertil Sex* 1991;19:635–638
20. 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
21. Vlad M, Walker D, Kennedy RC: Nuclei number in human embryos co-cultured with human ampullary cells. *Hum Reprod* 1996;11(8):1678–1686
22. Hartshorne GM, Bell SC, Waites GT: Binding proteins for insulin-like growth factors in the human ovary: Identification, follicular fluid levels and immunohistological localization of the 29–32 kD type 1 binding protein, IGF-bp1. *Hum Reprod* 1990;5(6):649–660
23. Liu LPS, Chan STH, Ho PC, Yeung WSB: Human oviductal cells produce high molecular weight factor(s) that improves development of mouse embryo. *Hum Reprod* 1995;10:2781–2786
24. Zoller LC: Quantitative analysis of the membrana granulosa in developing and ovulatory follicles. In *Ultrastructure of the Ovary*, G Familiari, S Makabe, PM Motta (eds). Boston, Kluwer Academic, 1991, pp 73–89
25. Schmidt CL, Kendall JZ, Dandekar PV, Quigley MM, Schmidt KL: Characterization of long-term monolayer cultures of human granulosa cells from follicles of different size and exposed in vivo to clomiphene citrate and HCG. *J Reprod Fert* 71:279–287