Cellular & Molecular Biology
Cell. Mol. Biol. 2015; 61 (8): 79-88
Published online December 24, 2015 (http://www.cellmolbiol.com)
Received on November 27, 2015, Accepted on December 20, 2015.
doi : 10.14715/cmb/2015.61.8.14

Increased pregnancy rate using standardized coculture on autologous endometrial cells and single blastocyst transfer : a multicentre randomized controlled trial

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Abstract

Despite excellent published results, the lack of well-designed, multicentre, randomized clinical trials results in an absence of general consensus on the efficacy of autologous endometrial cells coculture (AECC) in Assisted Reproductive Technology (ART). An open, multicentre, prospective, randomized controlled trial was designed to compare the pregnancy rate (PR) after the transfer of one blastocyst on day 5 after AECC to the transfer of one embryo on day 3 (control group). Patients were women aged 18 to 36, undergoing an ART cycle with no more than 1 embryo transfer failure. Sample size was calculated at 720 for a superiority trial involving an intermediate analysis at 300 patients. We present the results of the intermediate analysis that resulted in the study ending considering the observed difference. Three hundred thirty nine patients were randomized: 170 in the AECC group and 169 in the control group. The clinical PR per transfer was 53.4% with AECC and 37.3% in the control group (p=0.025). The quality of embryos was improved with AECC. These results suggest that implementation of the AECC technique to a large number of *In-Vitro* Fertilization (IVF) centres could lead to a substantial improvement in the proportion of successful assisted reproduction. The study was supported by the Laboratoires Genévrier, France.

Key words: Blastocyst, Endometrial Cell, Coculture, Pregnancy rate, Single embryo transfer.

Introduction

Despite many novelties in terms of techniques and culture media, the chance of successful pregnancy per embryo transfer still plateaus at 32.9% in Europe, 28.9% in France (1), and the proportion of patients that achieve their goal of having a biological child after several attempts is only 42% (2,3,4). Thus, the primary focus of in-vitro fertilization (IVF) teams remains to improve pregnancy rates.

In the 1990s, some IVF teams developed cell coculture, with the idea of supporting the development of embryos into blastocysts and of transferring them at a better time towards implantation window (5,6,7,8). Although some experts agreed that coculture and blastocyst-stage transfer improved clinical results (9,10,11,12,13,14,15,16), financial and technical, e.g. cost and issues of contamination have precluded widespread adoption of the method. In France, it was performed in 20% of cycles in 2013, according to the last national report (17), and only with culture media. A French monocenter controlled randomized study failed to show a difference in cumulative pregnancy rate (PR) between single blastocyst and cleaved embryo transfer (18). A recent metaanalysis (4) concluded that "that there is a small significant difference in live birth rates in favour of blastocyst transfer (Day 5 to 6) compared to cleavage stage transfer (Day 2 to 3). However, cumulative clinical PR from cleavage stage (derived from fresh and thaw cycles) resulted in higher clinical PR than from blastocyst cycles".

However, in all studies, blastocyst cultures were performed with culture media. For coculture itself, no well-designed prospective, multicentre, randomized clinical trial comparing endometrial coculture with blastocyst transfer to routine IVF techniques with cleaved embryo transfer has been published (19).

The *Instituto Valenciano de Infertilidad* (IVI) group has developed a safe and effective clinical program in which embryos are cocultured with pooled homologous endometrial cells from oocyte donors until the blastocyst stage and transferred to the patient (20). In 2010, the group compared the results of implanted blastocysts grown in sequential media to those issued from the endometrial coculture system (21). Both blastocyst formation and implantation rates were statistically increased when embryos were grown on feeder cells. However, the major weakness of the latter study was the absence of randomization and the fact that this was a retrospective monocentric study.

There is thus still a need for comparing blastocyst to cleaved embryos transfers and, in particular, blastocysts obtained through coculture. Moreover, it is also important to perform multicenter studies, since PR may great-

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ly differ between IVF centres and, apparently, still more following blastocyst transfers (4).

Here we report on a multicentre, prospective, randomized controlled trial, in which the primary objective was to compare PR following the transfer of one embryo on day 3 using conventional medium *vs.* one blastocyst on day 5 after autologous endometrial cells coculture (AECC). Furthermore, an innovative procedure was used in which culture of the endometrial cell monolayer was performed using endometrial cells from the patient and centralized in a unique facility, which should reduce variability in cell support between centres and improve the overall success rate of the technique. The intermediate results of our study are presented.

Materials and Methods

Trial design

This study was an open, multicentre, randomized (1/1 ratio), controlled, efficacy, clinical trial conducted in patients undergoing ovarian stimulation for an in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) comparing the transfers of a single embryo on day 3 and of a single blastocyst on day 5 obtained through a coculture on endometrial cells.

Participants

Among the 35 ART clinics willing to participate (34 French and 1 Belgium), 28 included at least one patient, of which 23 at time to be included in the intermediate analysis. Patients were women intending to get an IVF or ICSI cycle, fulfilling all of the following inclusion criteria: i) age ≥ 18 and ≤ 36, ii) having experienced no more than 1 embryo transfer failure, iii) with basal FSH level ≤ 12 IU/L within the 6 months prior to the study, iv) endometrial biopsy during the menstrual cycle preceding the ovarian stimulation (6 to 10 days after a documented ovulation) and v) negative serology for HIV-1/2, HBV, HCV, HTLV 1/2 and syphilis.

Exclusion criteria were: i) hypersensitivity to one of the culture media components (penicillin, human origin serum-albumin or fetal calf serum), ii) oocyte or sperm donation, iii) thawed embryos transfer, iv) women with endometriosis grade III and IV, chronic endometritis, hydrosalpinx, polycystic ovary, amenorrhea, anovulation, uterus with malformation, uncontrolled prolactinaemia, uterine synechia, uterine fibroma, exposition to diethylstilbestrol, uterine polyps, v) any endocrine disease.

The participants flow chart is presented in Figure 1.

Interventions

During inclusion visit (Visit 1), the investigators collected informed written consent from both patient and partner, as well as demographics, clinical gynaecological data, previous medical history, hormonal status, infection status and concomitant treatments. After checking for negative serology results, the investigator performed an endometrial biopsy (visit 2), 6 to 10 days after a documented ovulation, using a “pipelle de Cornier” (CCD laboratories, Paris, France). The biopsy was ~ 200 mg, and was performed in both groups. This also allowed control patients to be offered a coculture with AECC free of charge in case of transfer failure. Then,

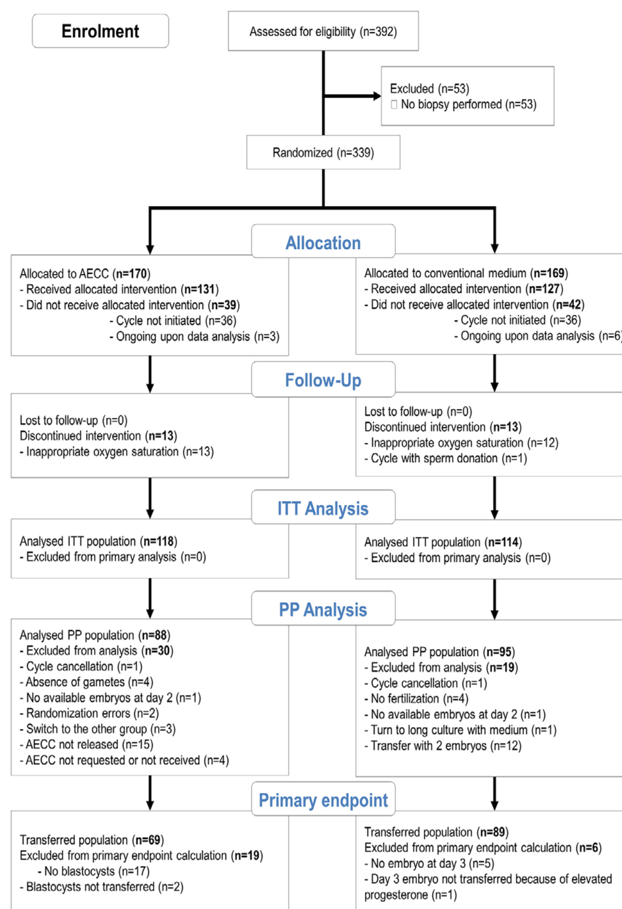

Figure 1. Flow chart of the study participants.

the patient was randomized to one of the two allocation groups (*i.e.* control or AECC group) according to a predefined randomization list. The biopsy was sent to Laboratoires Genevrier in order to be frozen (see below).

ART cycle was initiated 1 to 2 months later. Visits 3 (*i.e.* 3a, 3b, 3c and 3d) were four successive visits corresponding to the start of ovarian stimulation until the embryo transfer. Each physician conducted ovarian stimulation and induction of ovulation according to their usual routine. Oocytes were retrieved 36 hours after ovulation triggering by HCG. Embryo culture in the control group was performed with the conventional medium usually used by each individual IVF centre. For coculture group, embryos were cultured on conventional medium usually used by each individual IVF centre until Day 2. From Day 2 to Day 5, embryos were cultured on AECC. Embryo quality on day 3 (Veeck’s criteria, for both groups), blastocyst quality (Gardner’s criteria) and blastulation rate on day 5 (for the AECC group only) were assessed. Only one 6- to 10-cell embryo on day 3 with less than 30% fragmentation, or one blastocyst on day 5 or 6 with a good quality of inner cell mass was transferred. Good-quality supernumerary embryos or blastocysts were cryopreserved.

During the first follow-up visit (Visit 4), the investigator recorded the βhCG pregnancy result performed 2 weeks after embryo transfer. If the result was positive, the embryo’s cardiac activity and/or gestational sac presence were assessed during the last follow-up visit (Visit 5), 5 to 8 weeks after embryo’s transfer. Finally, the investigator called the patient within 2 weeks after expected delivery date to assess delivery and baby status.

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Cell culture

The endometrial biopsy was shipped to Laboratoires Genevrier (Sophia-Antipolis, France) at 4°C overnight, minced and frozen in liquid nitrogen upon reception. Twenty-four hours before oocyte recovery, endometrial tissue was thawed and digested 15 min at 37°C in 0.2% collagenase NB6 (Serva electrophoresis, Heidelberg, Germany) in BM1 medium (Eurobio, Courtabeuf, France). Epithelial and stromal cells were isolated as previously described (14,22,23), seeded in a four-well plate (BD Falcon, Franklin Lakes, NJ, USA) and cultured in CCM™-30 (Vitrolife, Göteborg, Sweden) supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK) (37°C, 5% CO_2). The AECC (Endocell®, Laboratoires Genévrier, Sophia-Antipolis, France) was composed of a mixed culture of stromal and epithelial cells containing 30–90% of autologous fragments of human endometrial epithelial glands and autologous human endometrial stromal cells, and 500 µL blastocysts culture medium per well. The sponsor of the study supplied the product to the IVF laboratory on day 2 of embryos. The culture process was validated (see below) and patented.

Before shipping back to the IVF centre, endometrial cell cultures were assessed for: i) microbiological contamination, ii) total confluence ≥ 40% and iii) % epithelial cells. Endometrial cell culture was performed in a clean room authorized by the French National Agency for the Safety of Drugs and Health Products (ANSM), in accordance with the good manufacturing practice (GMP) for therapeutic goods. Upon reception in the IVF centre, culture medium was replaced with CCM™-30 (27°C, overnight).

The AECC should be manipulated under aseptic condition. Upon receipt, the biologist might rinse the culture with 1mL of provided culture medium (CCM™-30, Vitrolife, Göteborg, Sweden), then incubate the plates with 1mL of CCM™-30 medium for at least 4 hours with 37°C, 5 (+1)% CO2, ~20% O_2 and saturated humidity conditions. The embryos could then be transferred for coculture on the AECC. In case of tri-gas incubator's use, the biologist should ensure that the O_2 concentration was 20%. Culture medium should not be replaced during the culture period, i.e. no media exchange was performed.

Endocell® validation

The AECC was validated prior to the clinical study by 3 means.

Immunofluorescence analysis

Endometrial cells were seeded in Labtek 8 wells (Nunc, Roskilde, Denmark). Cells were fixed 20 min in 3.7% formaldehyde (Sigma Aldrich, St Louis, MO, USA) and permeabilized/satured with 0.1% saponine (Sigma Aldrich, St Louis, MO, USA) and 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (30 min at room temperature). Cells were then incubated with anti Vimentine (DakoCytomotion) or anti pan-cytokeratin (H-240) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) during 1 hr at room temperature. Cells were then washed 3 times with 0.5% BSA PBS and incubated for 30 min with anti-mouse FITC (sheep) (DakoCytomotion, Glostrup, Denmark) or anti-rabbit Cy3 (donkey) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were then washed 3 times. DAPI (Sigma Aldrich, St Louis, MO, USA) (150 nmol, 3 min) was used to counterstain nuclei and observed on an Axioskop 40 microscope (Zeiss, Oberkochen, Germany).

Scanning electron microscopy

Cells were first fixed with 1.6% gluteraldehyde (Sigma Aldrich, St Louis, MO, USA) (in 0.1M sodium phosphate buffer) then in OsO4 (0.5%) for 15 min. Samples were dehydrated with solutions containing increasing amounts of acetone for 15 min each (50, 70, 96 and 100%) and finally washed with Hexamethydisilazane (5 min), dried overnight at room temperature, sputter coated with a thin layer of AuPd (Polaron E 5100, Quorum Technologies, Ringmer, UK) and analyzed with SEM (JEOL 6700F, JEOL Ltd, Tokyo, Japan).

Outcomes

The primary endpoint was the clinical pregnancy rate after the transfer of one blastocyst on day 5 after AECC (AECC group) *vs.* one embryo on day 3 using conventional medium (control group).

Secondary endpoints were: i) blastocyst quality on day 5 (Gardner criteria), ii) blastulation rate and comparison of iii) embryo quality on day 3 iv) clinical PR per started stimulation cycle, v) clinical pregnancy evolution (*i.e.* ongoing pregnancy, spontaneous abortion and prematurity rates per clinical pregnancy), vi) delivery rate per transfer and per started stimulation cycle and vii) multiple births and health status of the neonates (*i.e.* hypotrophy, perinatal mortality and malformation rates per neonate).

The quality of embryos on day 3 was assessed according to an evaluation scale derived from the Veeck's criteria: **Grade 0,** embryo with equal blastomeres, without fragmentation; **Grade 1,** embryo with equal blastomeres and ≤ 10% fragmentation; **Grade 2,** embryo with unequal blastomeres and 10% to 30 % fragmentation; **Grade 3,** embryo with equal or inequal blastomeres and 30% to 50 % fragmentation; **Grade 4,** embryo with extreme fragmentation (> 50%) (24). The quality of blastocyst was described according to the Gardner's criteria (25).

Sample size

The sample size was calculated for a superiority trial, according to the hypothesis of p=0.0294 in a bilateral testing with one intermediate analysis (26) and 1-beta=0.842, for a power equal to 80%. The hypothesis was a difference of 12 % in pregnancy rate per transfer, in favor of the blastocyst group, as shown in the French register on IVF: FIVNAT: 25 % for SET at day2-day3 and 37% for single blastocyst transfer (SBT) at day 5, which corresponds to pregnancy rates per initiated cycle of 20% and 30%, respectively. According to these hypotheses, the total number of patients to be included was 600 (300 per group). A percentage of 15% of dropouts was anticipated, so that the number of patients to include was 720 (360 in each group). Patients who discontinued the study were not replaced. An intermediate analysis was planned in the protocol when 300 patients had completed the study (150 per group), in order to end the study if a statistically significant difference was

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reached for the primary endpoint, and not to expose patients to the less efficacious treatment.

Randomization

The randomization list was prepared using a validated SAS® software by the data manager appointed for this study and stored in electronic format (Excel file) in a dedicate directory so as to grant the full confidentiality of it, in full respect of internal standard operating procedures. The list was organized in balanced blocks of 8 by centre. Two series of sealed envelopes were generated, each containing the patient's randomization number and the allocated group. Before starting the recruitment phase, one set of envelopes was supplied to each centre. A second set of envelopes was kept at the Sponsor site, in a secure and locked place. During visit 2, once the biopsy was performed, the investigator opened the envelop corresponding to the patient's randomization number and identified the treatment group (AECC or control group) to which the patient was allocated. Any discrepancy between the group the patient was allocated and the group she was finally treated had to be explained and reasons clearly documented.

Statistical analyses

The results presented here are those of the intermediate analysis planned in the protocol after 300 patients had completed the study. This analysis was planned in order to stop the study in case of the demonstration of the AECC superiority and to then avoid exposing additional patients to the inferior treatment.

The intent-to-treat (ITT) population was defined as all randomized patients having initiated a stimulation cycle for IVF/ICSI and having all required cell culture conditions and adequate fertilization, in both groups. It excluded non-initiated cycles, patients ongoing at the time of interim analysis, patients with inappropriate oxygen saturation periods and cycles with sperm donation. ITT population included the protocol violations (non respect of number of transferred embryos for example). The per protocol (PP) population included all patients with retrieved oocytes and embryos on day 2, for whom the treatment was properly performed and no more than 1 embryo / blastocyst was transferred. The safety population was defined as all randomized patients.

Normally distributed quantitative variables were described using mean ± standard deviation (SD) and compared between treatments using analysis of variance (ANOVA). Qualitative variables were described using number and frequency and compared between treatments using Chi-square or the Fisher's exact test ($n<5$). Tests were two-sided and considered significant at an alpha (α) level of 2.94% for the intermediate analysis of the primary endpoint. Statistical analyses and data processing were performed using SAS® Software 9.1.3, Service Pack 32 (SAS Institute, Cary, NC).

Ethics

The study protocol and any amendments were reviewed and approved by the French National Agency for the Safety of Drugs and Health Products (ANSM) and an independent ethics committee. This study was conducted in accordance with the Declaration of Helsinki as amended in Seoul (2008), the European Directive 93/42/CEE and its amendments (European Directive 2001/20/CEE), current international Good Clinical Practice guidelines (ICH Topic E6 (R1), July 2002 step 5 including Step 4 errata), and applicable regulatory requirements. The study was registered at www.clinicaltrials.gov under the number NCT01058603 and title "Efficacy of Endocell® vs Conventional Medium in the Treatment of Infertility".

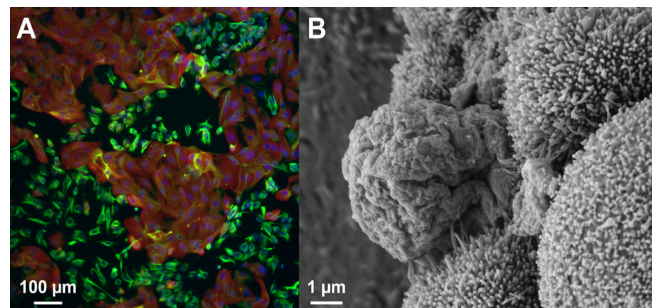

Figure 2. Epithelial cell characterization. **A,** Imunohistochemistry analysis. Stromal cells were stained with anti-vimentine antibody (green) and epithelial cells were stained with anti-cytokeratin antibody (red). **B.** Phenotype of epithelial cells during the implantation window was characterized by the presence of pinopodes by scan electron microscopy.

Results

Endocell® validation: in vitro characterization of endometrial cells

The presence of epithelial cells within a typical monolayer cell culture was characterized by cytokeratin immunostaining (Figure 2A). Characteristic phenotype of epithelial cells during the implantation window was determined by expression of $\alpha 5\beta 3$ integrin (data not shown) and by the presence of pinopodes onto their membrane using scan electron microscopy (Figure 2B). The functionality of endometrial monolayer was tested using a mouse embryo assay. A total of 63 mouse embryos were cocultured with endometrial monolayer in three different experiments. At 96 hours, the percentage of blastocyst development was 90%. These analyses allowed validation of the AECC process, which has received marketing authorization from the French National Agency for the Safety of Drugs and Health Products (ANSM).

Participant flow

The patients flow chart is presented in Figure 1. A total of 392 patients were assessed for eligibility to the study. Fifty-three (53) patients were not randomized because biopsy was not performed (mostly positive viral and/or bacterial sampling results).

Among the 339 randomized patients, 9 were ongoing at the time of data extraction and have been therefore excluded from this analysis. They were considered not to have initiated a cycle. Two hundred and fifty-eight (258) patients had an ART cycle (131 in the AECC group and 127 in the control group). Of these, 25 were excluded due to inappropriate oxygen saturation (13 AECC and 12 controls) and 1 because of sperm donation. The ITT population finally contained 232 patients (118 AECC and 114 controls). PP population included 183 patients (88 AECC and 95 controls). Most patients excluded from the PP population had either no AECC

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Table 1. Patient characteristics in ITT population.

	AECC group		Control group		
	N	mean ± SD	N	mean ± SD	*p*-value
Demographics					
Age (years)	118	30.8 ± 3.3	114	31.0 ± 3.0	*0.71*
Height (cm)	118	165.8 ± 6.8	113	166.0 ± 6.4	*0.80*
Weight (kg)	118	63.8 ± 13.3	113	62.6 ± 10.8	*0.47*
BMI (kg/m^2)	118	23.2 ± 4.7	113	22.7 ± 3.6	*0.33*
Hormonal status					
FSH at day 3 (IU/L)	118	6.8 ± 1.8	114	6.5 ± 1.7	*0.22*
Estradiol at day 3 (pg/mL)	113	52.5 ± 29.5	112	52.3 ± 39.9	*0.96*
Progesterone (ng/mL)	90	13.7 ± 8.9	93	13.3 ± 8.2	*0.75*
Sperm quality					
Sperm concentration (millions/mL)	114	49.5 ± 51.1	109	37.8 ± 41.6	*0.06*
Sperm progressive motility A+B (%)	114	36.8 ± 17.4	108	36.4 ± 19.3	*0.87*
Sperm normal forms (%)	107	23.0 ± 18.2	99	22.0 ± 19.0	*0.71*
Stimulations and biopsy					
Amount of gonadotropin used (IU)	116	1857 ± 677	113	1899 ± 797	*0.74*
Stimulation duration (days)	115	10.8 ± 1.8	113	10.4 ± 1.8	*0.16*
Day of biopsy	98	21.9 ± 1.7	102	22.2 ± 1.9	*0.29*
Endometrium thickness (mm)	105	10.9 ± 10.7	108	9.3 ± 2.4	*0.12*
Oocytes					
Number of oocytes collected	117	10.2 ± 5.5	113	10.7 ± 5.3	*0.48*
Number of oocytes injected/inseminated	117	8.9 ± 5.4	111	9.4 ± 5.1	*0.51*
Number of oocytes inseminated in IVF	117	4.3 ± 6.0	111	3.9 ± 6.2	*0.60*
Number of oocytes injected in ICSI	117	4.6 ± 4.8	111	5.5 ± 4.6	*0.18*
Number of embryos at day 2	117	5.9 ± 4.1	111	6.0 ± 3.6	*0.93*
Cleavage rate per ins/inj oocyte (%) [a]	113	67.7 ± 23.2	109	65.0 ± 24.6	*0.40*

N: Number of patients with data recorded

a. In patients with at least 1 inseminated/injected oocyte

released / received (n=19), or were transferred with two D3 embryos (n=12).

Recruitment

Patients were assessed for eligibility between February 2008 and April 2011. Data presented are those of the initially planned intermediate analysis. Indeed, in view of the higher difference than initially expected between groups, the study was prematurely stopped.

Baseline data

No significant difference was observed between groups in couple characteristics in the ITT (Table 1) nor PP populations (data not shown). In particular, age (30.8±3.3 vs. 31.0 ± 3.0 years in AECC and controls, respectively), body mass index (BMI), basal FSH (6.6±1.8 IU/L vs. 6.5±1.7 IU/L) and estradiol levels, were similar between groups. Neither was there any significant difference in endometrial biopsy day (21.9 ± 1.7 vs. 22.2±1.9), endometrium thickness or progesterone level on the day of biopsy (13.7 ± 8.9 vs. 13.3±8.2 ng/mL) or sperm analysis characteristics. Three stimulation protocols were used in both groups as follows: (1) agonists with short protocol, (2) agonists with long protocol, and (3) antagonists. The respective protocols were used for 4.3%, 52.1% and 43.6% of the patients in the AECC group, and in 2.6%, 52.6% and 44.7% of the participants in the control group (p=0.79). Urinary FSH, recombinant FSH and HMG were used in 25.4%, 66.9% and 7.6% in AECC, and 24.6%, 69.3%, and 6.1% in controls (p=0.92).

The technique used (IVF, or ICSI) did not differ between the AECC and the control group. IVF was used in 36.3% of the patients in the AECC group vs. 28.6% in the control group (*p*=0.13). Finally, stimulation results (*i.e.* total number of gonadotropin units, duration of stimulation, number of collected oocytes, number of embryos on day 2, cleavage rate and fertilization technique) were also similar between groups.

Outcomes

Pregnancy and delivery rates

In ITT population, the clinical PR per transfer (primary endpoint) reached 53.4% in the AECC group versus 37.3% in the control group (Table 2), thus 16% higher (p=0.025). The clinical PR per initiated cycle was not significantly higher in the AECC group (39.8% vs. 33.3%, p=0.30).

The number of spontaneous and medical abortion was similar in both groups (Table 2). Finally, there was a tendency towards a higher proportion of deliveries per transfer in favor of AECC group, close to significance (45.5% *vs.* 33.7%, p=0.10).

No statistically significant difference was observed between groups with regards to delivery outcomes (Table 3). The numbers of multiple deliveries, preterm deliveries and malformations were similar between groups. Nonetheless, the mean height of neonates tended to be higher in the AECC group than in the control group (49.7±1.9 cm *vs.* 48.4±2.6 cm, p=0.06). The mean weight was also slightly higher in the AECC group, but not significantly (3214±460 g *vs.* 3114±520 g, p=0.39).

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Table 2. Clinical pregnancies per transfer (primary endpoint) and other efficacy results.

	ITT			PP		
	AECC	**Control**	*p*-value	**AECC**	**Control**	*p*-value
Number of cycles	118	114		88	95	
Number of transfers	88	102		69	89	
Clinical pregnancies						
Per cycle, n (%)	47 (39.8)	38 (33.3) [a]	*0.30*	35 (39.8)	30 (31.6)	*0.25*
Per transfer, n (%)	47 (53.4)	38 (37.3) [a]	***0.03***	35 (50.7)	30 (33.7)	***0.03***
Discontinued pregnancies						
Miscarriage, n (%)	3 (6.4)	2 (5.4)	*0.85*	2 (5.7)	2 (6.7)	*1.00*
Ectopic pregnancy, n (%)	1 (2.1)	0 (0.0)	*1.00*	1 (2.9)	0 (0.0)	*1.00*
Medical abortion, n (%)	3 (6.4)	1 (2.7)	*0.63*	2 (5.7)	1 (3.3)	*0.89*
Deliveries						
Percentage per cycle, n (%)	40 (33.9)	34 (30.1)	*0.54*	30 (34.1)	27 (28.4)	*0.41*
Percentage per transfer, n (%)	40 (45.5)	34 (33.7)	*0.10*	30 (43.5)	27 (30.4)	*0.09*

a. One of the declared pregnancies was lost to follow-up, without any data on pregnancy outcome.

Table 3. Delivery outcome.

	ITT			PP		
	AECC	**Control**	*p*-value	**AECC**	**Control**	*p*-value
Number of deliveries	40	34		30	27	
Multiple deliveries, n (%)	2 (5.0)	0 (0.0)	*0.50*	1 (3.3)	0 (0.0)	*1.00*
Term of delivery (weeks), mean ± SD	39.6 ± 1.3	39.4 ± 2.4	*0.88*	39.5 ± 1.4	39.6 ± 1.2	*0.85*
Preterm, n (%)[a]	1 (2.5)	2 (5.9)	*0.59*	1 (3.3)	1 (3.7)	*1.00*
Gender						
Male, n (%)	21 (52.5)	16 (47.1)		17 (56.7)	13 (48.2)	
Female, n (%)	19 (47.5)	18 (52.9)		13 (43.3)	14 (51.9)	
Sex ratio	1.11	0.89	*0.64*	1.31	0.93	*0.52*
Height (cm), mean ± SD	49.7 ± 1.9	48.4 ± 2.6	*0.06*	49.9 ± 2.0	48.5 ± 2.5	*0.06*
Weight (g), mean ± SD	3214 ± 460	3114 ± 520	*0.39*	3212 ± 486	3093 ± 515	*0.37*
< 1500g, n (%)	0 (0.0)	1 (2.9)	*0.46*	0 (0.0)	1 (3.7)	*0.47*
< 2500g, n (%)	3 (7.5)	5 (14.7)	*0.32*	3 (10.0)	4 (14.8)	*0.58*
Apgar, mean ± SD						
At 5 min	9.97 ± 0.18	10.00 ± 0.00	*0.41*	9.95 ± 0.21	10.0 ± 0.0	*0.40*
At 10 min	10.00 ± 0.00	10.00 ± 0.00	*1.00*	10.0 ± 0.0	10.0 ± 0.0	*1.00*
Malformation, n (%)	3 (7.5)	3 (8.8)	*0.83*	2 (5.7)	3 (10.0)	*0.52*

a. before<37 weeks of gestational age.

Quality of embryos on day 3

In ITT population, no difference was observed between groups as to the number of cells per embryo (Table 4). However, the number of grade 3 embryos was higher in the control group than in the AECC group (respective mean±SD of 0.7±1.4 and 0.3±0.8, p=0.01). The control group also showed more embryos with fragmentation higher than 30% (*i.e.* grade 3 or higher, p=0.02). These data suggest that the quality of embryos was improved with AECC.

Blastulation rate and quality of blastocysts on day 5 according to Gardner's criteria

Among the 88 cycles with day 2 embryos cocultured, 17 resulted in no blastocyst, 70 provided at least one blastocyst (79.5%) and 1 provided a morula. 182 blastocysts were obtained (excluding the morula). Compared to the 550 embryos obtained in these patients on day 2 and cocultured on autologous endometrial cells, the blastulation rate was 33.1%. In total, 64.4% of blastocysts were of grade 3 or better (Table 5).

Among the 182 blastocysts obtained, 64 were transferred, 82 were frozen and 36 were discarded. In addition, four non-graded blastocysts and the morula were transferred, resulting in a total number of 69 transfers.

Ancillary analyses

Although 76% of biopsy tissues were contaminated by various commensal microorganisms upon arrival at the Genévrier laboratory, primary cell culture was possible in 84.5% of the total biopsy samples received. Due to patient tissue heterogeneity, the epithelial/stromal cell ratio at 48 hours of culture was highly variable and led to the composition of three groups: group a, < 15% of epithelial cells; group b, between 15% and 30% of epithelial cells; and group c, >30 % of epithelial cells. The overall mean percentage of epithelial cells was 32.9 ± 22.2%.

Because low expression of Leukemia inhibitory factor (LIF) has been described as a possible cause of implantation failure, LIF secretion was measured in separate endometrial cell monolayer cultures in parallel to the current clinical trial. No significant difference was found in LIF secretion between groups b and c, which had respective LIF secretions of 346.4 ± 197.7 pg/mL *vs.* 232.2 ± 54.1 pg/mL at day 2 and 4708.6 ± 1453.7 *vs.*

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Table 4. Quality of embryos at day 3, ITT population.

	AECC		Control		
	N	mean ± SD	N	mean ± SD	*p*-value
Number of cells per embryo [a]					
< 6 cells	94	1.7 ± 2.4	105	1.9 ± 1.8	*0.52*
≥ 6 cells	94	4.2 ± 3.3	105	4.1 ± 3.1	*0.95*
Quality of the embryo					
Grade 0	94	2.5 ± 2.5	105	2.6 ± 2.9	*0.75*
Grade 1	94	1.2 ± 2.4	105	1.0 ± 1.5	*0.46*
Grade 2	94	1.7 ± 2.7	105	1.3 ± 1.7	*0.27*
Grade 3	94	0.3 ± 0.8	105	0.7 ± 1.4	***0.01***
Grade 4	94	0.2 ± 0.8	105	0.3 ± 0.9	*0.36*
Embryos with Fragmentation ≤ 10 % (≤ Grade 1)	94	3.7 ± 3.3	105	3.6 ± 3.1	*0.85*
Embryos with Fragmentation > 30 % (≥ Grade 3)	94	0.5 ± 1.2	105	1.0 ± 1.9	***0.02***

N: Number of patients with recorded data

Results are expressed as mean ± SD

a. On cases where embryo quality at Day 3 was reported; Percentages are computed on cases with embryos at Day 3.

Table 5. Quality of blastocysts at day 5 (Gardner's criteria), ITT population.

	AECC (N=84)
Mean number of blastocysts per cycle, mean ± SD	**2.19 ± 2.30**
Grade 1	0.40 ± 0.60
Grade 2	0.36 ± 0.57
Grade 3	0.56 ± 0.87
Grade 4	0.73 ± 1.27
Grade 5	0.08 ± 0.50
Grade 6	0.00 ± 0.00
Grade 3-5	1.37 ± 1.81
Blastocyst with quality assessed, n (%) [a]	**180 (98.9)**
Grade 1	34 (18.9)
Grade 2	30 (16.7)
Grade 3	47 (26.1)
Grade 4	62 (34.4)
Grade 5	7 (3.9)
Grade 6	0 (0.0)
Grade 3-5	116 (64.4)

a. A total of 182 blastocysts was obtained.

4447.5 ± 2923.9 pg/mL at day 7. Group a showed lower LIF secretion but represented only 9.3% of the total endometrial culture started.

Finally, 85% of the biopsies shipped to the GMP laboratory were processed for coculture and 83% of these were shipped back to the IVF centre and potentially used by the IVF team for embryo coculture.

In order to evaluate a potential centre effect, we compared the results between the two most experienced centres that provided almost one third of cycles and those of other centres combined (30 *vs.* 58 cycles in AECC, and 30 *vs.* 72 in control group). A higher PR per transfer was observed in those centres for the AECC group 83.3% *vs.* 37.9%, p<0.001, ITT population), whereas no difference was observed regarding the technique usually performed (*i.e.* control conditions) and no difference was observed in the control group (33.3% *vs.* 38.9%, p=0.60).

Safety

A total of 100 adverse events (AEs) were reported in 69 patients: 59 in 41 patients in the AECC group and 41 in 28 patients in the control group. Among them, 17 were considered as serious:

six ovarian hyperstimulation syndromes (2 in the AECC group and 4 in the control group), 1 miscarriage (control group), 4 induced abortions (3 in AECC group, 1 in control group), 1 colopathy (AECC group), 1 preterm delivery risk (control group), 1 gestational diabetes (control group), 1 premature rupture of membranes (control group), 1 metrorrhagia (AECC group), 1 HELLP syndrome (AECC group). None of these events were related to embryo culture or AECC. Most were related to ART or study procedures. All patients recovered.

A total of 83 AEs were quoted as non-serious. In 2 patients, 3 AEs resulted in definitive discontinuation. No statistical difference was observed between groups with regard to the number or severity of AEs.

Discussion

This study is the first multicentre, randomized controlled trial comparing the efficacy of AECC and transfer on day 5 to that of culture on a conventional medium and transfer on day 3, the standard procedure used in IVF centres. Results in 232 patients showed that AECC significantly improved the quality of embryos and pregnancy rate per transfer (*i.e.* the primary endpoint). It can be stressed that there was no difference concerning the patients' characteristics, nor the stimulation protocols.

Indeed, contrary to sequential culture media with a defined and limited formulation, autologous endometrial cells are able to continuously synthesize a complete pattern of cytokines and growth factors including LIF (27,28,29,30,31,32,33,34). The fine balance between their positive and negative regulatory input positively affects embryo development, especially because cytokine and growth factor receptors are present on the early preimplantation embryo (32).

Several previous studies have also shown improved results with the AECC technique (19,35,36). In 2008, soon after the first patient was enrolled in the present study, a systematic literature review identified 17 prospective randomized trials reporting the use of coculture in human IVF (19). None of these 17 studies was multicentre. The lack of comprehensive and carefully controlled studies was a major reason for conducting the

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present study. In addition, we sought to better determine the underlying cause of any improvement in outcomes, whether due to the AECC procedure or to variation in the blastocyst transfer protocol.. Although additional studies are needed to further compare the two methods, our first objective was to determine the efficacy of AECC method. what we conclude. The biopsy itself was determined to not play a significant role as both groups had a biopsy in the previous cycle.

AECC increased the per transfer PR by 16% (p=0.025). In addition, AECC was associated with a decreased proportion of poor-quality embryos on day 3. This is in accordance with previous monocentric studies on the relative efficacy of AECC compared to usual techniques (19,36,37,38). Heterogeneity of the centres with regard to their usual techniques and experience with AECC most probably decreased statistical power, which further supports our statistically significant results. For instance, considering the primary endpoint, a significantly higher per transfer PR was observed with AECC as compared to control, but this difference predominantly involved the most experienced centres. This was not due to a general difference in blastocyst culture that all had, but possibly to the learning curve needed by the AECC technique implying several changes in the whole ART process. Moreover, the data presented are those of an intermediate analysis performed after 300 patients were included in the study. This was scheduled in the protocol, including the calculation of the required number of patients and the p value used for the statistical testing. The study was stopped in view of the significant difference between the AECC and control groups considering the primary endpoint. Although the sample size calculation showed that 720 patients should optimally be included, statistical significance for the primary endpoint was reached after randomization of only 339 patients. Admittedly, considering the PR per initiated cycle, the difference was lower than for the primary endpoint (per transfer PR)(Table 2). This is in agreement with most of the studies comparing blastocyst to cleaved embryo transfers (4,18) because in a proportion of the cases, the blastocyst stage is not obtained. However, the difference remains in a positive direction. It should be noted that this controlled study was not double blinded. This was not possible because transfers were made at day 3 and day 5, with different types of embryos (cleaved and blastocysts). Moreover, the primary outcome was the objective fact of clinical pregnancy.

During the study, a certain number of patients dropped out. These were evenly distributed between both groups and typically occur in most studies. Nonetheless, a putative bias in the study might be related to the patients (n=25) excluded from analysis of the primary endpoint (19 in the AECC group and 6 in the control group). The variation between groups is mostly due to the fact that the study consisted of a comparison of blastocyst transfer on day 5 after AECC with transfer of an embryo on day 3 after culture on conventional medium. This choice was made because the latter technique is the most often used in IVF centres, despite the formulation of new sequential media capable of supporting the development of viable human blastocysts (39). The validity of our results is supported by the higher quality and absence of any significant differences between groups with regards to the number of embryos on day 3. As this was an open study, our analyses suggests that little bias has occurred, and that the differences observed are likely attributed to improved outcome using the AECC technique. This is supported by the experimental observation of the much higher probability of obtaining a D3 embryo than a D5 blastocyst in similar conditions (40,41,42). To evaluate the precise influence of AECC, further studies should be conducted to compare the efficacy of D5 embryos transfer originating from either AECC or conventional culture conditions.

In addition, 15% of biopsies were discarded from the coculture program primarily due to inadequate biopsies, logistic problems, others issues and 17% of the cocultures were not used (<15% of epithelial cells, residual contamination during the process, logistic problems, lack of oocyte, or sperm, unexpected problems). These reasons are related to the heterogeneity of patients (and consequently of cultures), contamination of biopsies, distance from the IVF centre to the promoter's facilities and need for non-experienced centres to comply with the requirements of the AECC technique. Major difficulties of AECC occur due to the need to learn the technique especially regarding performing high-quality biopsies as well as in the precise timing it requires. In the present study, IVF centres had to implement new procedures with respect to the optimal intervals between ovarian stimulation, biopsy, oocyte retrieval, fertilization, coculture and transfer. The positive results show that these difficulties can readily be dealt with.

Interestingly, the difficulties encountered during the study allowed for the identification of sensitive points that can lead to improvements in the overall process of AECC. Further studies will benefit from these improvements and the pregnancy rate with the AECC technique should largely improve. Points of improvement were partly found in subgroup analyses. For instance, the pregnancy rate per transfer in the PP population was significantly higher if the endometrial biopsy was performed before day 9 following ovulation in the AECC group (25.0% *vs.* 62.5%, p<0.01). The same was observed when considering the delivery rate per transfer (20.8% vs. 52.5%, p<0.001). This supports the Spandorfer study (23), which demonstrated that culture with endometrial cells obtained at this time after ovulation allows better implantation rates than with cells collected later. This is probably related to the fact that the late luteal phase is close to the endometrial refractory phase when cells become apoptotic. On the other hand, Dominguez et al. (21) reported very good results when the endometrial biopsy is performed at the time of the oocytes recovery in a group of donors. Altogether, these results suggest that endometrial cell biopsy has to be performed earlier in the luteal phase.

Finally, although the coculture process was centralized in the AECC protocol used, part of the AECC technique had to be implemented in centres (*e.g.* biopsy). As previously discussed, this was associated with some technical difficulties increasing variability of the results between experienced and non-experienced centres. This suggests that increasing centres' level of expertise by the progressive learning of the AECC technique could be sufficient to rapidly and substantially increase the pro-

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portion of successful ART. Results suggest that today's generalization of the AECC technique to all the IVF centres would not result in decreased patient outcomes. On the contrary, whereas experienced centres showed increased successful ART when using AECC (83.3% *vs.* 33.3%, p=0.001), less experienced centres did not show decreased proportions of successful ART using AECC (37.9% *vs.* 38.9% using the usual technique, p=0.91). Furthermore no detrimental effect has been observed in children born after blastocyst transfer (43,44,45,46). As a consequence, implementation of AECC may rapidly result in a progressive and significant increase in the proportion of successful ARTs (2).

Conclusion

This first multicentre study of the efficacy of AECC and transfer on day 5 compared to transfer on day 3 after culture using conventional medium showed that AECC improves quality of the embryo on day 3 and pregnancy rate per transfer. The present results also suggest that implementation of the AECC technique to a large number of IVF centres could lead to a substantial improvement in the success of ART.

Author's roles

Drs Ohl, de Mouzon, Viville, Nicollet and Menezo were members of the coordination committee in charge of the study design, protocol drafting and revising the article.
Drs Ohl, Viville and Nicollet participated in the inclusion of patients.
Dr de Mouzon was the statistician of the study in charge of the analysis.
Anissa Benoussaïdh was the clinical project manager of the study, she participated to the draft and revision of the manuscript.
Jean-Noël Gouze was the manager of the biotechnology laboratory in charge of Endocell production, he participated to the draft and revision of the manuscript.

Acknowledgements

The authors wish to thank all the patients and centres that participated in this study and anyone who contributed to the development of the project. They also wish to thank Guillaume Hébert from SC Partners for his editorial support. Protocol of the study is available upon request.

Funding

The study was funded by the Laboratoires Genévrier, Sophia-Antipolis, France.

Conflict of interest

Drs Ohl, de Mouzon, Viville, Nicollet and Menezo received honoraria for their participation in the study. JN Gouze and A Boussaïdh are employees of the Laboratoires Genévrier.

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