Human Pregnancies After Transfer of Fresh (Four- to Eight-Cell) Versus Frozen–Thawed Blastocysts Resulting from Intracytoplasmic Sperm Injection

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Purpose: The objective of this study was to obtain expanded blastocysts following intracytoplasmic sperm injection (ICSI) and Vero-cell co-culture, cryopreserve them at this stage, and transfer the frozen–thawed blastocysts to obtain pregnancies.

Methods: Twenty-two couples with severe male-factor infertility or failed fertilization in a previous in vitro fertilization cycle were included in this study. ICSI was performed for all of them, and sperm-injected oocytes were immediately subjected to Vero-cell co-culture for varying intervals. Then 14 couples were treated by embryo transfer at the four- to eight-cell stage (Group I), whereas 8 couples were treated by transfer of frozen–thawed blastocysts (Group II).

Results: Percentages of cleaved embryos and term survival rates were 57.1 and 73.3% for Group I and 50.0 and 37.5% for Group II, respectively.

Conclusions: Blastocysts obtained after ICSI and Vero-cell co-culture can retain developmental competence after cryopreservation and thawing. Transfer of frozen–thawed blastocysts derived by these means holds promise for establishment of viable pregnancies.

KEY WORDS: blastocyst; co-culture; cryopreservation; intracytoplasmic sperm injection; pregnancy.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) has provided a treatment opportunity for couples who have had marginal or poor semen characteristics because of abnormal spermatozoal functions, or failed fertilization in

their previous in vitro fertilization (IVF) cycle(s). Although different methods such as partial zona dissection, zona drilling, increasing sperm number, and SUZI have been used to overcome male infertility, ICSI has proved to be far more effective than any of these other techniques (1). However, there have been strong controversies related to using ICSI as a human therapeutic technique. The quality of the embryos obtained after ICSI could not be correlated with the previous observations gathered from regular IVF, where there was a strong negative relationship between embryo quality and abnormal sperm parameters (2–5).

Improved embryo culture conditions after IVF or ICSI have provided an opportunity to culture human preembryos up to the blastocyst stage and further. Therefore, it has been suggested that the developmental arrest taking place at the time of genomic activation could be overcome by embryo co-culture (6). Co-culture has also been proposed to be a good tool for analyzing early embryonic development. Because embryos enter the uterus on day 4 or 5 after fertilization, it has been suggested that embryo transfer (ET) should be done at the blastocyst stage (7). Thus, blastocyst transfer not only allows better synchronization between embryo and uterus but also permits the IVF team to select better-quality embryos, to prevent multiple gestation (>2), to avoid ectopic pregnancies, and to avoid the acute symptoms of ovarian hyperstimulation syndrome after ET (8,9).

Different cell culture systems such as uterine fibroblasts (10), ampullary epithelial cells (8), endometrial cells (11), granulosa cells (5), and green monkey kidney epithelial cells (7,12,13) have been used to produce human blastocysts. In our studies we used Vero cells (green monkey kidney epithelial cells) because they produce constant rates of growth factors from the beginning to the end of the coculture period (Menezo, unpublished results).

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Cryobiology techniques became an essential part of IVF programs because ovarian stimulation often leads to the production of a high number of oocytes (13). Cryopreservation at the blastocyst stage is available with substantial success (7). Freezing of supernumerary embryos has enabled reduction of high-order embryo replacement and has allowed flexibility for ET cancellation when it was necessary (14,15).

Here, we report a preliminary experiment to evaluate supernumerary embryo formation following ICSI and Vero-cell coculture and subsequent development after blastocyst cryopreservation. Transfer of these frozen-thawed blastocysts resulted in development to term.

MATERIALS AND METHODS

Twenty-two couples with a history of infertility were included in this study. Indications for ICSI in these couples included at least one failed IVF and severe oligospermia. Patients with ovarian hyperstimulation ($n = 2$) and pregnancy failure after ET ($n = 6$) were included in group II.

Controlled ovarian stimulation was achieved by human menopausal gonadotropin (hMG) (Humegon, Organon, West Orange, NJ; or Pergonal, Serono Laboratories Inc., Randolph, MA) and follicle-stimulating hormone (FSH) (Metrodin; Serono Laboratories, Randolph, MA), following pituitary down-regulation with gonadotropin-releasing hormone (GnRH) analogues (Lupron; TAP Pharmaceuticals, Chicago, IL). Human chorionic gonadotropin (hCG; 5000 or 10,000 IU; Pregnyl, Organon) was administered 36 hr before transvaginal ultrasound-guided oocyte retrieval. Aspirates were collected into a 15-ml centrifuge tube (Falcon) containing 1.0 ml of tissue culture medium (TCM) 199 (0.5 ml) + phosphate-buffered saline (PBS) (0.5 ml) + heparin (20 IU/ml). Cumulus-oocyte complexes (COCs) were washed twice and checked for the presence of the first polar body before being transferred into the culture medium (Menezo B2 + penicillin + streptomycin).

Semen samples were collected by masturbation after 2–3 days of abstinence. Each ejaculate was washed once with TCM 199, then the pellet was resuspended in 100 μ l of B2 + antibiotic (16). The individual motile sperm cells used for ICSI were isolated by a sperm injection needle (Humagen; 15-degree angle) in Earle's medium (Sigma) containing 10% polyvinylpyrrolidone (PVP; ICN Biochemicals) and 0.5% bovine serum albumin (BSA) (sperm isolation medium).

Approximately 3 hr after retrieval the cumulus and corona cells were removed by brief (90-sec) treatment with hyaluronidase (176 IU/ml; Sigma) in Earle's medium containing 0.5% BSA. The oocytes that demonstrated an extruded first polar body were used for ICSI. The micromanipulation approach used was an inverted microscope fitted with Hoffman modulation optics and a heated stage. One 10- μ l droplet of sperm isolation medium containing sperm and five 5- μ l droplets of Earle's medium with 0.5% BSA and 1 mM HEPES containing eggs were placed on a warmed plastic dish (Falcon, Catalog No. 3002) and covered with mineral oil. The holding pipette and injection needle were introduced into these droplets at 15 degrees from the horizontal, with the angle of travel of the injection needle adjusted to parallel this angle of approach to minimize egg membrane damage.

Sperm medium (1–2 μ l) was placed next to a 10- μ l drop of sperm isolation medium and allowed to mix for 5 min. Under the inverted microscope, motile spermatozoa were visualized, then immobilized by breaking the sperm tail between the slide and the injection needle. Spermatozoa were aspirated into the injection needle, then the needle was carried over the medium with an oocyte, which was positioned with its polar body at the 12-o'clock or 6-o'clock position. Confirmation of sperm placement in the ooplasm was achieved either by stirring the ooplasm with the needle tip coupled with aspiration of ooplasm into the needle lumen or by repeated stabbing. Each egg was exposed to periods of less than 10 min on the micromanipulation microscope (4). The injected oocytes were washed three times with B2 and placed in four-well plates (NUNC, Kamstrup, Denmark) containing 1 ml of B2 plus 10% fetal bovine serum (FBS; Sigma) with Vero-cell co-culture.

Fertilization of the oocytes was assessed for the presence of two pronuclei at 16 to 18 hr, and for cleavage on the second day after injection. The Vero-cell co-culture system has been described previously (6).

Two or three good-quality embryos obtained after ICSI were transferred after 48 or 72 hr in 14 patients. The embryos for blastocyst transfer were co-cultured up to day 5, 6, or 7 until expanded blastocysts were formed. The blastocysts were cryopreserved with a slow freezing procedure reported previously (13,17). After thawing, the cryoprotectants were removed in two steps (12). Blastocysts were transferred to B2 plus 10% FBS with Vero-cell co-culture for 4–5 hr before ET. The endometrium was artificially synchronized using a protocol with oral micronized estradiol and

intramuscular progesterone (7). Pregnancy was confirmed by both beta hCG and ultrasound determinations.

Data were analyzed by use of the SigmaStat (Jandel Scientific, San Rafael, CA) software package. One-way repeated-measures analysis of variance was applied to assess statistical differences. The Bonferroni *t* test was used to determine differences among the groups. Differences of *P* less than 0.05 were considered significant.

RESULTS

Overall results of blastocyst development after ICSI are summarized in Table I. A total of 283 oocytes was injected after 3 hr of culture. Of these, 64.3% had two pronuclei (2 PN) 16–18 hr after ICSI, and 135 (74.2%) reached the eight-cellstage on day 3. After transfer of 59, 76 spare embryos were cultured with Vero cells for another 2–4 days. A total of 52 (68.4%) blastocysts was obtained and frozen. Thirty-two blastocysts (in Group II) were thawed and 28 (87.7%) were transferred, resulting in 10 pregnancies (45.4% of 22 patients overall).

Patient, oocyte, fertilization, and blastocyst formation data are summarized in Table II (four- to eight-cell-stage embryo transfer; frozen-thawed embryo transfer). Patient age and mean numbers of oocytes and injected oocytes were not different between the two groups. However, mean numbers of 2 PN and

Table II. Comparative Results of Embryo Development After Early Embryo Transfer (4–8 Cell) vs Transfer of Frozen–Thawed Blastocysts

	4- to 8-cell-stage embryo transfer	Frozen-thawed blastocyst transfer
No. of patients	14	8
No. of cycles	16	9
Age of female	29.3 ± 3.9	31.9 ± 2.8
No. of oocytes collected	261	167
Mean ± SD	16.3 ± 6.1	18.6 ± 6.7
Total No. of oocytes injected	163	120
Mean ± SD	10.2 ± 5.7	13.3 ± 4.6
Total No. of 2 PN	93	88
Mean ± SD	2.6 ± 1.8	11.0 ± 4.1
Fertilization rate (%)	57.1	73.3
Mean No. of embryos transferred on day 3	2.8 ± 1.2	0
Total No. of spare embryos	28	58
Mean ± SD	1.7 ± 1.9	6.4 ± 4.4
Total No. of blastocysts on days 5–7 (%)	20 (71.4)	42 (72.4)
Mean ± SD	1.2 ± 1.8	4.6 ± 3.4
Total number thawed	0	32
Total number survived (%)	0	28 (87.5)
Mean No. of frozen-thawed embryos transferred	0	2.2 ± 0.4
Pregnancies (% pregnancy rate per patient)	7 (50.0)	3 (37.5)
Ongoing pregnancies	3	—
Delivered	4	3

Table I. Overall Results After Intracytoplasmic Sperm Injection (ICSI)

No. of patients	22
No. of cycles	25
Age of female	30.2 ± 3.8
No. of oocytes collected	428
Mean ± SD	17.1 ± 6.3
Total No. of oocytes injected	283
Mean ± SD	11.3 ± 5.5
Total No. of 2 PN	182
Mean ± SD	7.2 ± 5.2
Fertilization rate (%)	64.3
Total No. of embryos on day of transfer	135
Mean ± SD	5.4 ± 3.9
Total No. of embryos transferred on day 3	59
Mean ± SD	2.24 ± 1.3
Total No. of spare embryos	76
Mean ± SD	3.04 ± 3.5
Total No. of blastocysts on days 5–7 (%)	52 (68.4)
Mean ± SD	2.08 ± 3.5
Pregnancies (% pregnancy rate per 22 patients)	10(45.4)
Ongoing pregnancies	5
Delivered	5

spare embryos and blastocysts obtained on days 5, 6, and 7 demonstrated significant differences between the two groups (*P* < 0.05). Although the data are not shown in Table II, most of the blastocysts were obtained on days 5 and 6, then frozen. The survival rate of frozen-thawed blastocysts was 87.5% (28 of 32). Comparison of pregnancy rates per patient or cycle revealed no differences (*P* = 0.05) between the two groups.

DISCUSSION

Successful pregnancies after transfer of frozen and thawed blastocysts obtained from ICSI are reported. It was already clear that high fertilization rates can be obtained by ICSI. There are still many factors and determinants of preimplantation development and implantation in practice where the clinician cannot readily distinguish the embryonic (paternal and maternal), the uterine, and the luteal (ovarian) influences, knowing that these effectors may be interrelated. Therefore, pregnancy rates per transfer do not necessarily reflect implantation potential (18). Recently,

some concerns have been raised regarding the quality of the embryos obtained by ICSI and the possibility of hidden genetic problems (18,20–22). Moreover, based on results obtained from regular IVF, a negative relationship was observed between abnormal sperm parameters and embryo quality (23–25); it was found that poor sperm correspond to poor embryo formation with poor developmental potential. This was confirmed in the blastocyst formation rates in vitro (2).

The interval between oocyte maturation and fertilization seems to be of critical importance. It is now well documented that embryos derived from reinsemination of oocytes, after fertilization failure in male-factor patients, have hardly any developmental potential. Edwards *et al.* (26) suggested that only competent embryos would reach the blastocyst stage. Nevertheless, in our trial blastocysts were obtained in both the group with severe male-factor infertility and the group with a failed IVF cycle.

There is an unexpectedly high proportion of blastocyst formation after ICSI. Direct injection prevents any delay in sperm penetration and shortens the fertilization process. The gained time may help to overcome developmental delays related to poor sperm quality. In cases of DNA-related defects [i.e., deletions in a coding region or anomaly in condensation (22)] or major epigenetic problems, ICSI is of no benefit, as the embryonic developmental problem remains present.

This preliminary study shows that blastocysts developing after ICSI can be cryopreserved with relatively good success. The sequence of these events does not seem to impair embryo viability. The methodology described combines the benefits of ICSI, co-culture, and cryopreservation at the blastocyst stage. Further studies are needed (a) to compare results obtained from transfers of cryopreserved early- and blastocyst-stage embryos produced following ICSI and (b) to evaluate if improved success rates with ICSI are due to a lack of delay in egg fertilization, thus avoiding the loss of maternal RNA that seems to occur during fertilization with low-quality sperm.

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