Donor Oocyte Cytoplasmic Transfer Did Not Enhance Implantation of Embryos of Women with Poor Ovarian Reserve

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Purpose: To determine whether donor oocyte cytoplasm transferred into the oocytes of women ≥ 40 years or with diminished ovarian reserve would enhance embryo quality, implantation, or pregnancy rates.

Methods: Study subjects included women ≥ 40 years (15) or with abnormal FSH levels (3). Healthy volunteers (18) produced oocytes for cryopreservation. Donor oocytes were thawed and cytoplasm from surviving oocytes was injected with a single sperm into the cytoplasm of recipient oocytes. Outcome measures included embryo quality scores, implantation, and pregnancy rates.

Results: Eighteen donors produced 213 oocytes for cryopreservation and 39/171 (22.8%) survived thawing. Eighteen recipients initiated 25 IVF cycles with embryo transfer in 20 cycles after cytoplasmic transfer (CT). Four cycles resulted in three biochemical losses and one aneuploid clinical loss. Embryo quality did not improve with CT compared to pre-CT IVF cycles in six recipients.

Conclusions: CT with cryopreserved donor oocyte cytoplasm did not enhance success in women with advanced reproductive age or low ovarian reserve.

KEY WORDS: Age factors; cryopreservation; cytoplasmic transfer; in vitro fertilization; ovarian reserve.

INTRODUCTION

Ovarian reserve, a term applied to the potential for successful live births, declines relentlessly as a woman ages (1,2). Unfortunately, diminished ovarian reserve affects an increasing number of women attempting pregnancy. Many of the mechanisms for this decline are poorly understood. Many researchers believe that aneuploid oocytes lead to embryos doomed to reproductive failure (3). Currently, effective treatments to correct this natural aging process do not exist.

While oocyte donation is a highly effective method for achieving viable pregnancies, its use requires that the recipient couple abandon their dreams of having a

genetically related child. Many couples choose oocyte donation, given the opportunity; however, financial and ethical issues prevent some from using this treatment option. An alternative treatment that would allow the recipient couple to use their own genetic material would be highly desirable.

Recent limited success with donor oocyte cytoplasmic transfer (CT) into the cytoplasm of recipient oocytes offered an opportunity to boost the implantation rate in patients who were thought to have normal ovarian reserve but poor embryo quality (4,5). This technique was based on research into the role of cytoplasmic factors that might influence oocyte maturation and activation (6–9). We hypothesized that while aneuploidy may be primarily responsible for decreased ovarian reserve of aging oocytes, the precise age at which cytoplasmic factors cease to be important is unknown. Thus, CT from healthy young oocytes

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may benefit some of these patients. We, therefore, initiated a clinical trial to determine whether women over 39 years or with abnormal ovarian reserve tests would benefit from CT.

MATERIALS AND METHODS

Patients

Recipient women had presumed diminished ovarian reserve either because they were 40 years or older or because they had elevated basal or clomiphene-induced FSH levels. Oocyte donors were screened for inclusion in a clinical oocyte donor program and were presumed to have normal fecundity. This study received Institutional Review Board approval. Both recipient women and oocyte donors signed an informed consent document.

Recipient and Donor Cycles

Recipients underwent standard GnRH suppression using a short-term or flare protocol and gonadotropin stimulation resulting in ultrasound-guided oocyte retrieval. The donors used oral contraceptives and GnRH suppression, followed by standard gonadotropin stimulation and ultrasound-guided oocyte retrieval. Donor oocytes were retrieved and cryopreserved prior to the recipient's cycle in order to avoid difficulties associated with synchronizing recipient and donor cycles. Since each recipient oocyte was scheduled for micromanipulation with CT of donor ooplasm, intracytoplasmic sperm injection (ICSI) was used to inseminate the oocytes regardless of sperm quality.

Oocyte Cryopreservation

After retrieval, the donor oocytes were placed in 1 mL of Hepes buffered earles (HBE) supplemented with 0.5% bovine serum albumin (BSA) Fraction V (Sigma-Aldrich, St. Louis, MO) plus 80 IU/mL hyaluronidase (Sigma-Aldrich, St. Louis, MO). The corona and cumulus cells were removed using a finely drawn pipette made in the laboratory. The capillary tube pipettes (Fisher Scientific, Fair Lawn, NJ) were pulled on a vertical puller model 730 (David Kopf Instruments, Tujunga, CA), fire-polished on a microforge MF-1 (Technical Products International, St. Louis, MO), connected to a 20- μ L Unopette reservoir (Beckton Dickinson, Franklin Lakes, NJ). The oocytes were then rinsed twice in HBE supplemented with 0.5% BSA. The oocytes were immediately

assessed for maturity and cryopreserved for future cytoplasmic transfer procedures.

The oocytes were quickly washed twice in Dulbecco phosphate buffered saline (DPBS; GIBCO Laboratories, Grand Island, NY) to remove traces of culture medium. Oocytes were then transferred into DPBS and 1.5 M 1,2-propanediol (PROH; Fisher Scientific, Fair Lawn, NJ) for 15 min. Oocytes were then transferred into cryomedia consisting of DPBS, 1.5 M PROH, 20% FBS (Fetal Bovine Serum, Gibco Laboratories, Grand Island, NY), and 0.1 M sucrose (Fisher Scientific, Fairlawn, NJ) for 15 min.

During the 15-min equilibration in cryomedia, individual oocytes were loaded into 0.25-mL aseptic straws, placed into a programmable rate freezer and held at 20°C until the completion of the 15-min period. The oocytes were cooled to -7°C at the rate of 2°C/min, seeded manually, and held at -7°C for 5 min. The oocytes were then cooled to -30°C at the rate of 0.3°C/min, held 1 min, and cooled to -140°C at the rate of 50°C/min. After reaching -140°C, the oocytes were plunged and stored in liquid nitrogen.

Oocyte Thawing

Straws were removed from the liquid nitrogen and allowed to thaw at room temperature for 40–60 s until all visible ice crystals disappeared. The oocytes were removed from the straw by cutting off the heat-sealed portion of the straw and pushing on the cotton plug with a stylet into a four-compartment petri dish. The oocytes were subsequently transferred successively into 50 μ L droplets of DPBS containing 1.0 M PROH and 0.2 M sucrose, 0.5 M PROH and 0.2 M sucrose, and 0.2 M sucrose at 5-min intervals. Each thaw medium was supplemented with 20% FBS. Each surviving oocyte was then cultured in human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA) supplemented with 10% FBS until used for CT.

Cytoplasmic Transfer

Approximately 4–5 h after egg retrieval, a dish was prepared for micromanipulation. The dish had eight 5 μ L droplets of HBE supplemented with 0.5% BSA encircling a 5- μ L droplet of 10% PVP (Sigma-Aldrich, St. Louis, MO) under preequilibrated mineral oil (Sigma-Aldrich, St. Louis, MO). The thawed donor's oocytes and the recipient's oocytes were placed in HBE droplets. A 1- μ L sample of sperm suspension was placed in a center droplet of 10% PVP to dampen sperm movement.

Micromanipulation was performed using a Nikon Diaphot 200 inverted microscope (Nikon Co., Tokyo, Japan) fitted with Hoffman optics and a Narishige IM6 injector (Narishige Co., Ltd., Tokyo, Japan). The holding pipettes and injection pipettes were made in the laboratory using glass pipettes (Drummond Scientific, Broomall, PA) and pulled on a horizontal puller (Sutter Instrument Company, Novato, CA). The holding pipettes were fire-polished and bent on the microforge. The injection pipettes were ground on a Narishige EG-4 micropipette grinder (Narishige Co., Ltd., Tokyo, Japan), spiked, and bent on the microforge.

A motile sperm cell was selected for injection and immobilized. Once immobilized, the sperm cell was placed tail first into the tip of the injection pipette. The pipette was then moved to the droplet containing the thawed donor oocyte. The polar body was positioned at the 12 or 6 o'clock position and the injection pipette was inserted at the 3 o'clock position. The ooplasm was aspirated to a distance of about 500–1000 μm in the pipette. Typically, a donor's oocyte was used for one to four of the recipient's oocytes, depending on the oocyte quality throughout the procedure. The pipette was then immediately moved to the droplet containing the recipient's oocyte. The polar body of the recipient oocyte was positioned at the 12 or 6 o'clock position using the holding pipette. The injection pipette was inserted at the 3 o'clock position. Once the membrane of the recipient's oocyte was pierced, the contents of the pipette (donor's ooplasm and sperm cell) were deposited into the patient's oocyte. After injection, the oocytes were graded and rinsed in 1 mL HBE supplemented with 0.5% BSA. The oocytes were then returned to fresh 20- μL droplets of Menezo B2 (SAGE Biopharm, Natick, MA) under preequilibrated oil. The recipient's oocytes were assessed for fertilization 16 h after injection.

Embryo Transfer

Two days after oocyte retrieval, the embryos were graded and assessed for cleavage. Embryo morphology scores were based on the symmetry and shape of individual blastomeres and the degree of fragmentation (10). The morphology scores ranged from 5 (best) to 1 (worst). After consulting with a physician, the patient determined the number of embryos transferred. The best embryos were transferred using a Wallace catheter (Marlow/Cooper Surgical, Shelton, CT). Pregnancy was defined as

a rising hCG level performed 13 days after oocyte retrieval.

Statistics

Statistical analysis was performed using SPSS (SPSS, Chicago, IL, Version 10). A paired *t* test was used to compare the cycle results of non-CT cycles to the cycle results obtained with CT. A *p* value of <0.05 was considered significant.

RESULTS

Recipient Cycles

Eighteen women initiated 25 CT cycles between October 1997 and July 1998. Fifteen women were ≥ 40 years old while three women were <40 years old but had abnormal ovarian reserve as judged by elevated FSH levels (≥ 12 mIU/mL). A summary of the cycle characteristics is provided in Table I. All 25 cycles progressed to retrieval. Two cycles failed to yield a viable mature oocyte. In three other cycles, the one or two mature oocytes failed to fertilize. Twenty cycles progressed to embryo transfer.

Oocyte Cryopreservation

Eighteen donors produced 213 eggs for cryopreservation. The number of oocytes retrieved ranged from 3 to 21 per donor. The oocytes were cryopreserved as mature with a polar body (177), mature without a polar body (23), and immature with a germinal vesicle (13).

Oocyte Thaw

The same personnel thawed 171 oocytes for use in CT. Thirty-nine oocytes (22.8%) survived thawing and they were used for the CT procedure. Individual

Table I. Summary of all 25 Initiated CT Cycles

	CT IVF ^a	Min	Max
Age	42.6 $\pm$ 3.9	35	49
Days stimulated	11.0 $\pm$ 2.5	7	17
Ampules FSH	66.5 $\pm$ 1.6	26	154
Peak E2 (pg/mL)	2099 $\pm$ 1241	370	4736
Total eggs	4.5 $\pm$ 3.0	0	11
Mature eggs	3.7 $\pm$ 2.6	0	11
Embryos	1.8 $\pm$ 1.9	0	7
Embryos per transfer	2.3 $\pm$ 1.8	1	7
Day 2 embryo blastomeres	3.5 $\pm$ 1.1	1	6
Day 2 embryo scores	3.4 $\pm$ 1.0	1	5

^a Data shown as mean $\pm$ SD.

Table II. Comparison of Non-CT and CT Cycles in Six Women

	Non-CT IVF Cycle ^a	CT IVF Cycle	<i>p</i>
Age	41.7 ± 2.4	43.1 ± 3.6	.02
Days stimulated	10.6 ± 2.2	10.9 ± 2.0	ns
Ampules FSH	53.3 ± 1.8	69.9 ± 1.8	ns
Peak E2 (pg/mL)	2326 ± 1306	2442 ± 1392	ns
Total eggs	7.4 ± 4.5	6.0 ± 4.0	ns
Mature eggs	5.8 ± 3.7	5.3 ± 3.2	ns
Embryos	3.8 ± 2.8	2.6 ± 2.2	ns
Embryos per transfer	3.2 ± 1.6	2.9 ± 2.1	ns
Day 2 embryo blastomeres	3.0 ± 1.3	3.5 ± 1.1	ns
Day 2 embryo scores	3.4 ± 1.6	3.2 ± 0.8	ns

^aData shown as mean ± SD.

donors demonstrated significant variability in oocyte survival ($p < 0.0001$). The survival rates per donor ranged from 0 to 83.3%. Six donors had only atretic oocytes after thaw (2, 3, 6, 6, 7, 11 oocytes each). The survival rate for the other 12 donors were 1–25% (7 donors), 26–50% (3 donors), 51–75% (1 donor) and 75–100% (1 donor).

Outcomes

Six women, who had CT embryos transferred, completed 16 IVF cycles at our institution prior to enrolling in the CT protocol. The cycle characteristics of those pre-CT cycles with their subsequent CT cycles are listed in Table II. The cycles were statistically similar in all respects. The embryos scores from the CT cycles were similar to the embryo scores from the pre-CT cycles.

Five cycles (25%) demonstrated an elevated hCG level. Four pregnancies were biochemical and in three cases the single elevated hCG rapidly declined to an undetectable level. A total of 45 embryos were transferred. One embryo implanted (2.2%) and progressed to a clinical pregnancy. This sole clinical pregnancy progressed to 8 weeks of gestation with visualization of embryonic heart motion. The pregnancy failed and karyotypic analysis of the products of conception revealed a 47, XY, +15 chromosome complement.

One woman, who failed to conceive from the CT embryos, subsequently underwent a standard IVF cycle and delivered healthy twins. Three other women conceived with the use of donor oocytes.

DISCUSSION

Reproductive aging leads to a progressive decline in fecundity culminating in sterility at menopause.

Clearly, from clinical (11,12) and basic (13) research, the oocyte from aging women is more likely to have an aneuploid chromosome complement. Resulting pregnancies generally fail to implant (lower cycle fecundity), or fail to develop (spontaneous abortion), and occasionally aneuploid pregnancies survive (i.e., Down's syndrome).

It is possible that there are other nonchromosomal factors that contribute to the progressive loss of fecundity (13). The role of cytoplasmic factors in the process of meiosis is complex and incompletely understood, but clearly important (7,9,15). The injected cytoplasm contains mitochondria that may provide a more normal milieu for early embryo development, mRNA that may enhance the recipient's pool, or other organelles or factors that may improve the oocyte such as the spindle apparatus (6,8,9,16). While transfer of donor cytoplasm is experimental, the few documented human pregnancies resulting from its use have been normal (4,5).

Despite clear evidence that much of the pregnancy wastage and lower fecundity associated with female aging is related to aneuploidy, the mechanism for failure in other patients remains unclear. Given the complex interactions between the cytoplasm and the nucleus, we felt that CT might benefit some women with low ovarian reserve. Unfortunately, cryopreserved donor cytoplasmic transfer into the oocytes of women with reduced fecundity failed to correct the factors responsible for poor implantation. Our experience of marginal biochemical pregnancies and a single clinical pregnancy, which proven to be aneuploid, led us to discontinue the protocol.

We conclude that CT using cytoplasm from cryopreserved oocytes does not improve fecundity in women with low ovarian reserve. The limited success of CT in younger women with poor quality embryos may still be a valuable adjunct for this select group of patients. Future cell biology insights or use of fresh donor oocytes may provide additional opportunities for this technology. In the meantime, we believe this treatment should remain a clinical investigative technique.

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