PHYSIOLOGY

Evaluation of the Meiotic Spindle Apparatus in Metaphase II Human Oocytes Following Cytoplasmic Donation¹

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Purpose: To determine if the removal of cytoplasm from metaphase II human donor oocytes damages the meiotic spindle apparatus.

Materials and Methods: Cryopreservation of metaphase II human oocytes was performed using a fast-freeze, fast-thaw protocol. Upon thaw, oocytes were incubated for 3–4 h and then used for cytoplasmic donation (test oocytes). Oocytes thawed but not used for donation served as controls. Test and control oocytes were fixed using a microtubule-stabilizing buffer. Tubulin was localized using antitubulin monoclonal antibody. Chromosomes were identified by counterstaining with DAPI.

Results: Forty-four oocytes had cytoplasm removed (test group) while 12 were not used for the procedure (controls). Twenty-three oocytes survived the donation procedure. Rates of normal spindle structure for the control and test groups were 21/23 (91.3%) and 12/12 (100%), respectively.

Conclusion: The removal of cytoplasm from a metaphase II human donor oocyte does not appear to significantly in-

crease the damage to chromosome alignment or to the spindle structure.

KEY WORDS: Cytoplasmic donation; human; meiotic spindle apparatus; metaphase II oocyte.

INTRODUCTION

Cytoplasmic transfer has recently become an alternate method as treatment for the restoration of normal developmental potential in oocytes with ooplasm deficiencies. Such deficiencies may be a factor in a woman's inability to conceive (1). Because this procedure is relatively new, little is known about the effect of removing cytoplasm on the ultrastructures of the oocyte, namely the meiotic spindle apparatus.

At ovulation, mammalian oocytes are arrested at metaphase of the second meiotic division, metaphase II, with the meiotic spindle apparatus composed of microtubules with maternal chromosomes attached. These microtubules are responsible for the separation of the sister chromatids during the second meiotic division after fertilization (2). In addition, microtubules play important roles in the postfertilization events of spindle rotation, polar body formation, pronuclear migration, and cytokinesis (3–5).

There has been some debate as to whether various forms of manipulation damage the microtubules of the meiotic spindle. Exposure of oocytes to adverse conditions, such as cryoprotectants and low temperatures, has been shown to disrupt the microtubules

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(2,6,7). However, a recent study conducted in mice by Eroglu and colleagues reported that cryopreservation does not impair the function of cytoplasmic microtubules in postfertilization events, provided a brief incubation period follows the thaw process (8). Similarly, Baka and coworkers reported that cryopreservation of prophase I human oocytes did not significantly increase abnormalities in the meiotic spindle (9). In the hamster oocyte, Asada *et al.* demonstrated that intracytoplasmic sperm injection need not result in significant damage to the meiotic spindle as long as the polar body was oriented away from the injection site (10).

There have been several reports in the literature that suggest that the transfer of certain cytoplasmic factors from healthy, good quality oocytes may influence or control various genetic, maturational, and developmental properties when donated to another. In a study performed in monkeys, Flood *et al.* found that the developmental potential of oocytes matured in vitro did increase by injecting them with cytoplasm of oocytes matured in vivo. In this same experiment, the developmental potential of the metaphase II oocytes, from which the cytoplasm was removed, was not compromised either, suggesting that structures required for proper development were not significantly altered by the procedure (11). Moreover, Cohen *et al.* reported the first human pregnancies following the transfer of cytoplasm from donor eggs to recipient eggs (12). In this instance, the genetic link to the mother is retained while the healthy cytoplasmic factors from the donor egg are transferred to the recipient oocyte with the intent to produce embryos of better quality. The researchers also state that the donor eggs were examined for intact metaphase plates, but their findings were not reported.

The aim of this study was to examine such "cytoplasmic donor" oocytes to determine if the removal of cytoplasm from a metaphase II human oocyte has an impact on the meiotic spindle apparatus. The meiotic spindle has been shown to be extremely important in the maturational changes of the oocyte to become haploid and also instrumental in events following fertilization. In the event that these metaphase II oocytes retain normal meiotic structures, they could possibly become yet another source of oocytes for routine donor egg programs from which the infertile female could achieve pregnancy. This additional oocyte source would eliminate the need for a subsequent donor cycle in the event cytoplasmic transfer does not produce a pregnancy. Of equal importance, the

evaluation of meiotic spindle damage may provide information on the risk of the inadvertent transfer of the chromosomal material from the donor oocyte to the recipient egg.

MATERIALS AND METHODS

Oocyte Donors

Cytoplasm was removed from cryopreserved Metaphase II eggs aspirated from donors who consented to the use of their oocytes in our Egg Donation Program. To qualify for the egg donation program, donors must be <33 years old; pass a physical and psychological examination; have good ovarian reserve; no evidence of HIV, hepatitis, or venereal diseases; and not be a heavy smoker.

Oocyte Cryopreservation

Cryopreservation of donor oocytes was performed using a fast-freeze, fast-thaw protocol. Egg freezing was performed in a programmable freezing machine (Planer Series II or III, Kryo 10, T S Scientific). Following aspiration, oocytes were allowed to incubate under standard culture conditions for 3–4 h. Cumulus and coronal cells were removed with hyaluronidase, using the standard ICSI procedure (13). Oocytes were cryopreserved individually in Nunc cryovials with 0.3 mL of 1.5 M propanediol with 0.2 M sucrose. Briefly, oocytes were placed in stepwise nonsucrose dilutions (0.0 M, 0.5 M, 1.0 M, 1.5 M) of 1,2-propanediol (PrOH) in modified Dulbecco's phosphate buffered saline supplemented with 0.3% bovine serum albumin for 10 min each (PBS, GIBCO Laboratories). Oocytes were transferred to a solution of 1.5 M PrOH + sucrose for 15 min. During this time period, oocyte integrity (reexpansion after initial shrinkage) was recorded. After the 15 min were over, oocytes were transferred to vials and cooled to -7°C at $-2^{\circ}\text{C}/\text{min}$. The cryovials were held at this temperature for 15 min during the manual seed, cooled to -30°C at a rate of $-0.3^{\circ}\text{C}/\text{min}$, plunged, and stored in liquid nitrogen until thawed. Thawing was achieved by gentle agitation of the vials in a 31°C water bath for 1 min. The cryoprotectant was removed by stepwise dilutions with sucrose (1.0 M, 0.5 M, 0.0 M PrOH) for 15 min each. Surviving oocytes were transferred to culture medium and incubated for 3–4 h before being used for cytoplasmic transfer.

Cytoplasmic Transfer, Sperm Injection, and Embryo Culture

Sperm preparation, oocyte identification, and oocyte embryo culture were performed as previously described (14). Gamete preparation and sperm injection for the cytoplasmic transfer procedure were performed as previously described (15). A single sperm was immobilized and drawn into the ICSI pipet. Briefly, this pipet was moved into a drop of medium containing the donor egg. While the egg was held in place with the holding pipet, the ICSI pipet was inserted into the donor egg in a region away from the first polar body to reduce contact with the meiotic spindle. Cytoplasm was drawn into the ICSI pipet to fill about 500 μm , as described by Cohen *et al.* (12). The ICSI pipet was moved to a drop of medium containing the recipient's egg and inserted into the egg, also in a region away from the first polar body. The donor cytoplasm was released into the egg along with the sperm. Injected oocytes were washed in fresh medium, transferred to dishes for incubation, and evaluated 1–2 h later for signs of damage and/or degeneration. Each donor oocyte was used for two recipient oocytes unless it degenerated following the initial removal of cytoplasm. Degenerate oocytes had dark, granular cytoplasm and/or ruptured oolemma.

Following the procedure, recipient oocytes were evaluated using the standard in vitro fertilization protocols ongoing in our program.

Fixation and Immunocytochemical Labeling

Following the cytoplasmic transfer procedure, all discarded donor oocytes, both test and controls, were stained as previously described (9). Oocytes were fixed for 20 min at 37°C in a microtubule stabilizing buffer (0.1 M Pipes pH 7, 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 2.5 mM ethylene glycol-bis (Beta-aminoethyl ether) *N, N, N', N'*-tetraacetic acid (EGTA) containing 2.0% formalin, 0.5% Triton X-100, 1 μM taxol). Subsequently, oocytes were washed three times in blocking buffer of phosphate buffered saline (PBS) with 2.0% bovine serum albumin (BSA), 2.0% powdered milk, 2.0% normal goat serum, 0.1 M glycine, and 0.01% Triton X-100, where they can be stored at 4°C for up to three days. To visualize the meiotic spindle, tubulin was localized by incubation in antitubulin monoclonal antibody (Amersham Corporation, Arlington Heights, IL) for 1 h at 37°C at a concentration of 1:1000 in PBS containing 0.1% BSA and 0.02% sodium azide. Oocytes were then washed for 1 h at room tempera-

ture in blocking buffer and further incubated in a 1:50 solution of fluorescein-conjugated goat antimouse immunoglobulin G (IgG; Amersham Corporation, Arlington Heights, IL) for 1 h at room temperature. Next, oocytes were washed three times in PBS containing sodium azide. To identify the chromosomes, a counterstain with 4'6'-diamidino-2-phenylindole (DAPI) (10 $\mu\text{g/mL}$; Molecular Probes; Eugene, OR) was performed for 30 min at room temperature. After washing with PBS, oocytes were mounted on slides in 50% glycerol in PBS containing 25 mg/mL sodium azide as an antifading reagent and were examined and photographed using a Nikon epifluorescent microscope.

Normal oocytes were those containing a distinct meiotic spindle showing an oblong structure. Chromosome morphology was considered normal if they were located exclusively on the metaphase plate. Abnormal structures included abnormally shaped spindles, partial or complete disorganization of the spindles, and dispersion of the chromosomes from the metaphase plate.

Statistical Analysis

Comparisons were performed between the test and control groups with regard to rates of meiotic spindle damage and chromosome alignment, using chi-square or Fisher's analysis.

RESULTS

A total of 105 oocytes, obtained from 11 donors, were thawed to serve as cytoplasmic donor eggs. Fifty-six (53.3%) of the oocytes survived the thaw process. Forty-four oocytes had cytoplasm removed for donation while 12 served as controls (were not used for the procedure). Twenty-three oocytes (52.3%) survived the cytoplasmic donation procedure and were evaluated for meiotic spindle morphology. Of the 21 oocytes that degenerated, 11 (50%) had cytoplasm removed for donation twice.

Evaluation of the data found that 21 of 23 (91.3%) test oocytes had intact normal meiotic spindle and chromosome alignment. Two (8.7%) demonstrated total dispersion of the chromosomes from the metaphase plate and complete disorganization of the spindles. One hundred percent (12/12) of the control oocytes demonstrated normal meiotic spindle apparatus. No significant difference was detected between rates of normal morphology between the two groups ($p = .53$).

DISCUSSION

Infertility can occur for a variety of clinical reasons. According to a study by Toner and coworkers, advanced female age significantly reduces one's chances of achieving a pregnancy following assisted reproduction (16). This problem can be minimized through the use of donor eggs. SART data has revealed that high pregnancy rates can be achieved with older patients using young oocyte donors, establishing that the decrease in pregnancy rates of older patients having IVF results from advanced oocyte age rather than advanced uterine age (17). In addition, some patients produce embryos of poor cleavage and morphological quality and previous studies have demonstrated that poor quality embryos have poor implantation and pregnancy rates (18–20).

Donor egg programs have been very successful in overcoming these two problems encountered by assisted reproduction patients. However, this method does not overcome the lack of genetic link to the mother, which is important to some seeking infertility treatment. Recently, the first human pregnancies following cytoplasmic transfer were reported (12). Lanzendorf and coworkers have also reported a pregnancy in which a patient with several failed IVF cycles, due to poor oocyte and embryo quality, achieved pregnancy after transfer of healthy cytoplasm from cryopreserved donor oocytes (15). Therefore, cytoplasmic transfer could be another viable option for those with advanced maternal age and/or poor embryo quality to achieve pregnancy while retaining the genetic heritage of the mother as well as the father.

In a study performed in monkeys, a pregnancy rate of 18% from metaphase II oocytes transferred after partial removal of ooplasm was reported (11). This result was not significantly different from the prophase I recipient oocytes that received the transfer of "healthy cytoplasmic factors" from those same metaphase II oocytes. The live birth rate per oocyte transferred was also not statistically different between the two groups. This study demonstrates that the ooplasm depleted monkey metaphase II oocytes were not severely compromised of developmental potential.

A normal second meiotic spindle is essential for the formation of euploid oocytes. It is known that normal spindle microtubules are essential for proper alignment and segregation of chromosomes. These microtubules are also important in many key events after fertilization. Results from the current study have shed light on the exact impact of cytoplasm removal on

the meiotic spindle apparatus. Results from this study yield evidence that in the human metaphase II oocyte, removal of cytoplasm need not damage this spindle apparatus allowing it to become another favorable source of donor oocytes. Similarly, these results may lead to another viable source for the recipient in a donor egg program to achieve pregnancy without having to go through another donor egg cycle. The oocytes used in the cytoplasmic transfer procedure could ultimately be inseminated for the patient and either utilized for embryo transfer or cryopreserved.

In addition, this study may provide information on the risk of the inadvertent transfer of nuclear material from the donor oocyte to the recipient oocyte, which is a topic of current debate. Recently, Brenner and coworkers (21) reported that the practice of transferring donor cytoplasm to a recipient egg could result in a mitochondrial population derived from the donor that remains after the procedure and may be replicated during fetal growth along with the mitochondria of the recipient. These authors concluded, however, that the minimal amount of mtDNA heteroplasmy determined by their study is not harmful since it spontaneously arises in normal populations. However, the American Association for the Advancement of Science (AAAS) has called for a "total moratorium" on human germ line gene therapy to determine its safety and medical ethics (22). The AAAS categorizes the cytoplasmic transfer procedure as a form of gene therapy and concludes that its practice should be ended. Therefore, studies like the present one could provide insight to a resolution over this controversial issue.

Based on the results of the current study, the policy of our program, to use each donor oocyte twice for cytoplasmic transfer, may need to be altered in order for these oocytes to be considered for insemination. Fifty percent of the oocytes that degenerated from the procedure had the cytoplasm removed twice. Also, of the two test samples that demonstrated abnormal spindle morphology, both had donated ooplasm twice. Therefore, it may be more advantageous to use an oocyte once for cytoplasmic donation prior to its insemination.

The fact that the oocytes were previously frozen did not appear to have an impact on the results of this study. Despite the lack of nonfrozen controls, the meiotic spindles of the control oocytes in this study showed normal morphology in all instances. This is supported by studies in both humans and mice in which the cryopreservation process was found not to have a significant effect on the morphology of the meiotic spindle apparatus (8,10,19).

In conclusion, the cytoplasmic transfer procedure may not adversely affect the meiotic spindle of oocytes, provided they are used only once to provide cytoplasm. Also, the risk of transfer of chromosomal material from donor egg to recipient seems to be minimal. Based on these results, the oocytes surviving the donation of cytoplasm could be available to the infertile patient undergoing cytoplasmic transfer as a "backup" donor egg cycle concurrently to her cytoplasmic transfer cycle.

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