

## Ooplasmic transfer in mature human oocytes

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**Ooplasmic transplantation aimed at restoring normal growth in developmentally compromised oocytes and embryos was evaluated in seven couples (eight cycles) with multiple implantation failures. Two approaches were investigated to transfer ooplasm from donor eggs at metaphase II (MII) stage into patient MII eggs: (i) electrofusion of a ooplasmic donor fragment into each patient egg (three cycles), and (ii) direct injection of a small amount of ooplasm from a donor egg into each patient egg (five cycles). Some donor eggs were used multiple times. Donor eggs were divided into two groups, one being used for ooplasmic extraction and the other one for egg donation. Cleaved embryos resulting from the latter were cryopreserved, where numbers and satisfactory development permitted. A second control group consisted of embryos derived from patient eggs after intracytoplasmic sperm injection without ooplasmic transfer. This was performed when sufficient number of eggs were available ( $n = 5$ ). Donor eggs ( $n = 40$ ) were evaluated cytogenetically after micromanipulation in order to confirm the presence of chromosomes. One egg was anuclear and the recipient embryos were not transferred. Normal fertilization was significantly higher after injection of ooplasm (63%) in comparison with fusion (23%). Pronuclear anomalies appeared enhanced after fusion with ooplasts. Embryo morphology was not improved in the three cycles with electrofusion and patients did not become pregnant. An improvement in embryo morphology was noted in two patients after injection of ooplasm and both became pregnant, but one miscarried. A third pregnancy was established in the repeat patient, without obvious embryo improvement. One baby was born and the third pregnancy is ongoing with a normal karyotype. Two other patients with male factor infertility had poor embryos after ooplasmic injection, but the donor embryo controls were also poor. The patients did not become pregnant and had no donor embryos frozen. Ooplasmic transfer at the MII stage may be promising in patients with compromised embryos; however, evaluation of ooplasmic anomalies and optimization of techniques will require further investigation prior to widescale application.**

**Key words:** electrofusion/intracytoplasmic injection/mature oocyte/mitochondrial fingerprinting

### Introduction

The role of the ooplasm in mammalian oocyte maturation and activation is well documented, but far from detailed understanding (Masui and Markert, 1971a,b; Balakier and Czolowska, 1977; Czolowska and Tarkowski, 1996; Eppig, 1996; Gross *et al.*, 1996). An equally critical importance of ooplasmic factors is postulated for the continued development of the zygote, particularly during early cleavage, when transcription of the embryonic genome is minimal (Clegg and Piko, 1983a,b; Piko and Clegg, 1983; Van Blerkom *et al.*, 1995a; Liu *et al.*, 1997). Dymorphisms and developmental irregularities are commonly observed in human oocytes and embryos in the course of assisted reproduction (Alikani *et al.*, 1995; Xia, 1997). Both genetic and non-genetic factors appear to play a role in their occurrence (Van Blerkom and Henry, 1992; Van Blerkom *et al.*, 1995b; Munné *et al.*, 1995). In individual cases the precise origins and causes are almost invariably obscure, and only glycoprotein synthesis prior to

the luteinizing hormone (LH) surge has been linked to maternal age (Cohen *et al.*, 1993). Some non-genetic anomalies that originate in the ooplasm rather than in the embryonic genome are, in a sense trivial, in nature. Nonetheless, they may and often do interfere with normal development and as a consequence may threaten the viability of the embryo concerned. The varying degree of fragmentation commonly observed in zona-enclosed embryos may be reasonably considered to represent a complex of such primarily ooplasmic dymorphisms, although it is known that fragments can be suppressed by altering culture conditions (Wiemer *et al.*, 1989). Attempts to overcome ooplasmic deficiencies and abnormalities in non-human mammals by egg or embryo manipulation at a subcellular level have been reported in the literature (Muggleton-Harris *et al.*, 1982; Kishimoto, 1986; Pratt and Muggleton-Harris, 1988; Flood *et al.*, 1990; Levron *et al.*, 1996).

The present report is an account of similar attempts in the

human. All the patient couples concerned had experienced multiple assisted reproductive cycles with implantation failure attributable to poor embryo development. Attempts to reverse anomalies by altering follicular conditions, applying co-culture, assisted hatching and fragment removal had failed (Cohen *et al.*, 1990, 1994). The aim of the work was to restore normal growth and viability in developmentally compromised embryos, where the underlying cause was judged to be ooplasmic deficiency. Two approaches were used to transfer ooplasm from donor eggs at MII into synchronous patient eggs: (i) electrofusion of an anuclear donor fragment into each patient egg, and (ii) direct injection of a small amount of ooplasm from a donor egg into each patient egg (Cohen *et al.*, 1997).

## Materials and methods

### **Internal review board considerations and patient selection**

The Internal Review Board of Saint Barnabas Medical Center, New Jersey, USA, approved the use of cytoplasmic transfer for patients with compromised embryonic development in May 1995. The board agreed to a two or three-tiered experimental protocol depending on the initial number of mature patient eggs. Donor and patient were to be simultaneously stimulated and only some donor eggs used for cytoplasmic extraction and insertion into patient eggs (experimental group). Apart from the patient eggs receiving donated ooplasm, others were to be set aside for standard in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (homologous or donor control group). This was applicable if there were a sufficient number of eggs available, because the clinical goal of pregnancy outweighs academic evaluation. Since all eggs were treated with hyaluronidase, ICSI rather than insemination was performed in all groups to optimize the chances of fertilization. The other donor eggs were injected with husband's spermatozoa (heterologous or patient control group). Embryos from the different groups were compared and if the cytoplasmically hybrid embryos were within normal standards, they were replaced. Morphologically normal embryos from the heterologous control were cryopreserved, consequently patients would still have the option of a later thaw and transfer resembling an egg donation cycle.

Selection of the seven patient couples (one patient had cytoplasmic transfer twice) was not necessarily based on maternal age, elevated concentrations of follicular stimulating hormone (FSH) or poor follicular response, but primarily on recurrent embryo anomalies. We preferred to sample candidates based on observed oocyte dysmorphisms, excessive embryo fragmentation, slow cleavage, multinucleation or other morphological anomalies. We attempted to avoid using patients who had elevated aneuploidy in their oocytes or only had a single previous attempt. This selection criterion was not applied in two instances due to patient-related concerns regarding the use of gamete donation. Unfortunately, neither patient became pregnant from the experimental technique. The two patients are included in this study in order to present all data objectively.

### **Follicular stimulation**

Synchronization of the stimulation and oocyte retrieval of recipients and donors was necessary to allow cytoplasmic transfer between mature oocytes. This required donors and recipients to receive human chorionic gonadotrophin (HCG) on the same evening. Stimulation for the donors and recipients was done using established stimulation protocols (Scott and Rosenwaks, 1989; Scott and Navot, 1994). The

substantial majority of the donors participating in the oocyte donation programme received their HCG on the 10th day of medication (cycle day 12 if medication began on cycle day 3). In contrast, the recipients had already undergone several gonadotrophin stimulations, which provided a basis for estimating the amount of time they would need to complete follicular stimulation (range 7–12 days). Synchronization was accomplished by calculating the anticipated difference in the duration of stimulation for the donor and recipient. For example, if the recipient typically took 12 days of medication to complete follicular stimulation, she began taking medication 2 days before the donor. The synchronization achieved between the donor and recipient stimulation was generally good. However, in those cases where some slight difference existed, the timing of HCG administration was determined by the follicular maturation of the recipient.

### **Equipment and culture media**

Micromanipulation procedures were performed on an inverted Olympus IX-70 (Tokyo, Japan) using a Hoffman modulation optics infinity lens system. Incorporated heating plates were set at 37°C. Bent microtools were used for all procedures. Holding pipettes and ICSI needles were used from Cooke (Australia) or Carolina Microtools (GenX, Connecticut, USA). Other pipettes were made in the laboratory. They were fabricated from cleaned, sterile Sutter glass tubes, 1.0 mm outer diameter and 0.75 mm inner diameter, pulled on a Sachs Flaming micropipette puller (model PC-84, Sutter Instrument Co, Navato, CA, USA). The ooplast pipettes were opened to a diameter of 50 µm and fire-polished on a microforge (model MF-9; Narishige, Tokyo, Japan) (Levron *et al.*, 1996). Zona dissection pipettes, which are closed at the tip, were prepared by pulling fine solid sterile glass tubing from pre-pulled pipettes on the microforge.

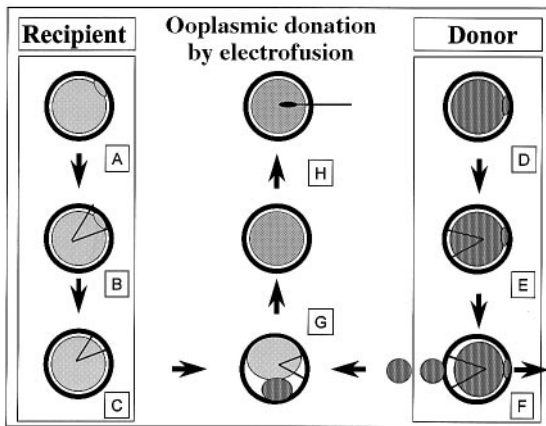
Oocytes were manipulated in 5 µl droplets with HEPES-buffered human tubal fluid supplemented with 6% human serum albumin (hHTF) under pre-equilibrated washed mineral oil (Squibb, Princeton, NJ, USA) in sterile shallow Petri dishes (Palermo *et al.*, 1994, 1995). Each egg was identified with a number and manipulated separately. The procedures were performed using one or two witnesses in order to ensure the strict separation of donor and patient eggs. Manipulated oocytes were individually washed in 10% maternal serum supplemented HTF (mHTF) and cultured for one day in order to observe pronuclear development. Further culture until day 3 or day 4 was performed using helper cells. Fertilized donor eggs (homologous control) which developed normally for 2 days were cryopreserved using propanediol as a cryoprotectant.

### **Egg treatment groups**

Approximately 4 h after egg retrieval, both donor and patient eggs were exposed to 0.1% hyaluronidase, and mature eggs were selected for injection. All corona cells were removed with fine bore pipettes. Donor eggs were split into two groups: the first group of eggs was used for ooplasmic extraction and the second group for ICSI (donor control). The patient eggs were divided into two groups, provided that there were enough eggs. The separation into two groups was done blindly under low magnification. The largest proportion was used for transfer of ooplasm. The second group was used for conventional ICSI (patient control).

### **Ooplasmic transfer by electrofusion of donor ooplast with intact MII oocyte (cycle 1–3)**

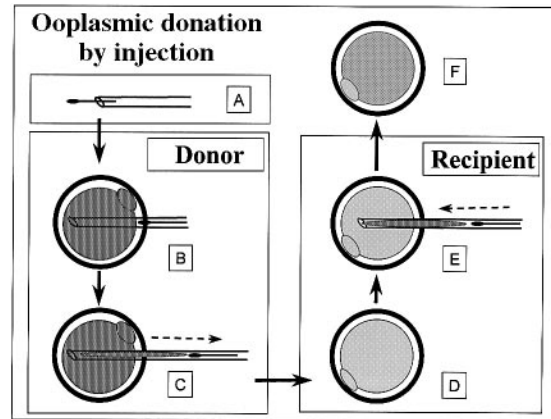
Eggs were exposed to 0.1% hyaluronidase (Palermo *et al.*, 1994) and zonae were opened mechanically using a microspear. Donor oocytes were exposed to hHTF medium containing cytochalasin B (CCB; Sigma Chemical Co, St Louis, MO, USA) (Willadsen, 1992; Levron *et al.*, 1996) for 10 min at 37°C. Partitioning of human MII oocytes



**Figure 1.** Ooplasmic transplantation by electrofusion with donated ooplast. (A) A patient egg is placed on the holding pipette. (B) A large gap is made over the zona pellucida using mechanical dissection. (C) The polar body is removed. (D,E) A gap is made opposite the polar body in the donor egg. (F) The egg is immersed in a solution containing membrane relaxants and ooplasts are removed. (G) The ooplast is placed underneath the zona of the recipient egg, and pipetted into an electrofusion chamber. (H) A spermatozoon is injected into the fused hybrid cell.

required variable cytochalasin B concentration depending on their sensitivity (~2.5 mg/ml). Ooplasts of various sizes were separated from donor eggs by withdrawing a portion of ooplasm enclosed in the plasma membrane (Levron *et al.*, 1995). Ooplasts were removed from areas contralateral to the polar body, in order to avoid nuclear material (Figure 1) (Levron *et al.*, 1995). This method was blind-tested by a geneticist using DNA stains and a numbering system randomly allocated by a third witness to the remaining oocytes, the polar bodies and/or large cytoplasts. Sufficient ooplasts were obtained for fusion with those patient eggs allocated for ooplasmic donation. Alignment and electrofusion in a mannitol solution (Levron *et al.*, 1996) was performed after insertion of the donor ooplast into the perivitelline space of the recipient egg from which the polar body was removed. This was done with a wide-bored polished microtool ~30–40 µm in diameter. The ooplast was sucked into the microtool and released once the tool was placed deeply into the perivitelline space.

The patient eggs were divided into two groups, provided that there were enough eggs. The separation into two groups was done blindly under low magnification. The largest proportion was used for transfer of ooplasm. The second group was used for conventional ICSI (homologous control). The zonae of the recipient eggs were opened mechanically over the polar body area using a microspear. The polar body was removed after re-positioning the oocyte on the holding pipette in such a way that the zona could be dissected using the closed microspear (Willadsen, 1986). The same position was used to insert the ooplast ~90° left of the area, which had contained the polar body. The zona was closed tight using the same tool. Electrofused cells were washed and incubated in mHTF for 40–90 min prior to ICSI. Spermatozoa were immobilized in 10% polyvinylpyrrolidone (PVP) for ICSI (Palermo *et al.*, 1992). The procedure was performed in hHTF while the short side of the aperture was at ~3 o'clock. The ICSI tool was moved through the artificial gap in order to avoid extrusion of ooplasm upon indentation of the zona during standard ICSI. All eggs were washed in mHTF, numbered and cultured separately after confirmation of the presence of intact metaphases in the donor oocytes from which ooplasts were removed.



**Figure 2.** Scenario for ooplasmic transplantation by injection. (A) An intracytoplasmic sperm injection (ICSI) needle is filled with polyvinylpyrrolidone (PVP) and a spermatozoon is immobilized and aspirated. The sperm cell is used as a marker to identify the position of aspirated ooplasm. (B) The membrane of a donor egg is broken with the polar body at the 2 o'clock or 4 o'clock position and the sperm cell at the tip. (C) Ooplasm is aspirated from areas opposite the polar body. (D) The needle is moved to a patient egg with the polar body positioned at 8 o'clock. (E) The membrane is broken and cytoplasm and spermatozoon are deposited into the egg. (F) The egg is cultured conventionally and co-cultured after confirmation of fertilization.

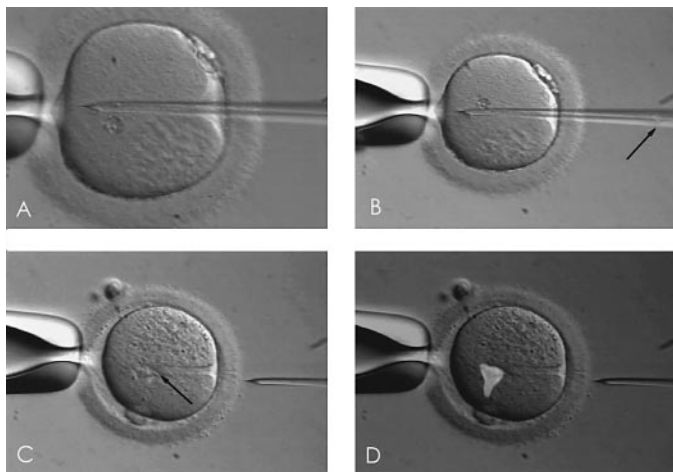
#### **Ooplasmic transfer by injection at the MII stage (cycle 4–8)**

One of the patients (B) who had ooplasmic fusion returned for another attempt. Four other patients also were selected for this group. Culture systems were the same as the three patients who had ooplasmic donation by electrofusion with ooplasts. Injection of ooplasm required optimal control of the suction in the microtool using 12% rather than 10% PVP.

The injection procedure is illustrated in Figure 2. One donor egg was pipetted into a 5 µl microdroplet under mineral oil containing hHTF. The same dish contained a 5 µl microdroplet with 12% PVP to which the prepared husband's spermatozoa were added. Two separate 5 µl droplets contained one patient oocyte each. Each droplet was clearly identified and donor and patient droplets were separated with non-toxic marking ink underneath the dish. One or more witnesses were present at all times and eggs were individually pipetted.

The ICSI pipette was filled from the tip with PVP solution. A sperm cell was selected and immobilized and placed ~30 µm from the tip. The tool was lowered into the droplet containing the donor egg. The egg was positioned with the polar body at 2 or 4 o'clock and the egg was pierced at 3 o'clock, in order to sample ooplasm at the contralateral side of the metaphase plate. The sperm cell was brought to the tip and suction control checked in order to ensure that there was no drift. The zona and membrane were penetrated as described by Palermo *et al.* (1996) using a stirring movement for membrane breakage rather than suction. This is the most gentle method of membrane breakage possible and increases the likelihood that the donor egg can survive two penetrations since it must undergo two extractions for insertion into two recipient eggs. Improved survival also increases the probability that the donor egg can be reliably checked for presence of chromosomes.

Ooplasm was sucked gently into the pipette using the sperm cell as a reference point. Blockage of the needle occurs rapidly but can normally be avoided by gently loosening the ooplasm with repeated use of suction and expulsion. This was repeated several times in different areas at the contralateral side in order to sample a representative area of the ooplasm. The procedure was performed at ×800–



**Figure 3.** Ooplasmic transfer in a human egg using injection. (A) The membrane is broken and the spermatozoon is positioned at the tip of the needle. (B) Ooplasm is aspirated. Arrow indicates sperm cell in needle. Its ultimate position is about twice the distance from the egg's zona. (C) The ooplasm and spermatozoon (arrow) are deposited into the recipient's egg. (D) The same as panel C, but the position of the injected ooplasm is highlighted.

1200 magnification, but  $\times 200$ – $300$  magnification was used for checking the amount of material in the needle by evaluating the position of the sperm cell in the pipette. Ooplasm was sucked into the pipette to a distance of  $\sim 500$ – $1000$   $\mu\text{m}$ . Suction was halted and neutralized by checking stability of the sperm position in the needle and ooplasm at the tip after withdrawal from the oocyte. This represents  $\sim 7$ – $14\%$  of the ooplasmic volume. The pipette was rapidly moved to the droplet containing the patient egg and its polar body positioned at 8 o'clock in order to deposit the donor ooplasm as close as possible to the chromosomal apparatus and maximize development. The membrane was pierced gently and ooplasm was aspirated and loosened locally in order to ensure proper deposition of donor ooplasm into the patient egg (Figure 3). The same donor egg was pierced at the original site of entry after sperm retrieval and the procedure was repeated using a second patient egg. All allocated patient and donor eggs were micromanipulated, washed and incubated as described above. Eggs were washed in mHTF, numbered and cultured separately after confirmation of the presence of intact metaphases in the donor oocytes from which ooplasm were removed.

#### Verifying the presence of metaphase chromosomes

Oocytes, cytoplasts and polar bodies were fixed individually as described previously (Munné *et al.* 1995), but the amount of fixative was reduced in order to minimize the risk of chromosome loss. Metaphases and chromosomes were checked with 4',6-diamidino-2-phenylindole (DAPI) stain and results immediately reported to the micromanipulation team. The geneticist performing the analyses had no knowledge of cell characteristics and origin.

#### Assessment of oocyte and embryo morphology

The morphology of the oocytes was scored and results entered in the database (EggCyte, West-Orange, NJ, USA). Anomalies of the zona pellucida, polar body and ooplasm were scored. Ooplasmic anomalies included, but were not limited to, central clustering of organelles, large dense bodies, granularity, organelle polarization, pyknotic nuclei, scattered dense material, vacuoles and clustered smooth endoplasmic reticulum (Van Blerkom and Henry, 1992; Alikani *et al.*, 1995).

Zygote pronuclei were counted and anomalies such as faint appearance, uneven size and irregular nucleolar complexes were entered in the database. Nuclei of blastomeres were visualized and multinucleation was recorded in each. Fragmentation was assessed by (i) estimating the amount of fragments expressed as a percentage, and (ii) characterizing the pattern or type of fragmentation (Warner *et al.*, 1997). Other embryonic variables are poor cell adhesion, uneven cell division and cytoplasmic anomalies.

#### Culture and manipulation of embryos

Fertilization and growth was assessed twice each day following egg retrieval. All fertilized and activated eggs were cultured separately and progress was followed using the original identification system. Zygotes from cycles 1–6 and 8 were pipetted in 4-well dishes (Nunc, Copenhagen, Denmark) containing monolayers of bovine epithelial cells without mineral oil cover in an incubator set at  $4.5\%$   $\text{CO}_2$  in air. Zygotes from the patient control group were also co-cultured. Donor control embryos from conventional egg donation were cultured without co-culture. All zygotes from cycle 7 were cultured without co-culture. Embryos from all groups were photographed and scored on day 2 and day 3.

Embryos which were selected for replacement were zona drilled for assisted hatching using acidified Tyrode's solution (Cohen *et al.*, 1993). Fragment removal was performed at  $\times 1200$  magnification using a  $12$   $\mu\text{m}$  pipette. Re-positioning the embryo with the hole anywhere between 1 and 5 o'clock was repeated several times in order to provide access to fragments located in areas not adjacent to the hole. Fragments and pipette tips were kept in the same focal plane to reduce the risk of blastomere damage. The pipette was moved between cells in order to target fragments contralaterally located to the hole. Most fragments of replaced embryos were removed. This procedure is standard practice in our laboratory for fragmented embryos that are to be replaced. For most of the patients described here, this procedure and co-culture had already been performed previous cycles without success.

#### Nuclear and mitochondrial DNA fingerprinting

The hypervariable region of the mtDNA was amplified by polymerase chain reaction (PCR) on the donors blood cells, the recipient patient blood cells and the amniocytes of the fetus from patient D. The 443 bp fragment was amplified from the donor/recipient blood using the primers L15997 and H16440 (Miller *et al.*, 1996). The PCR steps were: denaturation at  $94^\circ\text{C}$  for 30 s; annealing at  $55^\circ\text{C}$  for 1 min and extension time at  $74^\circ\text{C}$  for 3 min. L15997 primer was 5'-CACCATTAGCACCCAAAGCT-3' and the H16440 primer was 5'-TGATTTACGGAGGATGGTG-3'. The DNA from each sample was sequenced to determine the base pair differences in order to distinguish between the mtDNA of the donor cells, the recipient cells, and the amniocytes.

In order to increase the chances of detection of any amplified mtDNA from the donor, we had to ligate the PCR product from amniocytes directly into the TA cloning vector, pCR2.1 (Invitrogen, Carlsbad, CA, USA). There were three possible recombinant orientation plasmids: pCR2.1/Donor, pCR2.1/Recipient/F, pCR2.1/Recipient/R. The pCR2.1/Donor represents the construct which contains the insert amplified from the donor blood cells mtDNA. The pCR2.1/Recipient/F and pCR2.1/Recipient/R construct represents the two possible orientations from the inserts amplified from the recipient's mtDNA. Ligating resulted in 119 individual clones of the amniocyte mtDNA. These were analysed by restriction analysis using the enzyme *TaqI*.

**Table I.** Aetiology and history of assisted reproduction treatments of patients undergoing ooplasmic donation

Patient	Cause of infertility	Maternal age	Previous embryo transfers	Previous co-culture cycles	Previous assisted hatching cycles	Previous cycles with fragment removal	Previous cycles with aneuploidy diagnosis	Previous cycles with poor development
A	ovarian dysfunction/translocation	40	7	0	3	–	1	0
B1	D.O.R	36	4	2	4	2	0	4
C	D.O.R	34	1	0	1	0	0	1
D	D.O.R anovulatory	39	4	1	1	1	0	4
E	ovulatory dysfunction/oligospermic	31	7	2	3	3	0	7
F	D.O.R	37	4	2	4	3	0	4
B2	D.O.R	38	5	3	5	3	0	5
G	oligospermic	39	3	0	3	1	0	3

D.O.R = diminished ovarian reserve.

## Results

### General observations during micromanipulation and fertilization

Seven patients (A–G) were selected for ooplasmic donation. One (B1) returned for a second attempt (B2), after the first procedure was unsuccessful (Table I). All eight cycles were initiated and synchronized with eight donors. Follicular stimulation was started in 11 cycles, but synchronization could not be maintained in three, and these were cancelled in the latter half of the follicular phase. Cycles A, B1 and C were performed during a single day using electro-fusion with donated cytoplasts. The causes of infertility were ovarian-related in all patients, but one male partner (E) had severely affected sperm counts of 0–100 000 spermatozoa per ejaculate and another (G) had moderate oligozoospermia and teratozoospermia. The use of patients A and C for this particular research protocol is questionable, since they did not fit the initial selection criteria. Although patient A had seven failed embryo transfers, embryo development until day 3 appeared normal. The patient miscarried twice as a consequence of a maternal translocation. Aneuploidy was determined in extracted blastomeres of 14 embryos in one cycle and chromosomal anomalies were suspected in 13 of them. Six of the embryos had monosomy 13 or 21. It is obvious that ooplasmic donation at the MII stage would be superfluous. The other patient D had only one previous attempt at IVF and poor embryos were recovered. Inclusion of both patients in this trial, however arbitrary, was based on patient-related concerns. The other five patients (B, D, E, F and G) had at least three unsuccessful previous attempts each with consistently poor embryo development and alternative protocols such as assisted hatching, co-culture with bovine uterine epithelial cells and fragment removal (Table I). In all, 85 embryos had been transferred in the seven patients in 36 previous cycles, without implantation.

### Ooplasmic transfer by ooplast fusion

In all, 26 patient and 45 donor eggs were mature. Homologous controls were not used for patient B and C since both had only 5 mature eggs (Table II). Only 9/45 (20%) of the donor eggs were needed for ooplast production. Two to five ooplasts were produced from each of the nine eggs. The egg remainders still contained intact metaphases after DAPI. The ratio of donor eggs used for each recipient egg ranged

from 0.3 to 0.6. Control donor eggs were injected with the husband's spermatozoa, and embryos from each patient were cryopreserved for later use (Table II). The extracted polar bodies of patient B were checked and the two most morphologically intact ones were injected into two enucleated donor eggs. Both eggs survived and one showed an enlarged single pronucleus with a single polar body.

All but one of the 22 eggs fused with ooplasts within 1 h of the electrofusion pulse. Their ooplasm appeared normal when spermatozoa were injected. Only 23% of the eggs developed two normal pronuclei and a second polar body but another 46% (Table III) showed evidence of fertilization in that the second polar body was retained (digynic), had faint pronuclei or a single enlarged pronucleus. The latter configurations were accompanied by extrusion of the second polar body. A single pronucleus was removed from the digynic zygotes and all three survived and cleaved (Cohen *et al.*, 1993). Patient A had only a single normally fertilized zygote, but all four of her control eggs injected with spermatozoa also had pronuclear anomalies (Table III). Patient C could not be evaluated when comparing the potential effects of cytoplasmic transfer on preimplantation development (Table IV). The sum of embryo characteristics of the other two cycles was evaluated, and it showed a potential improvement in terms of reduced fragmentation after cytoplasmic transfer, while cleavage rate appeared not to be affected in these patients. The comparison may not be essential, since patient A had a high proportion of morphologically normal embryos in previous cycles. A total of 12 embryos were replaced in the patients, but only five had normal fertilization (Table III). The three patients did not test positive for pregnancy.

### Ooplasmic transfer by injection

A total of 59 patient and 90 donor eggs were mature. Homologous controls were not used for cycles B2 and G since the patients had few mature eggs (Table II). Only 29/90 (32%) of the donor eggs were needed for ooplasmic injection. Each of 19 donor eggs was used twice for ooplasmic extraction. Ten donor eggs were only used once for the procedure. The eggs from which ooplasm was extracted were checked with DAPI and all but one (3%) still contained intact metaphases. Embryos resulting from ooplasm extracted from this one egg were excluded. Control donor eggs were injected with the

**Table II.** Distribution of mature donor and patient eggs for ooplasmic transfer and use of homologous (patient eggs without ooplasmic transfer) and heterologous (donor eggs used for egg donation) control groups

Patient	Donor eggs for transfer	Recipient patient eggs	Ratio of donor/patient eggs	Enucleated donor egg injected with patient PB	Homologous patient control eggs	Heterologous donor control eggs	Donor embryo frozen
<b>Electrofusion with ooplast</b>							
A	4	12	0.3	0	4	6	5
B1	2	5	0.4	2	0	22	13
C	3	5	0.6	0	0	6	5
<b>Ooplasmic injection</b>							
D	7	14	0.5	0	6	8	4
E	6	12	0.5	0	3	20	0
F	5	9	0.6	0	2	11	6
B2	7	7	1.0	0	0	17	3
G	6	4	0.7	0	0	14	0

PB = polar body.

**Table III.** Formation of pronuclei (PN) after ooplasmic transfer using electrofusion or injection, in comparison with homologous control groups

Patient	Proportion with 2PN	Proportion with 3PN	Proportion with 1PN or abnormal PN	Proportion lysed	Proportion with 2PN in control	All PN anomalies in control
<b>Electrofusion with ooplast</b>						
A	1/12	2/12	3/12	3/12	0/4	4/4
B1	2/5	1/5	2/5	0/5	–	–
C	2/5	0/5	2/5	0/5	–	–
Total	5/22 (23%) <sup>a</sup>	3/22 (14%)	7/22 (32%) <sup>b</sup>	3/22 (14%)		
<b>Ooplasmic injection</b>						
D	4/14	3/14	2/14	3/14	5/8	0/8
E	8/12	1/12	1/12	0/12	2/3	0/3
F	8/9	0/9	0/9	0/9	2/2	0/2
B2	7/7	0/7	0/7	0/7	–	–
G	3/6	1/6	0/6	0/6	–	–
Total	30/48 (63%) <sup>a</sup>	5/48 (10%)	3/48 (6%) <sup>b</sup>	3/48 (6%)		

<sup>a</sup>*P* < 0.005.

<sup>b</sup>*P* < 0.05.

**Table IV.** Development of embryos after ooplasmic transfer in comparison with control groups and historical records

Patient	Proportion of day 3 embryos with <20% fragmentation			Proportion of day 3 embryos with more than five cells				
	Previous cycles	Ooplast transfer	Patient control	Donor control	Previous cycles	Ooplast transfer	Patient control	Donor control
<b>Electrofusion with ooplast</b>								
A	13/17	7/7	2/2	cryo d2	14/17	6/7	2/2	cryo d2
B1	4/20	3/4	–	8/12	4/20	1/4	–	9/12
Total	17/37 <sup>a</sup>	10/11 <sup>a</sup>			18/37	7/11		
<b>Ooplasmic injection</b>								
D	2/23	1/7	0/4	cryo d2	8/23 <sup>b</sup>	6/7 <sup>b</sup>	0/4	cryo d2
E	4/22	2/8	0/2	3/18	5/22	1/8	0/2	6/18
F	1/10	5/8	0/2	5/9	0/10 <sup>c</sup>	4/8 <sup>c</sup>	0/2	7/9
B2	4/20	0/7	–	11/14	4/20	1/7	–	11/14
G	3/13	1/2	–	4/5 <sup>d</sup>	2/13	1/2	–	1/5
Total	11/75	8/30	0/8		17/75	12/30	0/8	

<sup>a</sup>*P* < 0.05.

<sup>b</sup>*P* < 0.05.

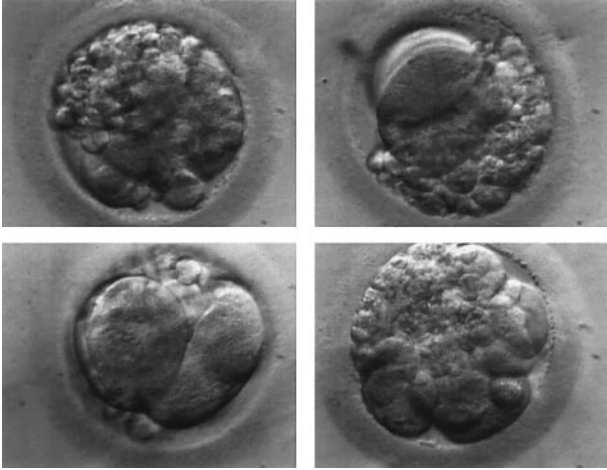
<sup>c</sup>*P* < 0.05.

<sup>d</sup>All blastomeres were multinucleated.

husband's spermatozoa and embryos from three of the patients were cryopreserved for later use (Table II). The donor embryos from patients E and G developed as poorly as did their embryos in previous cycles when using their own eggs. It was also noted in both patients that the embryos from ooplasmic

injection developed poorly, as did the homologous eggs when injected with spermatozoa only.

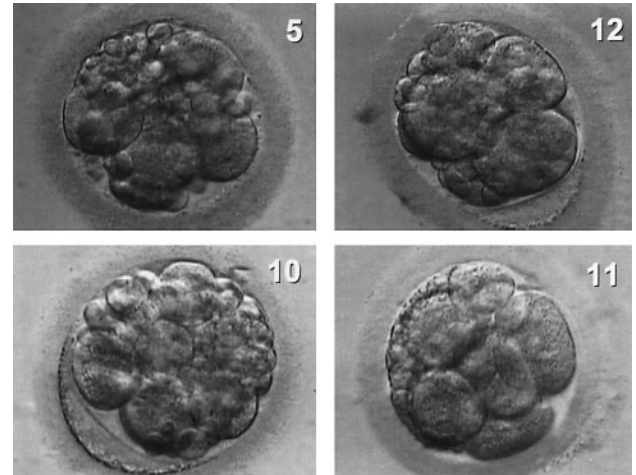
Only 6% of the eggs lysed, which is similar to the general incidence of 5% after ICSI in our programme. The proportion of eggs with normal morphology in the patient group was



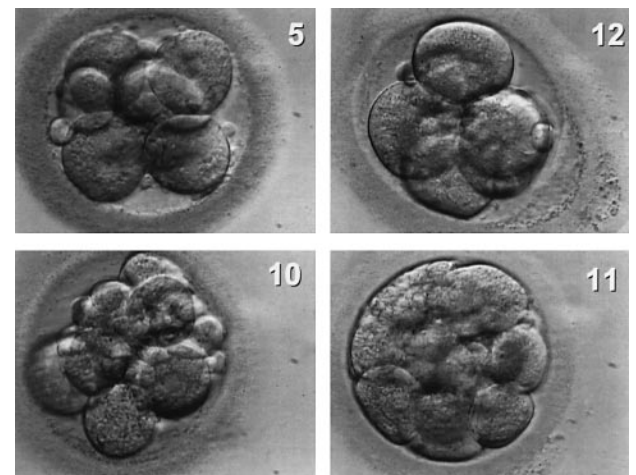
**Figure 4.** Four homologous control embryos from patient D obtained by intracytoplasmic sperm injection (ICSI) without ooplasmic transfer. Note excessive fragmentation on day 3 and few normal blastomeres.

10% (6/59), which compared poorly with the 71% (61/86) normal eggs in the donor group ( $P < 0.001$ ). The incidence of normally fertilized eggs was 63% after ooplasmic injection, which compared positively ( $P < 0.005$ ) with those eggs fused with ooplasm (23%) (Table III). The incidence of digynic zygote formation (10%) after injection was similar to the incidence after fusion (14%), but the other pronuclear anomalies were clearly reduced after injection: 32% versus 6% for fusion and injection respectively ( $P < 0.05$ ).

A single pronucleus was removed from the three digynic zygotes of patient D and all three survived and cleaved. Two of these were replaced after cleavage concurrent with normally fertilized embryos. The third enucleated zygote did not divide. Development rate of embryos from ooplasmic injection in this patient was significantly faster (Table IV) than those of previous cycles or in the patient embryos only injected with spermatozoa. The latter control embryos developed very poorly. Fragments were removed from these embryos in order to visualize blastomeres and staining confirmed the presence of only one embryo with two blastomeres on day 4 following egg retrieval. Once fragmentation, development rate, multinucleation and cytoplasmic conditions were evaluated in the three groups of embryos in patient D, it became clear that the embryos from ooplasmic transfer were comparable with donor embryos, while the homologous embryos were significantly less normal (Figures 4 and 5). The patient had four embryos transferred from which fragments were removed (Figure 6). Two normally fertilized embryos had eight and seven cells with 25% and 20% fragmentation respectively, 74 h post-egg retrieval. In all, 15% of the fragments were removed from the 8-cell embryos after assisted hatching and all fragments were removed from the 7-cell. When they were transferred 3 h later, both embryos had divided into 9- and 12 cells. The only other replaceable embryos were derived from digynic zygotes from which a single pronucleus was removed; a 7-cell embryo with 25% fragmentation and a 6-cell embryo with 20% fragmentation. All fragments were successfully removed from these embryos, but they did not divide until replacement 3 h later. The



**Figure 5.** Four replaced embryos from patient D after ooplasmic donor injection. Note that the general morphology is improved compared to control embryos shown in Figure 4. The numbers represent embryo identifications, which are similar to the identifications shown in Figure 6 after fragments have been removed.



**Figure 6.** These are the same replaced embryos as shown in Figure 5 after assisted hatching and fragment removal. Note that embryos #5 and 12 were digynic, but enucleated.

replacement procedure was complicated by elevated amounts of mucus and unexpected cervical resistance. Two embryos returned during the second attempt. They were washed and re-loaded and transferred separately. The patient had a positive pregnancy with a single fetal heartbeat. Amniocentesis at week 16 was uneventful and revealed an 46,XX girl. Birth at week 40 and neonatal development were uneventful.

Patient F had five ooplasmically hybrid embryos transferred derived from normally fertilized zygotes (Table V). Their development ranged from the 5- to 8-cell stage 75 h post-retrieval. Fragments were removed from the two most fragmented ones. It was decided to transfer more than the usual number of two to four embryos, because of the high number of previous failures. The transfer was uneventful and the patient had three positive HCG tests within the normal range, but ultrasonography could not confirm the presence of a sac.

Patient B2 had six replaceable embryos, but the cleavage rate was low, with five still at the 4-cell stage 76 h post-egg

**Table V.** Embryo replacement and implantation after cytoplasmic transfer

Patient	No. embryos replaced	Average fragmentation rate	Average fragmentation after removal <sup>a</sup>	Average number of cells	Positive pregnancy tests	Clinical outcome <sup>b</sup>
<b>Electrofusion with cytoplast</b> (replacement on day 4)						
A	6	5%	na	±12	no	np
B1	3	13%	5%	8	no	np
C	3	3%	na	9	no	np
<b>Cytoplasmic injection</b> (replacement on day 3)						
D	4	23%	4%	9	yes	1 girl born
E	1+3 <sup>c</sup>	14%	12% <sup>d</sup>	8	no	np
F	5	12%	9%	8	yes	miscarried
B2	6	30%	0%	5	yes	2 sacs, 1 fhb
G	2	27%	3%	6	No	np

<sup>a</sup>Not applicable.

<sup>b</sup>np = not pregnant; fhb = fetal heart beat.

<sup>c</sup>Only one viable embryo from cytoplasmic transfer; supplemented with three embryos from egg donation.

<sup>d</sup>More fragments formed after fragment removal.

**Table IV.** Sequence differences among the donor, the recipient mother's and the fetus mtDNA of patient D in the region from positions 15997 to 16395

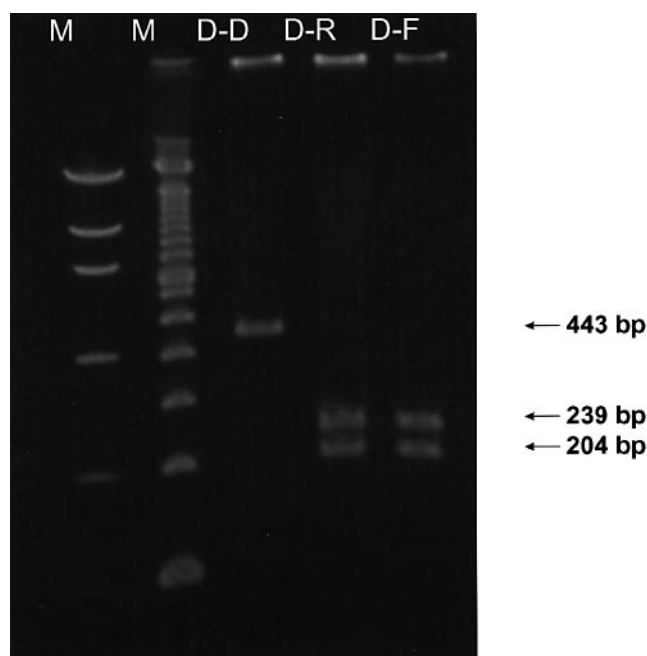
Sample	Base pair		
	16192	16219	16311
Cytoplasmic donor	T	A	T
Patient mother	T	G	C
Fetus	T	G	C

retrieval. Fragmentation rate was 25–40%. The embryos were not morphologically better than those obtained during previous cycles. All fragments were successfully removed from the six embryos. Until replacement 3 h later, none of the embryos divided further. It was decided to replace all six, since the patient had failed five previous times. Two fetal sacs were found on ultrasound, but only one developed a heartbeat. The singleton pregnancy is now in its second trimester following normal results and karyotype at amniocentesis.

**Evaluation of pregnancy and fingerprinting results**

In patient D, the hypervariable region of the mtDNA was amplified by PCR on the donor cells, the recipient cells and the amniocytes. The 443 bp fragment was amplified from the donor/recipient and fetal blood using the primers L15997 and H16440. DNA sequence analysis showed there are two base pair differences between the donor and recipient cells at positions 16219 and 16311; but there was no difference between the mother and the fetus (Table VI). The initial sequencing results failed to show that the fetus carries the mtDNA from the donor. However, since the quantity of donor ooplasm injected into the recipient was <15%, it is possible that this small amount of donor mtDNA would not be detected by direct sequencing of the PCR product, even if it were present.

A more sensitive assay was undertaken for detection of possible donor mtDNA in the fetus. Initially, a search was done for a restriction enzyme polymorphism that distinguished the mtDNA from the ooplasmic donor and the recipient patient. It was found that there is a restriction site, *TaqI*, present in



**Figure 7.** Polyacrylamide gel showing polymerase chain reaction (PCR) products of case D of mtDNA of donor (D–D), recipient patient (D–R) and fetus (D–F).

the PCR products from the recipient, but no *TaqI* on those from the donor. The sample was digested with *TaqI*, from the donor (D–D), from the recipient (D–R) and from the fetus (D–F) and the fragments separated on a 6% polyacrylamide gel. Results are presented in Figure 7: (i) D–D gave 443 bp after *TaqI* digestion, indicating there is no *TaqI* site for the PCR product from the donor cells; (ii) D–R gives a 240 bp and 203 bp after *TaqI* digestion, showing there is one *TaqI* site for the PCR product from the recipient; and (iii) D–F produces a 240 and 203 bp DNA product showing that the amniocytes analysed carried the same amplified product from the recipient. Although there is no visible band of the donor, like the DNA sequencing data, very low amounts of product not detectable by ethidium bromide staining could nevertheless be present.

In order to increase the chances of detection of any amplified mtDNA from the donor, the PCR product from the amniotic fluid was ligated directly into the TA cloning vector, PCR2.1. In this way, every copy of the amplified PCR product had an equal chance of being cloned and analysed. Individual clones ( $n = 119$ ) were analysed based on the different *TaqI* restriction patterns. 101 clones contained the correct size of inserts with the *TaqI* enzyme. There were three possible recombinant plasmids: pCR2.1/Donor, pCR2.1/Recipient/F, pCR2.1/Recipient/R. The pCR2.1/Donor represents the construct which contains the insert amplified from the donor mtDNA. The recipient *TaqI* restriction analysis gives two distinct patterns representing the two possible orientations the insert can ligate into the pCR2.1 vector. Based on the different *TaqI* restriction patterns, it was found that all 101 clones contained the same pattern as the ooplasmic recipient patient.

## Discussion

The aim of the present study was to augment the development and viability of embryos by improving the quality of unfertilized eggs suspected of having non-nuclear deficiencies by supplying them with ooplasm from normal donor oocytes. Two approaches were used to transfer ooplasm to recipient eggs, electrofusion and injection. The results show that both were technically and biologically feasible, at least to the extent that they both allowed fertilization and further development of a proportion of the eggs. It appeared that in some patients cytoplasmically hybrid zygotes showed improved development compared to zygotes from untreated eggs from the same patients. Furthermore, of the 30 embryos replaced, four implanted and two developed beyond the fetal heartbeat stage, whereas none of the embryos from the same patients implanted in previous cycles.

The direct injection approach was not only relatively simple, but entailed minimal disturbance of the usual ICSI routine. Even more encouraging is the positive clinical outcome in two of the five patients in this group, although the possibility that the same result might have occurred without ooplasmic transfer cannot, of course, be ruled out, despite consistent failure of previous attempts. One disadvantage of the injection method is that it allows only transfer of polarized material and in restricted amounts. In the present study, it was determined that ~7–14% of the ooplasm was transferred, but the calculation is based on amount of ooplasm in the needle, which may not have the same concentration of material as in native ooplasm.

None of the embryos replaced in the electrofusion group implanted, and at first sight the results with this approach look less promising than those obtained with injection. This is perhaps not surprising, since electrofusion is the technically more complex and radical of the two; although it is by far the more versatile. For this reason it may become the method of choice in future attempts to redress ooplasmic imbalance and deficiencies. But before that can happen, it will be necessary to optimize both the individual steps, which make up the complete procedure and their relation to one another in the context of specific clinical situations. In the present study, it was undoubtedly a disadvantage that electrofusion was carried

out prior to sperm injection, since the electrofusion almost invariably activates the oocyte. At the time of ICSI, all of the eggs in the electrofusion group were visibly in the process of polar body extrusion. Although it was interesting to observe that at least in some instances, apparently normal male pronuclear development could still take place, the non-physiological timing probably did not help towards a positive clinical outcome. A more effective approach would have been to perform ICSI prior to electrofusion. Alternatively, the electrofusion technique might be modified or applied in such a way that it does not activate the egg. With bovine eggs this is easily achieved, provided the oocytes concerned have only reached MII within the last few hours (S. Willadsen, personal observation). The post-maturation age of the oocyte also appears to be an important determinant for the ease with which mouse eggs are activated parthenogenetically (Kaufman, 1982).

The criteria used in selection of patients are crucially important, since ooplasm transfer clearly could not be expected to cure problems that are either causally unrelated to the constitution of the ooplasm of the mature egg or have become irreversible by the time the procedure is carried out. The category of eggs that are suitable for this technique are those having a normal nuclear genome, but ooplasm that is abnormal or deficient due to maternally mediated factors. Even if a precise diagnosis were presently possible, it could not be performed at the time when treatment must be instituted to be effective, and especially not for individual eggs. It is obvious that investigation over a larger series and more diverse sets of patients is needed in order to substantiate preliminary conclusions drawn here. Nevertheless a number of important issues require further debate.

The risk of transmitting potentially harmful factors into the host ooplasm should be weighed, since both the donor and recipient egg are opened and exposed to exterior conditions. It is likely that these are not much different from conditions created during standard ICSI, where problems with PVP have been implied but are largely unfounded, as data for larger amounts of clinical results have become available (Ray *et al.*, 1995; Schwartz *et al.*, 1996). The application of media modelled after intracellular conditions such as those used during patch clamping, may also have to be considered (Mazzanti *et al.*, 1990). One of the hypothetical impairments could be genomic transgenesis, if reverse transcriptase was present. This is nevertheless extremely unlikely, since reverse transcriptase enzymes are unusual DNA polymerases which have only been detected in retroviruses (Baltimore, 1970).

The supposition that this procedure is a form of genetic manipulation or gene therapy confuses the issue, even if only justifiable from a puritan point of view, since it involves incorporation of foreign ribosomal DNA, mRNA and mtDNA. Classic transgenesis involves incorporation of external DNA into the nuclear genome, the likelihood of which must be considered, albeit perhaps remote in these instances of ooplasmic transfer. Gene manipulation may have to be redefined since it can be argued that specific mRNA, protein inhibitors, growth factors and other compounds can be incorporated in the ooplasm via injection,

aimed at affecting processes such as those regulating cell cycle, apoptosis and housekeeping genes. Permanent cellular changes may occur after altering in-vitro conditions, although they appear to affect viability during pre-implantation and implantation stages exclusively rather than cause inheritable alterations.

More valid than the argument of germ cell-line therapy, may be the concern of creating chimaeric mitochondrial populations, although there is no evidence that that adversely affects development. Preliminary investigations of mitochondrial populations after nuclear transplantation for genomic multiplication in farm animals has thus far only detected mitochondrial hybrids in one animal (S. Willadsen, unpublished results). Mixtures of initially diverse populations of mitochondria at the zygote stage, are apparently prone to mechanisms involving specific mitochondrial demise. Indeed, both the sperm mitochondria and the majority of ooplasmic mitochondria cannot be detected in advanced embryonic stages. The fact that mitochondrial DNA is maternally inherited in mammals appears well founded and provides a means of testing relationships between populations and genetic drift. In the mouse, ~200 of the initial population of  $1 \times 10^5$  mitochondria are found in oogonia of the next generation (Jenuith *et al.*, 1997). The rapid segregation of mitochondria in the female germline provides a means for calculating the risk of transmitting mitochondrial disease. The recent discovery of paternal mitochondria in mitotic cells deserves immediate attention and confirmation, since it is possible that mitochondrial fingerprinting has been too sensitive in previous studies (Gyllesten, 1991). Transplantation of pronuclei or germinal vesicle nuclei of women at risk of transmitting mitochondrial disease into normal ooplasts of enucleated recipient cells should provide a unique opportunity, since it may discontinue any transmission of these genetic diseases in affected families.

The question as to how developmental competence of dysfunctional oocytes may be affected using ooplasmic transfer is difficult to answer in the light of the limited knowledge available for assessing ooplasmic and nuclear status in single cells. Although oocytes may display a multitude of dysmorphisms, their effect on development and more specifically the consequences of well described aberrations such as pyknotic areas and clustered golgi on cytoplasmic competence are unknown. Correlation of other anomalies such as clustered organelles and granularity with aneuploidy have been described, and are important when they occur during MI (Van Blerkom and Henry, 1992). The relationship between dysmorphisms and specific events after fertilization is considerably less well understood (Alikani *et al.*, 1995). Although the oocytes of both clinically pregnant patients described here were morphologically abnormal, we can only guess whether these anomalies may have affected development. Moreover, it cannot be reliably ascertained that cytoplasmic incompetence was overcome by the addition of small amounts of presumably normal ooplasm. The relativity of volume-related changes should be discussed as well, though it should not be underestimated, considering that even the paternal cytoplasmic component has an overwhelming contribution in spite of its proportionally minuscule volumetric size (Palermo *et al.*,

1994). Although the amount of injected ooplasm was limited in this work, the volume injected is estimated to be equivalent to that of blastomere from an 8-cell embryo.

There are four routes by which the ooplasmic domain is hypothetically affected after ooplasmic donation: (i) mitochondria from the donor oocytes may provide a more physiologic milieu in the initial phases of embryo development, in spite of the fact that the same mitochondria may not survive later; (ii) the internal pool of mRNA may be jeopardized and boosted after cytoplasmic transfer (Bachvarova and DeLeon, 1980; Bachvarova *et al.*, 1985). This is especially likely, since mRNA levels are dependent on maternal sources until complete genomic expression of the embryo days after fertilization. It is also likely because oocytes from different women may have initially different levels of mRNA; (iii) other organelles or organizing units such as the spindle apparatus may be specifically affected. This might be reversed by ooplasmic transfer, provided that enough of the material is incorporated; and (iv) the ooplasmic transfer may have very specific consequences by altering only a single mechanism. Oocytes from women with germinal vesicle or zygote arrest, for instance, may be assisted by cell cycle regulating factors incorporated in transferred ooplasm.

The current results emphasize the importance of the sperm cytosolic component after fertilization (Schatten *et al.*, 1986; Sathanathan *et al.*, 1991; Navara *et al.*, 1994; Palermo *et al.*, 1994; Simerly *et al.*, 1995; Moomjy *et al.*, 1996), since the cytoplasmically hybrid as well as the donor embryos from the oligospermic patients had equally poor development. One male factor patient produced multinucleation in donor as well as homologous embryos. The donor embryos were either mosaic or haploid following fluorescence in-situ hybridization. This presumably indicates deficiencies in the assembly of the spindle apparatus at syngamy or anomalies during pronuclear organization. In either way, these paternally related embryonic phenomena are likely the result of centriolar incompetence, although DNA fragmentation in the sperm cell cannot be excluded. Interestingly, the proportion of midpiece anomalies of spermatozoa from both these patients was elevated.

It is evident among several non-mammalian species that the ooplasm is polarized. Cytoplasmic transfer via incorporation of cell parts rather than nuclear transplantation poses interesting questions about ooplasmic polarity and its consequences. It was recently postulated by Edwards and Beard (1997), through comparative data of *Caenorhabditis elegans*, *Xenopus* and *Paracentrosis lividus*, that mammalian embryos separate their germline, endoderm and trophectoderm very early in development. They suggested that this could occur via a minor axis to overcome the equalizing distribution of factors at the 2-cell stage through the first meridional division. This secondary axis could then easily be overcome in blastomeres that reverted to totipotency after nuclear transfer or splitting. The success of the latter would be explained through cells being either committed or allocated to a certain lineage. Totipotency in this model appears to have a narrow molecular basis and an uncertain physiological significance in the undisturbed embryo. The models postulated by Edwards and Beard (1997) and also Antczak and Van Blerkom (1997) are intriguing, since they

argue for molecular as well as structural gradients in the human ooplasm. If this were so, then ooplasmic transfer could interfere with development. This would be even more serious if it were to be found that injected or fused material did not translocate once perfused in the ooplasm (J. Van Blerkom, personal communication). Using these suppositions, it is important to realize that in our cytoplasmic injection approach, cellular material, from the vegetal pole opposite the polar body, was extracted and deposited near the animal pole in the recipient eggs. This apparently has not jeopardized development, at least these few instances. It is possible that polarization phenomena can be manipulated in these artificial models, and this may, again hypothetically, promote development. Both injection as well partition techniques (Levron *et al.*, 1995) should provide a mode of studying polarity in human oocytes by combining different areas from different eggs. Although the work by Antczak and Van Blerkom (1997) may indicate that disruption of polarization can have adverse developmental consequences, it appears from our results so far that this is not so in the manipulated oocyte.

In conclusion, this is believed to be the first instance where ooplasm donation has been used with the aim of augmenting embryo viability in humans. While such treatment is tentative at this time, when comparatively little is known about the pathophysiology of the human oocyte, it seems to imply that these or similar procedures, such as nuclear transplantation at the germinal vesicle stage, will eventually prove valuable in assisted human conception. The fact that donor eggs or spermatozoa introduce a completely new set of mtDNA and donor chromosomes, and that for most prospective parents the relative importance of third party mtDNA is an unknown issue, may overshadow the demand for these new procedures. A discussion related to the drive to have one's own genetic children, may have to be considered against the pros and cons of ooplasmic manipulation with emphasis on the potential of creating three-parental individuals from a cell-biological point of view. It is likely that, in the light of unrestricted practice, such debate may be waived in lieu of progress. In our opinion, the presented technology is highly experimental and it would be wise to delay its widespread medical application until further studies in animal models and donated human material indicate the best approaches.

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