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Spontaneous and artificial changes in human ooplasmic mitochondria

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Our research has focused on promoting the development of compromised embryos by transferring presumably normal ooplasm, including mitochondria, to oocytes during intracytoplasmic insemination. Because of the enigma of mitochondrial heteroplasmy, the mixing of populations of oocyte cytoplasm has provoked considerable debate. We are currently investigating oocyte mitochondrial (mt) DNA mutations and the effects of ooplasmic transplantation on mitochondrial inheritance and mitochondrial functionality. Ageing human oocytes could accumulate mtDNA deletions, which might lead to detrimental development. Elimination of abnormal, rearranged mtDNA, such that the offspring inherit only normal mitochondria, is postulated to occur by a mtDNA 'bottleneck'. Among compromised human oocytes (n = 74) and early embryos (n = 137), investigations have shown the occurrence of ΔmtDNA^{4977}, the so-called common deletion, to be 33% among oocytes and 8% among embryos. Using a nested polymerase chain reaction (PCR) strategy of long followed by short PCR, another 23 novel mtDNA rearrangements were found: various rearrangements were present in 51% of the oocytes (n = 295) and 32% of early embryos (n = 197). The difference in the percentage of mtDNA rearrangements between oocytes and embryos was significant ($P < 0.0001$) and implies that there could be a process of selection as fertilized oocytes become embryos. There was no significant relationship between the percentage of human oocytes or embryos that contained mtDNA rearrangements and age. The first series of ooplasmic transfers have been performed in women with repeated implantation failure associated with slow and morphologically abnormal development of their embryos. In a total of 23 attempts in 21 women, eight healthy babies have been born and other pregnancies are ongoing. By examining the donor and recipient blood samples it is possible to distinguish differences in their mtDNA fingerprint. A small proportion of donor mitochondrial DNA was detected in samples with the following frequencies: embryos (six out of 13), amniocytes (one out of four), placenta (two out of four), and fetal cord blood (two out of four). Ooplasmic transfer can thus result in sustained mtDNA heteroplasmy representing both the donor and recipient.

Key words: embryos/mitochondrial DNA/mtDNA rearrangements/oocytes/ooplasmic transfer

Introduction

Interest in the relationship between reproduction and mitochondria has recently been renewed because of the possibility of transferring ooplasm containing mitochondria from one oocyte to another (Cohen *et al.*, 1997, 1998). It has been proposed that the resulting offspring are the recipients of genomes from three people: nuclear DNA from the mother, nuclear DNA from the father, mtDNA from the mother, and some mtDNA from the donor. Although ooplasmic transplantation does not involve the transfer of any nuclear DNA, there is controversy about whether mitochondrial diseases may be transmitted via the donor ooplasm or contained in the compromised recipient oocyte. However, it is presumed that the frequency of mutated mtDNA would be low or possibly absent in the resulting oocyte, and this would rule out the possibility of transmission of mitochondrial disease. Since little is understood about the maintenance of mitochondrial heteroplasmy and its nuclear regulation during human development (Robertson, 1999), the mixing of mitochondrial populations has provoked debate.

Our research has focused on how the ooplasmic transplantation technique, including the transfer of mitochondria, can restore normal growth and viability in developmentally compromised oocytes and embryos. The mechanisms involved are still enigmatic but could relate to mitochondria, although not necessarily exclusively. Other cellular components might be as effective after ooplasmic augmentation, including the pool of stored messenger RNAs, cell cycle regulating factors, housekeeping gene products and cryptic factors such as a human homologue to Qa-2, the protein that regulates rate of embryo division in the mouse (Cao *et al.*, 1999). Here we discuss our preliminary progress with the clinical work as well as our efforts to perform mitochondrial fingerprinting on oocytes that have either failed to fertilize or embryos that have arrested or failed development after ooplasmic transplantation. Results of mitochondrial inheritance in the pregnancies and babies are also presented. The concept of heteroplasmy is further investigated by studying mitochondrial rearrangements in compromised oocytes and embryos donated to research.

Spontaneous rearrangements in mtDNA

There are now >150 known mtDNA rearrangements, which include deletions, insertions and duplications (Wallace *et al.*, 1993). Such mutations in mtDNA are responsible for a number of catastrophic neuromuscular diseases, including Kearns–Sayre syndrome (KSS), chronic progressive external ophthalmoplegia (CPEO), and Pearson's syndrome (Moraes *et al.*, 1989; Zeviani *et al.*, 1989; and see Christodoulou, 2000). mtDNA rearrangements are inevitably harmful, causing cellular energy deficiencies and, in high doses, resulting in clinical disorders that affect the brain, heart, skeletal muscle, kidney, bone marrow, and pancreatic islet cells. One, predominant mtDNA rearrangement is found in 50% of patients with KSS syndrome. This deletion, called the 'common deletion', is a 4977 bp deletion (ΔmtDNA^{4977}) located between nucleotides 8482 and 13 460.

Mitochondrial DNA rearrangements have been shown to accumulate with age, and appear prevalent in post-mitotic, non-dividing tissues such as muscle and brain (Cortopassi and Arnheim, 1990; Ikebe *et al.*, 1990; Corral-Debrinski *et al.*, 1991; Hattori *et al.*, 1991; Kitagawa *et al.*, 1993). When ageing tissues accumulate mtDNA rearrangements, and when the percentage of mtDNA rearrangements reaches a significant threshold level, a measurable reduction in the efficiency of oxidative phosphorylation will occur (Richter *et al.*, 1988; Hattori *et al.*, 1991). Oocytes are non-

dividing cells with their meiotic division arrested for up to 50 years, and like other cells not subject any longer to mitotic competition, can accumulate mtDNA rearrangements with age (Keefe *et al.*, 1995). The presence of other mtDNA rearrangements and their potential correlation with reproductive senescence is currently being investigated in our laboratory.

In metaphase II (MII) oocytes, individual mitochondria with mtDNA rearrangements might be unable to produce adequate energy because of a decline in oxidative phosphorylation enzyme components. A lack of compensatory copies of wild-type mtDNA within individual mitochondria of the mature MII oocyte (Giles *et al.*, 1980; Hauswirth and Laipis, 1985; Chen *et al.*, 1995) could render the mitochondrion particularly sensitive to the effects of mtDNA mutations, through oxidative damage. It is these alterations in mitochondrial morphometry (i.e. reduced functional mitochondria) that could correlate with declining fertility, since the process of fertilization and subsequent preimplantation embryo development requires an abundant amount of stored ATP. Studies have shown that mouse oocytes and preimplantation embryos containing <2 pmol ATP do not develop beyond the 8-cell stage (Van Blerkom *et al.*, 1995). Since replication of mtDNA does not occur between the mature MII oocyte and the 64–128-cell stage in the mouse, the original estimate of 100 000 mitochondria in the mouse oocyte are distributed between all 128 cells of the blastocyst (Pikó and Taylor, 1987; Ebert *et al.*, 1988; Meirelles and Smith, 1998).

mtDNA rearrangements in human oocytes and embryos

Previously our laboratory has published results on a particular mtDNA mutation (ΔmtDNA^{4977}, the 'common deletion') in compromised human oocytes and embryos determined by using a nested polymerase chain reaction (PCR) strategy (Brenner *et al.*, 1998). This mutation was found in 33% of human oocytes (n = 24/73) and in 8% of human embryos (n = 11/137) (Figures 1, 2 and Table I). We have also investigated other mtDNA mutations in human oocytes and embryos (Barritt *et al.*, 1999). Two-thirds of the mitochondrial genome was amplified using two rounds of PCR, long PCR followed by short PCR, with multiple sets of primers. The first round of extra-long PCR (elPCR) amplified the entire larger 10.2 kb arc of mtDNA between the primers (Figures 3 and 4). Five second-round PCR reactions amplified regions within the first round elPCR reaction product (Figure 5). Short amplification cycles in the second round PCRs showed no bands unless mtDNA rearrangements were present to bring the primers closer together.

Using this long-PCR/short-PCR strategy, 149 (51%) of 295 compromised human oocytes showed fragments that were characteristic of mtDNA rearrangements (Figure 5 and Table I). Fragments characteristic of ΔmtDNA^{4977} were observed in 101 (34%) of the 295 oocytes. Of the 149 oocytes showing at least one fragment characteristic of a rearrangement, 92 showed multiple fragments characteristic of rearrangements (92/295 = 31%). Of the 197 compromised embryos analysed, 64 (32%) showed fragments that were characteristic of mtDNA rearrangements (Table I). Fragments characteristic of ΔmtDNA^{4977} were observed in 42 (21%). Of the 64 embryos showing at least one fragment characteristic of a rearrangement, 28 showed multiple fragments characteristic of rearrangements (28/197 = 14%). χ^2 analysis revealed there was a significant difference between oocytes (149/295 = 51%) and embryos (64/197 = 32%) for the presence of any mtDNA rearrangement ($P < 0.0001$). A significant difference between oocytes (101/295 = 34%) and embryos (42/197 = 21%) was also demon-

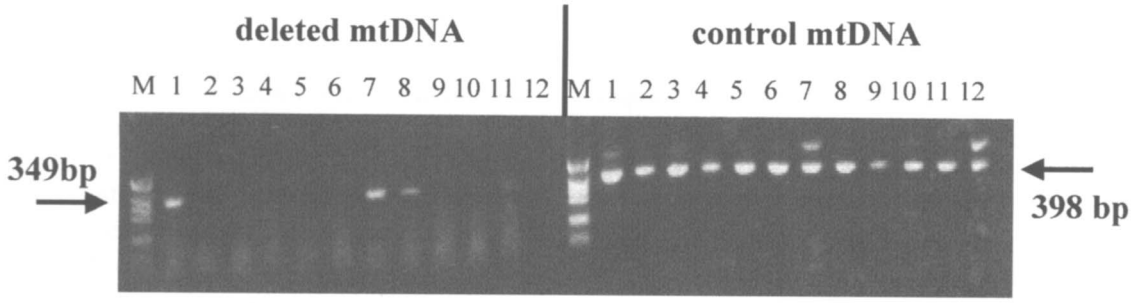

Figure 1. ΔmtDNA^{4977} and normal ('control') mtDNA present in human oocytes. Nested polymerase chain reaction (PCR) products were separated on 2% agarose gel and stained with ethidium bromine. Lane M = 100 bp DNA 'ladder' (marker). Lanes 1, 7, 8 and 11 (left side) demonstrate a 349 bp fragments produced by the ΔmtDNA^{4977} rearrangement when amplified with primers MT-1 and MT-4 (which were flanking the mitochondrial 'hot spot') in order to identify the 4977 bp deletion and thus amplify a 349 bp amplicon in single human oocytes (Brenner *et al.*, 1998). The nested normal control mtDNA PCR products (right side) were amplified using primers MT-2 and MT-3 (producing a 398 bp amplicon) and was a 398 bp amplicon (right side) and can be seen in each lane (1–12). (Reproduced from Brenner *et al.*, 1998, with permission).

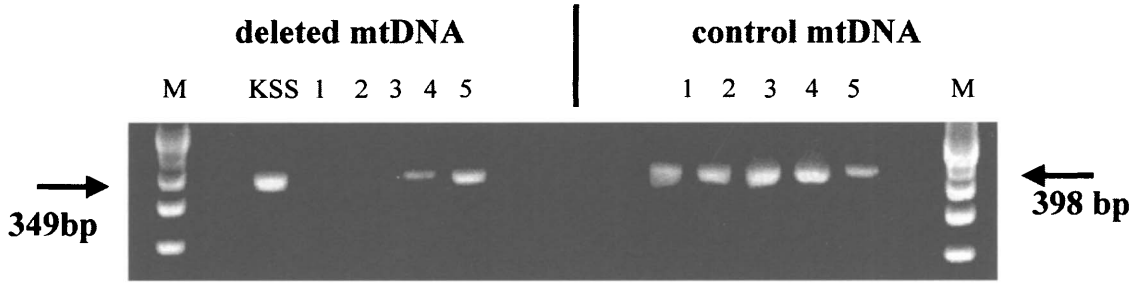

Figure 2. ΔmtDNA^{4977} and normal ('control') mtDNA present in human embryos. Lane M = 100 bp DNA 'ladder' (marker). Lane KSS = positive control cell line from a patient with Kearns–Sayre syndrome showing the effect of the ΔmtDNA^{4977} rearrangement in these polymerase chain reaction (PCR) conditions. Lanes 4 and 5 (left side) demonstrate the 349 bp fragment indicative of the presence of the ΔmtDNA^{4977} rearrangement in individual embryos. The nested normal control mtDNA PCR products (right side) were 398 bp long and can be seen in each lane (1–5). The nested PCR strategy to detect the KSS mtDNA mutation and control in human embryos was the same as that described for Figure 1. (Reproduced from Brenner *et al.*, 1998, with permission).

Table I. Mitochondrial (mt) DNA rearrangements in human oocytes and embryos. Values in parentheses are percentages

	Nested PCR	Long/Short PCR		
	ΔmtDNA ^{4977} rearrangement	ΔmtDNA ^{4977} rearrangement	any mtDNA rearrangement	multiple mtDNA rearrangements
Oocytes	24/73 (32.8)	101/295 (34.2)	149/295 (50.5)	92/295 (31.2)
Embryos	11/137 (8.0)	42/197 (21.3)	64/197 (32.5)	28/197 (14.2)
Level of significance[a]	$P < 0.0001$	$P = 0.0028$	$P < 0.0001$	$P < 0.0001$

PCR = polymerase chain reaction.
[a]Significant difference between oocytes and embryos.

strable with χ^2 analysis ($P = 0.0028$) for ΔmtDNA^{4977}. A significant difference between oocytes (92/295 = 31%) and embryos (28/197 = 14%) containing multiple mtDNA rearrangements was also demonstrable ($P < 0.0001$). In other words, a significant decrease in the prevalence of individual mtDNA rearrangements, available ΔmtDNA^{4977} and multiple mtDNA rearrangements, was demonstrable between (compromised) oocytes and

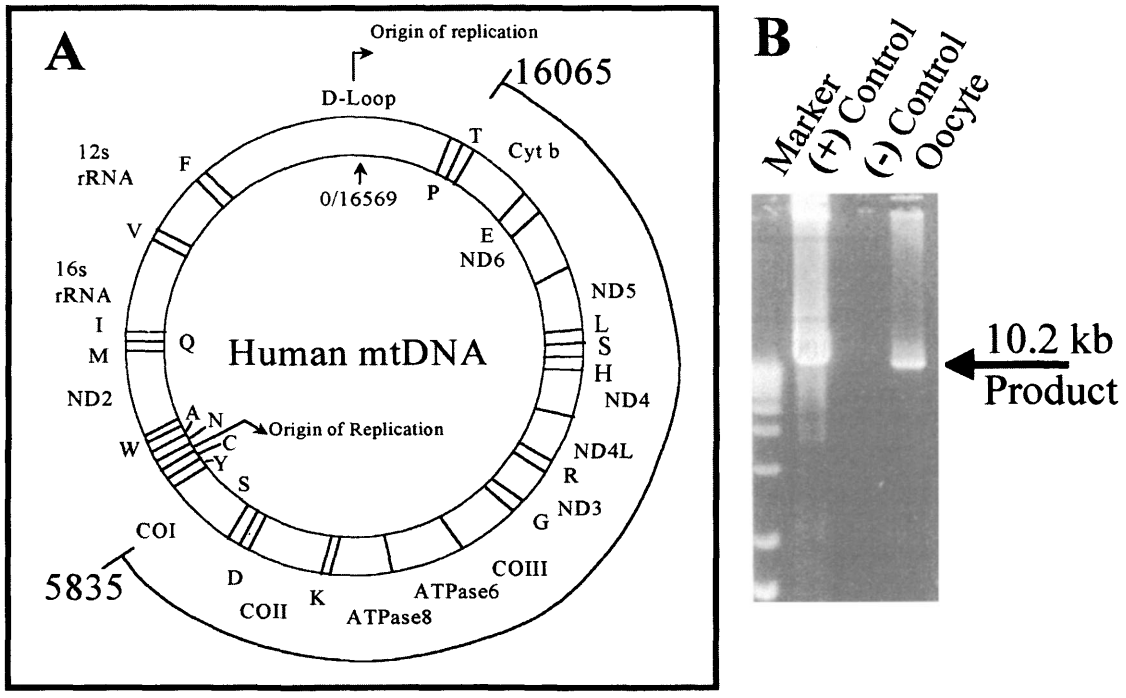

Figure 3. First round 'extra-long' polymerase chain reaction (elPCR) reactions with normal results (see text for details). (**A**) Diagram of the mtDNA genome showing the region that was amplified in the first round PCR reactions with primers starting at nucleotides 5835 and 16065. The 10.2 kb region between the primers is represented by the outermost arc, and represents two-thirds of the mtDNA genome covering the area where deletions most often occur. (**B**) 0.8% agarose gel showing 1st round reaction products. Lane 1 = marker, with the top band representing a 12 kb fragment, and lower bands marking locations for known smaller fragments. Lane 2 = positive first round control reaction, from elPCR core kit, showing an expected 20 kb band for verification of reagent quality. Lane 3 = negative first round control reaction (containing water and showing no bands). Lane 4 = first round reaction on a human oocyte, showing a single band consistent with a homoplasmic amplification of the undeleted 10.2 kb region. (Reproduced from Barritt *et al.*, 1999, with permission).

day 3 embryos. mtDNA mutation differences between oocytes and embryos may support the idea of the fertilization 'bottleneck'; however, one has to be careful about drawing conclusions since the majority of the MII oocytes and embryos were not normal due to the obvious need for the normal oocytes and embryos in the fertility clinic. Moreover, a study of bottleneck mechanism and timing is dependent on the determination of quantifiable mtDNA mutations in single cells, rather than the presence of any mutation. DNA sequencing reactions were performed to verify that DNA amplicons were mtDNA rearrangements. The results have identified 23 novel mtDNA rearrangements from 21 of the samples analysed (Table II). The rearrangements include 23 deletions, two insertions and one duplication.

Oocyte mtDNA rearrangements and age

The investigations described above (Brenner *et al.*, 1998; Barritt *et al.*, 1999) have identified the presence of ΔmtDNA^{4977} in 30–35% of human oocytes that were immature at oocyte retrieval. A correlation with age could not be confirmed. Similarly, among 184 tested MII oocytes, the mean patient age with mtDNA rearrangements (30.5 $\pm$ 0.6 years: mean $\pm$ SEM) was significantly lower (Mann–Whitney rank sum test; $P < 0.0001$) than the patient

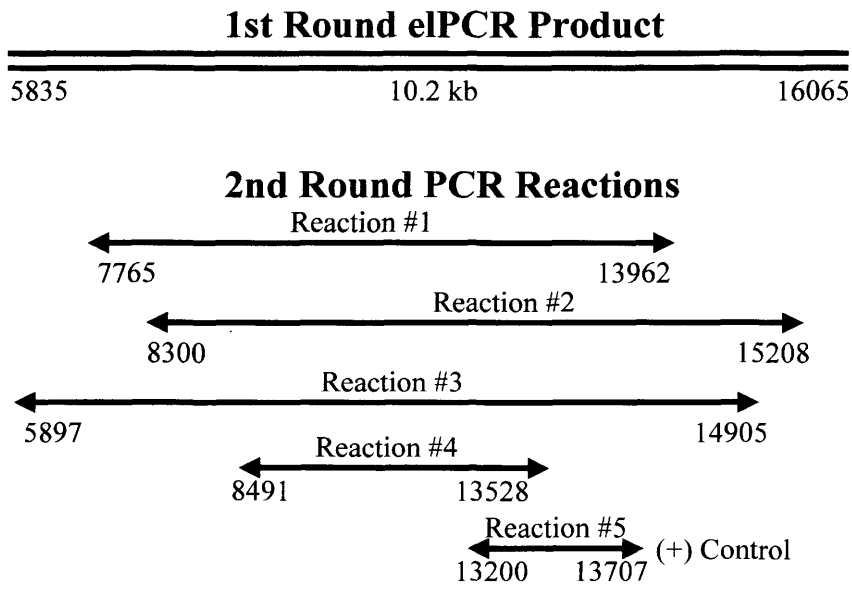

Figure 4. Primer design to detect deleted mtDNA fragments within the 10.2 kb region investigated. Five second round polymerase chain reaction (PCR) reactions are represented by lines between the start and finish nucleotides for the primers used. For the second round, PCR conditions were set so that only fragments of <5 kb would be amplified (smaller than the nucleotide distance between primers in sets 1–4), so that only deleted segments will amplify and produce stainable bands. Primer set 5 produced a 507 nucleotide fragment from normal mtDNA to serve as a positive control. (Reproduced from Barritt *et al.*, 1999, with permission). elPCR = extra-long PCR.

age of MII oocytes without mtDNA rearrangements (33.4 ± 0.3 years). This finding is interesting because it demonstrates fewer rearrangements in older patients. There was clearly no age-related increase in the presence of mtDNA rearrangements in human oocytes. The presence of mtDNA rearrangements in oocytes from patients aged <38 years was 84 out of 192 (43.8%); the presence of mtDNA rearrangements in oocytes from patients aged ≥38 years was 14 out of 40 (35.0%) (P = 0.31). Among 196 tested early embryos, the average age with mtDNA rearrangements (33.2 ± 0.5 years) was not significantly different from the patient age of embryos without mtDNA rearrangements (33.2 ± 0.4 years; MWRST; P = 0.88). The presence of mtDNA rearrangements in embryos from patients aged <38 years was 48 out of 155 (31.0%); in embryos from patients aged >38 years, it was 16 out of 42 (38.1%; P = 0.38).

Ooplasmic transplantation by intracytoplasmic injection

Our laboratory has attempted 23 ooplasmic transplantations by injection. In all, 21 couples were consulted for ooplasmic donation (after multiple previous conventional IVF attempts had failed because of poor embryo development) and agreed to this study. Ovarian reserve and fertilization did not appear to be compromised in these patients. The protocol had been approved by the Internal Review Board of Saint Barnabas Medical Center (1995 protocol) on the condition that not all donor oocytes would be used for the experimental procedure, but that some would be injected with the husband's spermatozoa and frozen when appropriate for the purpose of later performing conventional oocyte donation. Donor and recipient women were stimulated in such a way that their respective oocyte retrievals would fall within a few hours of each other.

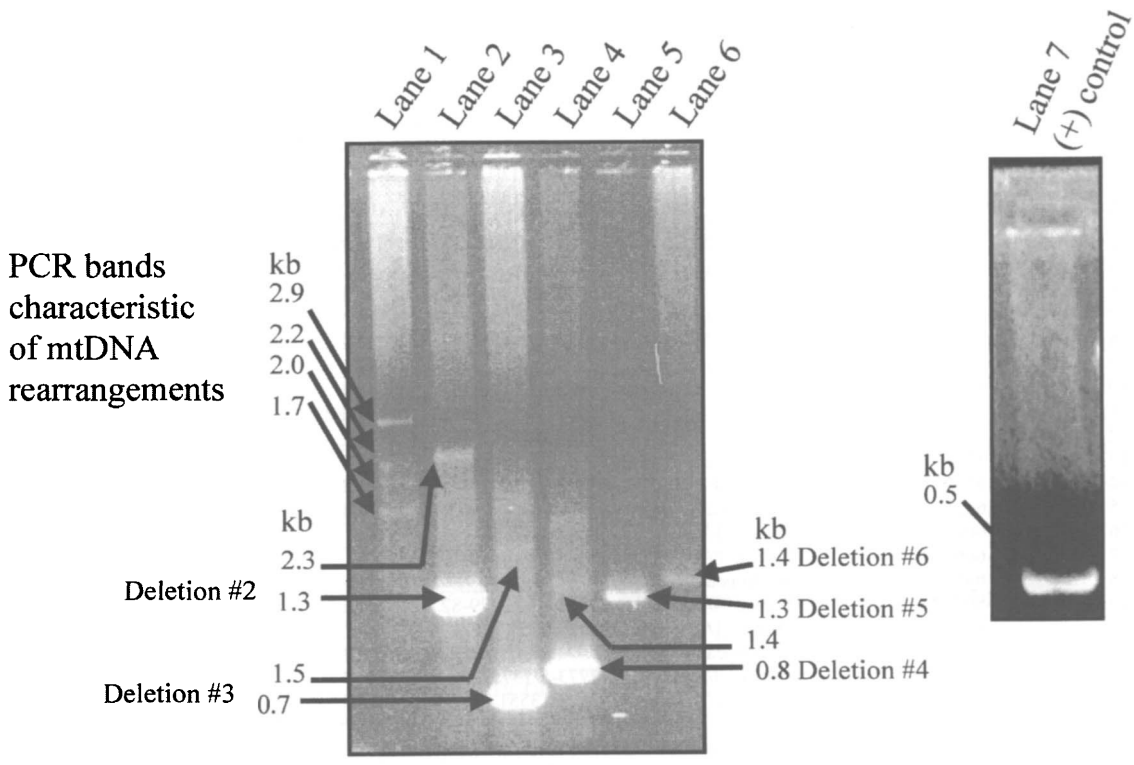

Figure 5. Illustrative second round polymerase chain reaction (PCR) reaction products on individual human oocytes. A 0.8% agarose gel with approximate kb determinations labelled. Multiple mtDNA rearrangements may occur in single oocytes and embryos; there may therefore be multiple bands of different sizes with second round PCR reactions. Some examples of mtDNA rearrangements with each primer sets are shown. Lane 1 = primer set 1, showing multiple bands characteristic of multiple rearrangements (1.7, 2.0, 2.2, and 2.9 kb). Lane 2 = primer set 3, showing two bands characteristic of rearrangements (1.3 and 2.3 kb). Sequencing reactions on the 1.3 kb band determined it was a 7855 bp deletion (deletion 2 in Table II). Lane 3 = primer set 1, showing multiple bands characteristic of rearrangements (0.7 and 1.5 kb); sequencing of the 0.7 kb band fragment showed it resulted from a 5463 bp deletion (deletion 3 in Table II). Lane 4 = primer set 2, showing multiple bands characteristic of rearrangements (0.8 and 1.4 kb); sequencing of the 0.8 kb band showed it resulted from a 6088 bp deletion (deletion 4 in Table II). Lane 5 = primer set 3, showing a single band characteristic of a rearrangement (1.3 kb); sequencing showed it resulted from a 7903 bp deletion (deletion 5 in Table II). Lane 6 = primer set 3, showing a single band characteristic of a rearrangement (1.4 kb); sequencing showed it resulted from a 7785 bp deletion (deletion 6 in Table II). Lane 7 = Second round reaction product with primer set 5 from a single oocyte, showing a single band representing the positive control fragment (0.5 kb); sequencing confirmed it was the amplified normaal mtDNA control fragment. (Reproduced from Barritt *et al.*, 1999, with permission).

(There has been no indication that the timing within a few hours can alter the outcome.) The oocytes were pre-treated similarly to those of patients for conventional ICSI. Ooplasmic transplantation was achieved in three steps.

First, a sperm cell was immobilized and placed in a standard intracytoplasmic sperm injection (ICSI) needle. The amount of polyvinyl pyrrolidone (PVP) used may be increased in order to reduce drift of membrane-free ooplasm later. During the second step, small amounts of donor ooplasm are removed by suction from the vegetal pole of MII stage oocytes (Cohen *et al.*, 1997, 1998). Approximately 5–15% of the ooplasm is sucked into the needle with the sperm cell marking the top of the column. The amount per oocyte varied slightly but, so far, the variation has not appeared to affect the outcome. Most donor oocytes are used twice, accommodating two recipient oocytes. The cytoplasmically depleted donor oocyte is then fixed for con-

Table II. Mitochondrial DNA rearrangements in human oocytes (taken from Barritt *et al.*, 1999)

Deletion number	Size of deletion (bp)	Type and size of repeat	Repeat location	Stage of development	Type of insemination
1	5989	Direct, 7/10	7943-52/13931-38	MII	IVF
2	7855	Direct, 7/8	6422-28/14275-82	MII	IVF
3	5463	Direct, 5/6	8143-47/13602-07	MII	ICSI
4	6088	Direct, 6/7	8793-99/14880-85	MII	ICSI
5	7903	No Repeat		GV	No spermatozoa
6	7785	Direct, 2/2	6979-80/14881-82	MII	ICSI
7	7862	Direct, 7/7	5367-73/13844-50	MI	No spermatozoa
8	8876	Direct, 5/7	5981-87/14857-63	MII	IVF
9	6390	No Repeat		GV	ICSI
10	5572	Direct, 3/3	8593-95/14165-67	MII	IVF
11	7730	Direct, 4/4	6041-44/13766-69	MII	IVF
12	6110	Direct, 1/1	8860/1470	MII	IVF
13	8796	No Repeat		MII	IVF
14	5531	Direct, 4/4	8200-03/13731-34	MII	IVF
15	6023	No Repeat		MII	IVF
16	6066	Direct, 1/1	8343/14458	GV	ICSI
17	6065	Direct, 8/8	8448-55/14512-19	MI	IVF
18	6451	No Repeat		MI	IVF
19	5902	Direct, 4/4	7878-81/13779-82	MII	IVF
20	6243	Direct, 1/1	8329/14571	GV	ICSI
21	6537	No Repeat		MII	IVF
22	5985	Direct, 3/3	7773-5/13757-9	MII	IVF
23	4973	Direct, 1/1	8579/13551	MII	IVF

Direct repeats (duplications) of three or more bp were observed in 12 sequences, and six sequences had no repeats (see Barritt *et al.*, 1999 for further information). GV = oocyte containing a germinal vesicle; ICSI = intracytoplasmic sperm injection; IVF = insemination with 150 000 to 500 000 spermatozoa; MI = metaphase I oocyte with no polar body; MII = metaphase II oocyte with one polar body.

firmation of the presence of its full chromosomal content. Accidental transfer of chromosomes has occurred twice from 258 oocytes; this was confirmed both by the cytogeneticist examining the depleted donor oocyte immediately after the procedure and by the embryologist examining the recipient oocyte the next day: after visualizing two polar bodies there were two pronuclei accompanied by extra subnuclei in a cortical location.

The third step involves careful breakage of the oolemma and injection of the donated, foreign ooplasm and the sperm cell. Care is taken to visualize, and to avoid leakage from, the injection area. The ooplasm in the recipient cell is 'loosened' prior to injection. Ooplasm and sperm cell are inserted into the ooplasmic area adjacent to the polar body. This is done because preliminary studies have shown improved development after positioning the metaphase plate close to the injected cell (Blake *et al.*, 2000); there could also be a benefit from exposing the perinuclear area and the sperm cell directly to foreign ooplasm. In spare oocytes, injected ooplasm stained with a fluorescent vital dye for mitochondria showed incorporation of mitochondria into the cytoplasm, with slow dispersal after several hours (J.A.Barritt, unpublished observations).

The procedure, based on ICSI, is relatively simple mechanically, but nevertheless requires observation by witnesses under conditions arranged to reduce the possibility of accidentally mixing two populations of oocytes. The incidence of cell damage after the procedure was 9.1%. This was significantly higher than the damage rate of 4.8% after ICSI in our laboratory over the same period. For the 23 procedures, 258 oocytes were injected. Seventy-four percent fertilized and 68% reached

day 3 of development. A total of 76 embryos were replaced in the 23 procedures (mean = 3.3): 12 embryos (16%) developed to display a heart beat. Fertilized donated oocytes were frozen successfully in 11 cycles. The failures after ooplasmic transplantation were caused either by poor embryo development as a result of male factor or by asynchronous follicular phases between the donor and recipient (Cohen *et al.*, 1997, 1998).

Since 1997, 12 clinical pregnancies have been achieved after 23 attempts in 21 women from transfer of embryos derived from ooplasmic transplantation. Two were twin pregnancies. Eight apparently normal healthy infants have been born from singleton pregnancies. One pregnancy ended in a miscarriage in the first trimester and the conceptus was karyotyped as 45,X. One fetus of the first twin pregnancy had developed abnormally on ultrasonographic examination at 15 weeks; amniocentesis also showed 45,X. In general, some fetuses with numerical chromosome disorders can develop well. It is possible that certain conditions, perhaps such as ooplasmic augmentation, could favour these embryos. There is no proof at this point that the percentage of congenital anomalies is increased after ooplasmic injection (the sample size is still small), but, given the pregnancy outcomes to date, we have altered our consultation process to reflect this possibility, and to consider a number of theoretical, scientifically based concerns.

One could consider aneuploidy assessment on cyto-recipient embryos prior to embryo transfers, but such embryo biopsy micromanipulation procedures might adversely affect these fragile embryos. These embryos develop at a rate that, on average, achieves just 4.7 cells on day 3, significantly lower than the day 3 average of 6.5 cells observed in other embryos after ICSI. These embryos' average fragmentation rate of >35% ordinarily indicates poor cellular survival (Alikani *et al.*, 1999) and an increased chance of mosaicism (Munné *et al.*, 1994, 1997). We are strongly considering polar body biopsy.

Currently there are eight healthy babies and the ongoing pregnancies are being closely monitored. A remarkable 50% clinical pregnancy rate has been achieved in a group of people who, with very poor embryo development, had previously repeatedly failed conventional IVF attempts. Because of this striking empirical success, we are now investigating all genomic aspects of ooplasmic transfer, especially mitochondrial inheritance, mitochondrial function, and the presence of mtDNA mutations among the offspring.

Mitochondrial DNA inheritance and mtDNA heteroplasmy

To establish differences in sequence between donor and recipient, DNA sequencing of the hypervariable D-loop region of the mitochondrial genome (i.e. mtDNA 'fingerprinting') is performed. This fingerprinting enables determination of their relative contribution to any subsequent heteroplasmic mtDNA population after ooplasmic transplantation, thus effectively quantifying cytoplasmic inheritance. Differences between donor and recipient mtDNA sequences are determined with blood samples and are then used as markers for mtDNA inheritance in embryos, amniocytes, placenta and fetal cord blood among offspring.

Recent investigations have shown (Brenner *et al.*, 2000) that after ooplasmic transfer, a mixture of donor and recipient mtDNA can be found in the discarded cyto-recipient embryos (n = 6/13), amniocytes (n = 1/4), placenta (n = 2/4), and fetal cord blood (n = 2/4). Investigations of amniocytes at 16 weeks of gestation have shown that donor mtDNA can be absent or the amniocytes can have a mixture of mtDNA from the donor and recipient. In placental tissues, both donor and recipient mtDNA has been found. Fetal cord blood

from offspring also contained donor mtDNA, although the donor mtDNA accounts for only a small amount of the total mtDNA. Although the data are still too few to enable generalizations, it is clear that donated mitochondria may not only survive but are able to proliferate in the embryo and fetus; thus the inheritance of mtDNA can be artificially altered by the transfer of small volumes of ooplasm. Ooplasmic transfer can result in sustained mtDNA heteroplasmy representing both the donor and recipient. These results show that the donor-derived mitochondrial populations persist after ooplasmic transfer and may be replicated during fetal development. Continuing investigations are aimed at determining the quantity of donor mtDNA and the segregation of this mtDNA genome in fetal tissues from the ooplasmic transplantation pregnancies.

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References

Alikani M., Cohen J., Tomkin G. *et al.* (1999) Human embryos fragmentation *in vitro* and its implications for pregnancy and implantation. *Fertil. Steril.*, **71**, 836–842.

Barritt, J.A., Brenner, C.A., Cohen, J. *et al.* (1999) Mitochondrial DNA rearrangements in human oocytes and embryos. *Mol. Hum. Reprod.*, **5**, 927–933.

Blake, M., Garrisa J., Tomkin G., and Cohen J. (2000) Sperm deposition site during ICSI affects fertilization and development. *Fertil. Steril.*, **73**, 31–37.

Brenner, C.A., Wolney, Y.M., Barritt, J.A. *et al.* (1998) Mitochondrial DNA deletion in human oocytes and embryos. *Mol. Hum. Reprod.*, **4**, 887–892.

Brenner, C.A., Barritt, J., Willadsen, S. and Cohen J. (2000) Mitochondrial DNA heteroplasmy after human ooplasmic transplantation. *Fertil. Steril.*, in press.

Cao, W., Brenner, C.A., Alikani, M. *et al.* (1999) Search for the human homologue of the mouse *Ped* gene. *Mol. Hum. Reprod.*, **5**, 541–547.

Chen, X., Prosser, R., Simonetti, S. *et al.* (1995) Rearranged mitochondrial genomes are present in human oocytes. *Am. J. Hum. Genet.*, **57**, 239–247.

Christodoulou, J. (2000) Genetic defects causing human mitochondrial respiratory chain disorders and disease. *Hum. Reprod.*, **15** (Suppl. 2), 28–43.

Cohen, J., Scott, R., Schimmel, T. *et al.* (1997) Birth of infant after transfer of anucleate donor oocyte cytoplasm into recipient eggs. *Lancet*, **350**, 186–187.

Cohen, J., Scott, R., Alikani, M. *et al.* (1998) Ooplasmic transfer in mature human oocytes. *Mol. Hum. Reprod.*, **4**, 269–280.

Corral-Debrinski, M., Stepien, G., Shoffner, J.M. *et al.* (1991) Hypoxemia is associated with mitochondrial DNA damage and gene induction. Implications for cardiac disease [comments]. *J. Am. Med. Assoc.*, **266**, 1812–1816.

Cortopassi, G.A. and Arnheim, N. (1990) Detection of a specific mitochondrial DNA deletion in tissues of older humans. *Nucleic Acids Res.*, **18**, 6927–6933.

Ebert, K.M., Liem, H. and Hecht, N.B. (1988) Mitochondrial DNA in the mouse preimplantation embryo. *J. Reprod. Fertil.*, **1**, 145–149.

Giles, R.E., Blanc, H., Cann, H.M. *et al.* (1980) Maternal inheritance of human mitochondrial DNA. *Proc. Natl Acad. Sci. USA*, **77**, 6715–6719.

Hattori, K., Agawa, T., Kondo, T. *et al.* (1991) Age dependent increase in deleted mitochondrial DNA in human heart: possible contributory factor to presbycardia. *Am. Heart J.*, **122**, 866–869.

Hauswirth, W.W. and Laipis, P.J. (1985) Transmission genetics of mammalian mitochondria: a molecular model and experimental evidence. In Quagliarello, E. (ed.), *Achievements and Perspectives of Mitochondrial Research.* Elsevier, Amsterdam, pp. 49–59.

Ikebe, S., Tanaka, M., Ohno, K. *et al.* (1990) Increase of deleted mitochondrial DNA in the striatum in Parkinson's disease and senescence. *Biochem. Biophys Res. Commun.*, **170**, 1044–1048.

Keefe, D.L., Niven-Fairchild, T., Powell, S. *et al.* (1995) Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women. *Fertil. Steril.*, **64**, 577–583.

Kitagawa, T., Suganuma, N., Nawa, A. *et al.* (1993) Rapid accumulation of deleted mitochondrial deoxyribonucleic acid in postmenopausal ovaries. *Biol. Reprod.*, **49**, 730–736.

Meirelles, F.V. and Smith, L.C. (1998) Mitochondrial genotype segregation during preimplantation development in mouse heteroplasmic embryos. *Genetics*, **148**, 877–883.

Moraes, C.T., DiMauro, S., Zaviani, M. *et al.* (1989) Mitochondrial DNA deletions in progressive external ophthalmoplegia and Kearns–Sayre syndrome. *N. Engl. J. Med.*, **320**, 1293–1299.

Munné, S., Weier, H.U.G., Grifi, J. and Cohen, J. (1994) Chromosome mosaicism in human embryos. *Biol. Reprod.*, **51**, 373–379.

Munné, S., Magli, C., Adler, A. *et al.* (1997) Treatment-related chromosome abnormalities in human embryos. *Hum. Reprod.*, **4**, 780–784.

Piko, L. and Taylor, K.D. (1987) Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Dev. Biol.*, **123**, 364–374.

Richter, C., Park, J.W. and Ames, B.N. (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl Acad. Sci. USA*, **85**, 6465–6467.

Robertson, J.A. (1999) Reconstituting eggs: The ethics of cytoplasm donation. *Fertil. Steril.*, **71**, 219–221.

Van Blerkom, J., Davis, P.W. and Lee, J. (1995) ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Hum. Reprod.*, **10**, 415–424.

Wallace, D.C., Lott, M.T., Torroni, A. *et al.* (1993) Report of the committee on human mitochondrial DNA. *Genome Priority Rep.*, **1**, 727–757.

Zeviani, M., Servidei, S., Gellera, C. *et al.* (1989) An autosomal dominant disorder with multiple deletions of mitochondrial DNA starting at the D-loop region. *Nature*, **339**, 309–311.