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BRIEF COMMUNICATION

Mitochondria in human offspring derived from ooplasmic transplantation

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Ooplasmic transfer from fertile donor oocytes into potentially compromised recipient patient oocytes has led to the birth of nearly 30 babies worldwide. Cytoplasmic transplantation has caused apprehension, since the mixing of human ooplasm from two different maternal sources may generate mitochondrial (mt) heteroplasmy (both recipient and donor mtDNA) in offspring. This investigation traced the mitochondrial donor population both during the ooplasmic transfer technique and in the bloods of two 1 year old children using mtDNA fingerprinting. Donor ooplasm stained for active mitochondria was transferred into recipient ooplasm and the mitochondria were visualized by confocal microscopy after the microinjection procedure and fertilization. Heteroplasmy was found in the blood from each of the children. This report is the first case of human germline genetic modification resulting in normal healthy children.

Key words: assisted reproduction/human oocytes/mtDNA fingerprinting/mitochondrial heteroplasmy/ooplasmic transplantation

Introduction

Ooplasmic transfer from fertile donor oocytes into developmentally compromised oocytes from patients with recurrent implantation failure has led to the birth of 15 healthy babies (Cohen *et al*., 1997, 1998; Brenner *et al*., 2000; J.A.Barritt and J.Cohen, unpublished results). The ooplasmic transfer technique introduces potentially beneficial components from donor oocytes, which may restore normal growth and viability (Cohen *et al*., 1998; Van Blerkom *et al.*, 1998). The transfer of small amounts of donor ooplasm (5–15%) probably includes mRNAs, proteins, mitochondria, as well as other factors and organelles. Mixing two different maternal sources of ooplasm may generate mitochondrial DNA (mtDNA) heteroplasmy in the offspring. It has been suggested that these children are potentially the recipients of two nuclear DNA (nDNA) genomes, one from each parent, and two mtDNA genomes, one each from the mother and the donor.

In this centre's ooplasmic transplantation programme, 12 clinical pregnancies were obtained after 28 attempts in 25 women. The clinical pregnancy rate was higher than expected (12 out of 28 cases), in a patient population with low fecundity. In some clinical cases significant improvement in embryonic development was seen after ooplasmic transfer. The basis for this work is the supposition that embryonic failure may be related to hitherto unknown cytoplasmic pathology. It is probable that a minimum threshold of ATP content is required for normal development, because cellular activities, e.g. chromosomal segregation, normal mitosis and physiological events during preimplantation development, require ATP. Furthermore, it is believed that there are significant differences in net ATP content in mature oocytes between patients (Van Blerkom *et al*., 1995).

Previously this laboratory has determined patterns of mitochondrial inheritance in embryos, amniocytes and fetal tissues after ooplasmic transplantation using the technique of mitochondrial DNA fingerprinting (Brenner *et al.*, 2000). In addition to recipient maternal mtDNA, a small proportion of donor mtDNA has been 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). So far, there is no reason to consider the minimal proportion of detected hypervariable mtDNA heteroplasmy as harmful, particularly since it can occur spontaneously in normal individuals (Howell *et al*., 1992; Bendall *et al.,* 1995; Wilson *et al.,* 1997). Currently, there are 15 healthy children being monitored following the cytoplasmic transfer procedure. Because of the striking empirical success of this technique, the genomic aspects of ooplasmic transfer are being investigated, especially mitochondrial inheritance, as it relates to the presence and maintenance of mtDNA heteroplasmy among the offspring. Here, the question of heteroplasmy is addressed in two 1 year old children.

Materials and methods

Confocal microscopy

After Internal Review Board (IRB) approval for the study of discarded non-viable tissues, consenting couples allowed the use of donated spare abnormal oocytes and spermatozoa from failed IVF cycles. Mitochondrial organelles were examined by confocal image analysis during a simulated ooplasmic transplantation experiment. MitoTracker Orange CMTMRos (Molecular Probes, Eugene, OR, USA) was used to label donor mitochondria with functionally active membrane potentials. Recipient oocytes and spermatozoa were not stained. Of the labelled donor cytoplasm, ~5–15% was microinjected into the unlabelled recipient oocytes to mimic a cytoplasmic transfer procedure (Cohen *et al*., 1997, 1998). Recipient oocytes (injected with stained donor ooplasm; $n = 26$), mitochondrial-stained donor oocytes (positive control; $n = 5$) and unstained oocytes (negative control; $n = 6$) were examined. Oocytes were imaged (×600 magnification), 10 min, 24 and 48 h after microinjection using an Olympus IX-70 inverted microscope fitted with an Olympus Fluoview laser scanning system (Olympus, Tokyo, Japan). A Kr/Ar laser was used to produce an excitation at 551 nm range and emission was measured at 576 nm (red emission). The oocytes were scanned at 5 µm intervals for both Nomarski and fluorescent images and the individual slices were displayed.

mtDNA fingerprinting

With IRB approval for clinical trials, 28 ooplasmic transplantation procedures in 25 couples were attempted. These couples participated in this study after previous conventional IVF attempts had failed due seemingly to persistent poor embryonic development. Each couple was counselled and consented to the known and unknown risks of this procedure and to the collection and study of any materials associated with this experimental technique. To determine the patterns of mitochondrial inheritance in the blood from two children after ooplasmic transplantation, mtDNA fingerprinting was performed as described previously (Brenner *et al*., 2000). The hypervariable region of the mitochondrial genome was amplified from the donor, recipient and the offspring's blood using the polymerase chain reaction (PCR) and the product was then DNA sequenced to determine the mitochondrial fingerprint (Brenner *et al*., 2000). The mitochondrial fingerprints (DNA chromatographs) of blood samples obtained from two children, aged 9 months and 14 months after paediatric evaluations, were then compared with the donor and recipient mtDNA fingerprints.

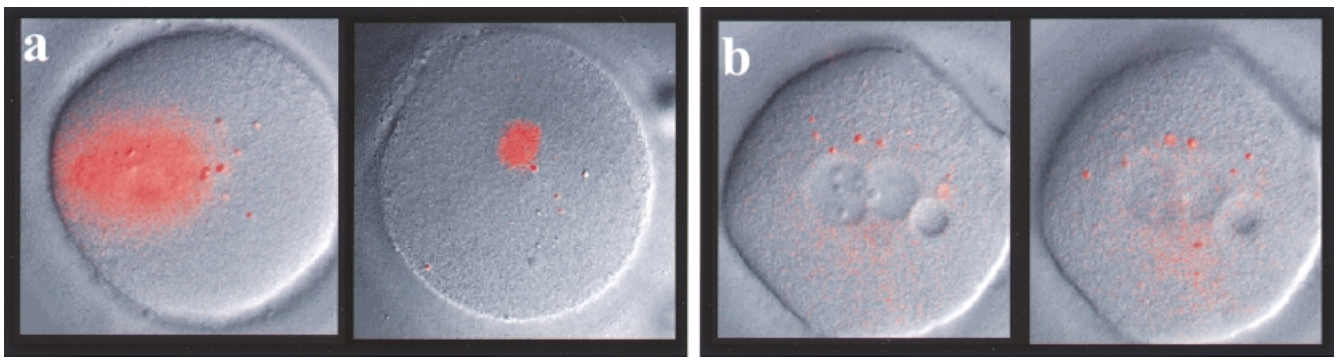

Figure 1. Confocal overlaying Nomarski images through (**a**) two different oocytes 10 min after ooplasmic injection with stained donor ooplasm (red) and (**b**) a three pronuclear (3PN) zygote 24 h after ooplasmic injection with stained donor ooplasm.

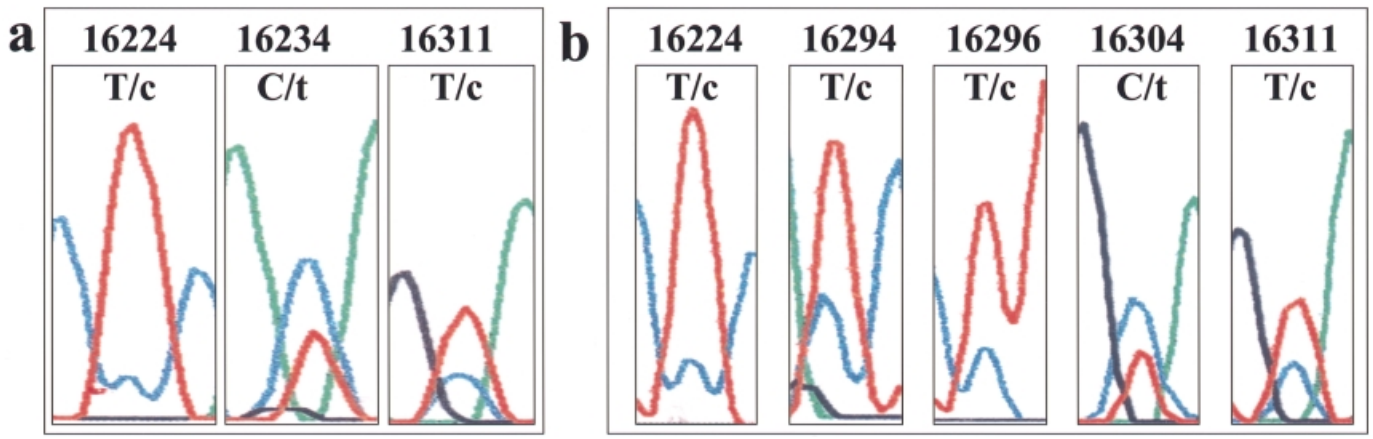

Figure 2. DNA chromatographs comparing mitochondrial DNA (mtDNA) sequences of the bloods from (**a**) the 9 month old offspring and (**b**) the 14 month old offspring. Numbers represent mtDNA fingerprints in (**a**) three sites and (**b**) five sites. Individual base pairs are represented in coloured peaks as follows: blue = cytosine, red = thymine, green = adenine and black = guanine. Uppercase C and T represent the predominant base pairs at each position. In cases of heteroplasmy, lowercase c and t represent the minor DNA sequences.

Results

Confocal microscopy

Confocal investigation of active mitochondria after experimental ooplasmic transplantation demonstrated that 10 min after injection, all recipient oocytes exhibited stained donor mitochondria in small, localized regions of the ooplasm at the injection sites (Figure 1a). By 24 h after injection, diffuse staining was observed throughout the recipient cytoplasm and particularly in the peri-nuclear region (Figure 1b). By 48 h, the labelled mitochondria segregated into separate blastomeres and cellular fragments of the embryos (data not shown). Positive control oocytes exhibited diffuse mitochondrial staining throughout the cytoplasm while negative control oocytes contained no visible fluorescence.

mtDNA fingerprinting

mtDNA fingerprinting of two of the children born following ooplasmic transfer demonstrated that in addition to the maternal mtDNA, there was a small proportion of donor mtDNA observed in their blood. DNA chromatographs from the blood of the 9 month old child, showed a minor quantity of donor mtDNA demonstrated by the DNA sequence at positions 16224, 16234 and 16311 in the mitochondrial hypervariable region (Figure 2a). Additionally, in the 14 month old child, donor mtDNA was detected at positions 16224, 16294, 16296, 16304 and 16311 in the mitochondrial hypervariable regions (Figure 2b).

Discussion

During human ooplasmic transplantation, active mitochondria were transferred from donor oocytes to recipient oocytes. These transferred mitochondria were detected by confocal analysis, 24 and 48 h post-injection within the developing preimplantation embryo. These active donor mitochondria were found in both blastomeres and cellular fragments, but were not evenly distributed throughout every cell. These findings suggest that since the donor mitochondria function normally when they are passed on to many recipient blastomeres they may provide the mitochondrial 'boost' necessary for embryonic development to prevent IVF failures (Van Blerkom *et al*., 1995, 1998). Alternatively, transfer of donor mitochondria to compromised oocytes may inhibit apoptotic mechanisms (Perez *et al.,* 2000).

Confocal analysis of preimplantation embryos showed distribution of mitochondria to a population of the cells and fragments. Similarly, the mtDNA heteroplasmy detected in the amniocytes, placental and fetal cord blood samples (Brenner *et al*., 2000) suggests that differences in the distribution of the donor mitochondria to fetal tissues may vary. The analysis of the distribution of the donor mitochondria in both embryonic and extra-embryonic tissues will require further investigation. Furthermore, the determination of whether donor mitochondria are actively segregated or randomly segregated within developing preimplantation embryos, embryonic and extra-embryonic tissues may even shed some light on the mitochondrial bottleneck theory (Hauswirth and Laipis, 1982).

mtDNA fingerprinting analysis performed on blood samples from 1 year old children following ooplasmic transfer detected mitochondrial polymorphisms in which both alleles were present in the hypervariable region of the mitochondrial genome. It has been previously estimated (Nickerson *et al.,* 1997) that the minor allele must be present in at least 30% of the sample to be detected on the DNA chromatograph and therefore the donor allele must be highly represented in the blood samples of the offspring. Furthermore, highly sensitive molecular analysis using allele specific–PCR (AS–PCR) or molecular beacons will be necessary to determine the frequency of donor and recipient mtDNA heteroplasmy in the blood samples or buccal smears derived from babies of the ooplasmic transplantation procedures.

These are the first reported cases of germline mtDNA genetic modification which have led to the inheritance of two mtDNA populations in the children resulting from ooplasmic transplantation. These mtDNA fingerprints demonstrate that the transferred mitochondria can be replicated and maintained in the offspring, therefore being a genetic modification without potentially altering mitochondrial function. Furthermore, there has been no alteration of nuclear DNA inheritance in the fetal cord blood from these babies (Brenner *et al*., 2000).

Presently, there is no reason to consider the minimal proportion of detected donor mitochondria observed in the offspring as harmful, since it is known to occur naturally in normal individuals (Ivanov *et al*., 1996). Also, there is an important discrepancy between benign heteroplasmy after mixing two potentially normal populations of mitochondria, as reported here, compared to pathological heteroplasmy which occurs during the ageing process or in patients with mitochondrial disease. We consider that the perceived risk to offspring from cytoplasmic transfer is minimal, and therefore we continue to perform these clinical procedures, albeit as an experimental protocol. However, the regulation of the donor's mitochondrial population and how it is maintained by the recipient's nuclear genome must be continually evaluated in the offspring from cytoplasmic transfer.

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