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# Epigenetic and experimental modifications in early mammalian development: Part II

# Cytoplasmic transfer in assisted reproduction

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**This report details the use of cytoplasmic transfer in human oocytes. The introduction of a small amount of ooplasm from a donor oocyte or zygote may alter the function of oocytes, with probable deficiencies. Cytoplasmic transfer from fertile donor oocytes or zygotes into compromised oocytes from patients with recurrent implantation failure after assisted reproduction has now led to the birth of nearly 30 healthy babies worldwide. Transfer of small amounts of cytoplasm probably involves mRNAs, proteins and mitochondria, as well as other factors and organelles. Even though the use of cytoplasmic transfer has been employed in several IVF clinics—and pregnancies have resulted—it is not known definitively whether the physiology of the early embryo is affected. This review outlines the experimental cytoplasmic transfer techniques and postulates the future impact in assisted reproduction.**

*Key words:* cytoplasmic transfer/human oocytes and embryos/human reproduction/IVF/ooplasm

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## Introduction

The complex role of ooplasm in human oocyte maturation and activation is not well understood, and efforts are underway to improve our understanding of oocyte development at both cellular and molecular levels. Clearly, events such as meiotic maturation, fertilization and the activation of the embryonic genome involve both nuclear and cytoplasmic processes. The ooplasmic factors include messenger RNA, maternally stored proteins, stockpiles of energy substrates, other energy-production components and many factors yet to be determined. A select group of patients continually experience poor embryo development and implantation failure during IVF procedures. In this group of patients, attempts to improve embryo development have included altering stimulation protocols, intracytoplasmic sperm injection (ICSI), co-culture, alternative culture methods, assisted hatching and fragment removal, ultimately leaving the use of donor gametes as the only option to overcome their infertility. However, the development of an ooplasmic transfer technique, which allows for the introduction of potentially beneficial ooplasmic components from donor oocytes, may now restore normal growth and viability to their own developmentally compromised embryos. This review explores the development of this and other very similar techniques, and provides a projection for their future use.

## Clinical experience

A variety of studies has been published during the past 15 years, all of which have been involved with an attempt to overcome ooplasmic deficiencies and abnormalities in non-human mammals by oocyte or embryo manipulation at the subcellular level (Muggleton-Harris *et al*., 1982; Pratt and Muggleton-Harris, 1988; Flood *et al*., 1990). During the early 1990s, members of our laboratory first began to pursue research in this area, and this led to an investigation involving the formation of mouse zygotes after fusion with synchronous and asynchronous cytoplasts (Levron *et al*., 1996). Synchronous transfer is defined as the replacement of cytoplasm from a donor oocyte to a recipient

oocyte at the same developmental stage, whereas asynchronous transfer is referred to as the replacement of cytoplasm from one developmental stage to a different stage of development [i.e. tripronucleate (3-PN) cytoplasm to a metaphase II (M-II) oocyte]. This work provided the basis for the first attempts at human ooplasmic donation to overcome multiple failed IVF cycles attributable to poor embryo development (Cohen *et al*., 1997, 1998). Other laboratories have performed additional studies using frozen–thawed donor oocytes as ooplasmic donors and 3-PN embryos as cytoplasmic donors (Huang *et al*., 1999; Lanzendorf *et al*., 1999).

After institutional review board (IRB) approval, a limited experimental trial was begun at our centre using two different techniques—electrofusion and injection—to transfer distinct amounts of ooplasm from donor to recipient oocytes (Cohen *et al*., 1997, 1998). The donor and recipient were simultaneously stimulated in such a way that their respective oocyte retrievals would fall on the same day. Ooplasmic transplantation was performed by transferring ~5–15% of donor ooplasm to recipient oocytes, either by cytoplast formation of the donor oocyte and electrofusion of the cytoplast to the recipient oocyte followed by ICSI; or by using an ICSI needle to extract donor ooplasm and injecting the donor ooplasm along with a single spermatozoon into the recipient oocyte (Cohen *et al*., 1997, 1998). Evaluation of the resulting embryos was carried out and transfer of the best embryos was performed on day 3 of culture after assisted hatching and fragment removal.

As a result of eight experimental clinical cases, our institution decided to pursue further investigations into the ooplasmic transplantation by injection technique alone. This technique was relatively simple, and entailed minimal modification of the usual ICSI routine. Ooplasmic transplantation by injection is achieved in three steps on an IX-70 inverted microscope (Olympus, Tokyo, Japan) using Narishige micromanipulators (Narishige, Tokyo, Japan). First, a spermatozoon was immobilized and placed in a standard ICSI needle. Second, ~5–15% of the donor ooplasm was removed by suction from the vegetal pole (opposite the polar body) of donor M-II oocytes, with the spermatozoon marking the top of the column. Donor ooplasm was taken from a single donor oocyte one or more times as required. The third step involved careful breakage of the oolemma of the recipient oocyte and injection of the donor ooplasm and spermatozoon near the animal pole. Care was taken to visualize, and to avoid leakage from, the injection area. Ooplasm in the recipient cell was 'loosened' prior to injection by gentle suction, causing a partial mixture of donor and recipient cytoplasm in the injection needle. Ooplasm and spermatozoon were then expelled into the ooplasmic area adjacent to the polar body, because preliminary studies had shown improved development after positioning the metaphase plate close to the injection site (Blake *et al.*, 2000).

To date, our laboratory has attempted 33 ooplasmic transplantation cases, after multiple previous conventional IVF attempts had failed because of poor embryo development. The electrofusion technique was performed in three patients with a low incidence of normal pronucleus formation, with essentially no improvement in embryo development or a clinical pregnancy (for more detailed information on the electrofusion technique, see Cohen *et al*., 1998). The injection technique was attempted 30 times in 27 couples. In these 30 cases normal fertilization and pregnancy rates resulted in one miscarriage, 10 singletons, one twin and one quadruplet birth, and one ongoing singleton pregnancy (Cohen *et al*., 1997, 1998; Brenner *et al.*, 2000; Barritt *et al.*, 2000, 2001) (Table I). The first trimester spontaneous miscarriage was diagnosed as a 45,XO. The 45,XO karyotype is the most common chromosomal abnormality associated with abnormal developmental morphology at the time of ultrasonography (Byrne *et al*., 1985). As much as 70% of spontaneous first trimester miscarriages are associated with chromosomal abnormalities and 45,XO is the single most common aneuploidy within this group with an incidence of 20–25% (Strom *et al.*, 1992; Simpson, 1990) The overall incidence of a 45,XO karyotype in amniocentesis and chorionic villus sampling analysis is approximately 0.2 and 0.4% respectively (Gravholt *et al.*, 1992).

Also, a singleton delivery originated as a twin pregnancy diagnosed as a normal 46,XX and abnormal 45,XO. The abnormal fetus was terminated at 16 weeks leaving the normal singleton

**Table I.** Cytoplasmic transfer cases performed

| Type of cytoplasm transferred to recipient oocytes | No. of procedures | Pregnancies achieved | Offspring delivered | Reference(s) |
|---|---|---|---|---|
| Synchronized fresh oocytes by electrofusion | 3 | 0 | 0 | Cohen *et al.* (1998) |
| Synchronized fresh oocytes by injection (USA) | 30 | 13[a] | 16 | Cohen *et al.* (1997, 1998); Brenner *et al*. (2000); Barritt *et al*. (2000, 2001) |
| Synchronized fresh oocytes by injection (Israel) | 15 | 5 | 6 | J.Levron *et al.* (pers. commun.) |
| Synchronized frozen oocytes by injection | 4 | 1 | 2 | Lanzendorf *et al.* (1999) |
| Asynchronous 3-PN zygotes by injection | 9 | 4 | 5 | Huang *et al.* (1999) |

[a]One pregnancy resulted in a miscarriage.

pregnancy. Out of 17 viable pregnancies in this series of women, the incidence of chromosomal anomalies is 1/17 or 5.9%. While this is higher than the rate of 1–6% major congenital abnormalities observed in the natural population (New York State Dept. of Health, 1999), other issues such as the increase in sex chromosome aneuploidy observed following ICSI may be involved, as well as maternal age.

It is possible that the increase in the incidence of chromosomal abnormalities, as seen in both the miscarriage and viable pregnancy described above, is real. It is conceivable that there may be an improved developmental potential of hybrid cytoplasm in chromosomally normal as well as abnormal embryos. Obviously the sample size is too limited to be able to draw conclusions in this regard. Our patients remain informed of this and other potential risks. This represents a clinical pregnancy rate (13 of 30 cases) in a group of patients with a history of repeated IVF failure and very poor embryo development. Figure 1d shows a single case as an example demonstrating the potential improvement in embryo development.

J.Levron and co-workers (personal communication) performed ooplasmic transfer cases at Tel Hashomer in Israel, and had comparable pregnancy results for this type of patient using the identical technique. Fifteen cases of ooplasmic transfer by injection resulted in five pregnancies and six healthy offspring (Table I).

A modification to our protocol was attempted clinically in four trial cases using frozen–thawed donor oocytes instead of fresh donor and recipient cycles (Lanzendorf *et al.*, 1999), and this resulted in one twin pregnancy and birth (Table I). The authors stated that frozen oocytes eliminated the need for cycle stimulation synchronization, but the generally poor results with clinical oocyte freezing (Trounson and Kirby, 1989; Gook *et al.*, 1995; Mandelbaum *et al.*, 1998; Tucker *et al.*, 1998) and the effects on the nucleus and the ooplasm may have taken their toll. Though damage to the spindle has been described after cold-shock and freezing in the human (Kola *et al.*, 1988), ooplasmic anomalies have been implicated as well after cryopreservation of activated mouse oocytes (Levron *et al.*, 1998).

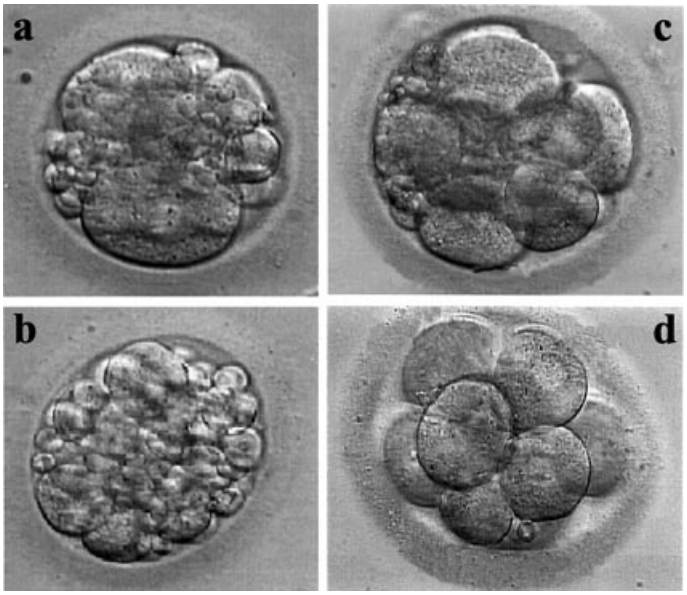


**Figure 1.** Human day 3 embryos from assisted reproduction procedures of one patient. (**a**) Recipient oocyte fertilized with husband's spermatozoon; (**b**) recipient oocyte fertilized with donor spermatozoon; (**c**) donor oocyte fertilized with husband's spermatozoon; (**d**) recipient oocyte injected with donor ooplasm fertilized with husband's spermatozoon. Original magnification, ×400.

Another modification of our protocol was attempted clinically using asynchronous transplantation, by transferring smaller volumes (~5%) of the cytoplasm, between spare donor 3-PN zygotes and recipient M-II oocytes (Huang *et al.*, 1999). These investigators used asynchronous cytoplasmic transfer, despite potential drawbacks seen with this scenario in the mouse (Levron *et al.*, 1996). Also, one of the suggested physiological effects of cytoplasmic transplantation is that total and specific mRNA transcripts reduce over time until genomic activation of the embryo (Clegg and Piko, 1983; Steuerwald *et al.*, 2000). Supplementing a possibly anomalous oocyte with the pool of messenger RNA of older zygotic cytoplasm will therefore bring lowered levels of mRNAs, which we would not expect to work so well for improving embryonic development. In spite of this, the initial results seen using this asynchronous cytoplasmic transplantation have been intriguingly successful (Huang *et al.*, 1999). Asynchronous transfer was attempted in nine patients, who had not achieved a single pregnancy in 85 previous IVF attempts. Four clinical pregnancies resulted in five healthy infants (Table I). While there may be a slight advantage to visualizing the nucleus in 3-PN zygotes, we feel that cytogenetic validation is still required to provide absolute proof that donor nuclear DNA has not been accidentally transferred. One reason is that incomplete sperm decondensation is common in these types of zygotes (Alikani *et al.*, 1992).

An additional problem which may be associated with asynchronous zygotic cytoplasmic transfer is that one can only be assured of polyspermy and thereby clear visualization of nuclei when two spermatozoa are purposefully injected, rather than trust to the uncertainties of chromosomally abnormal donor zygotes being produced. In any case, this protocol does not avoid the need for synchronization of donor and recipient cycles.

## Clinical research

Our laboratory has focused on how the ooplasmic transplantation technique can restore normal growth and viability in developmentally compromised oocytes and embryos. The mechanisms involved are still enigmatic, and it remains unclear as to which cellular components are transferred in the donor ooplasm. Cellular components that might provide a beneficial developmental effect include the pool of stored messenger RNAs and proteins that may regulate oocyte function and embryo development, as well as mitochondria that provide the necessary ATP for essential cellular functions.

Cytoplasmic transfer has provoked considerable interest because of the possibility of mixing mitochondria from one oocyte with another during ooplasmic donation, and possible resulting heteroplasmy of mitochondrial DNA (mtDNA). Although ooplasmic transplantation does not involve the transfer of any nuclear DNA, there is still controversy about the mtDNA that may be transmitted via the donor ooplasm to any resulting offspring. It has been proposed that the resulting offspring are the

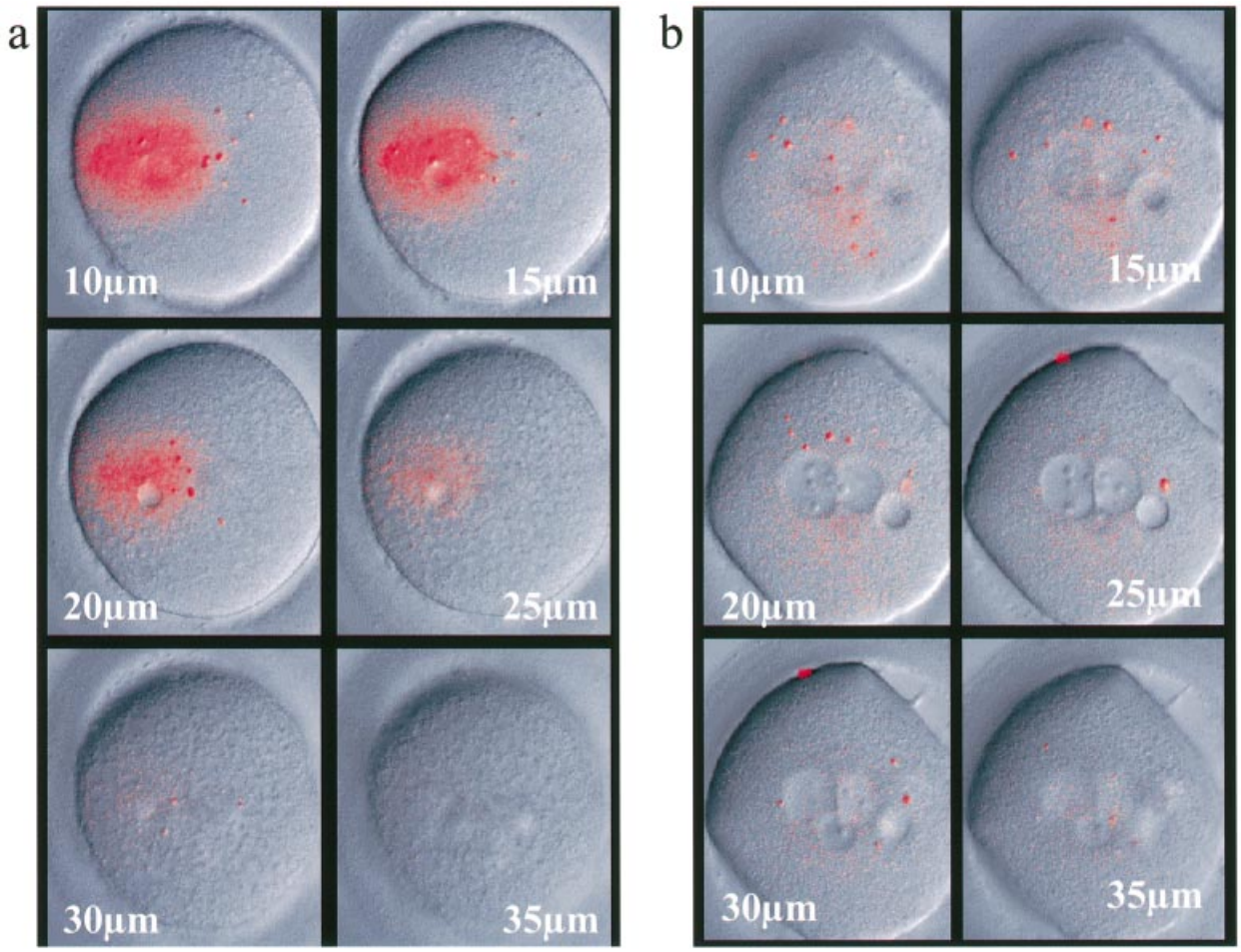


**Figure 2.** Confocal overlaying Nomarski images at 5 µm intervals through (**a**) an oocyte 10 min after ooplasmic injection with stained donor ooplasm. The donor ooplasm was stained with MitoTracker Orange CMTMRos (labels active mitochondria), which can be seen to localize to the injection sites. Confocal overlaying Nomarski images at 5 µm intervals through (**b**) a 3-PN zygote (with a single vacuole) 24 h after ooplasmic injection with stained donor ooplasm. The donor ooplasm was stained with MitoTracker Orange CMTMRos, which can be seen to aggregate in a general way around the pronuclei. The mitochondria are seen diffused throughout the cytoplasm. Original magnification, ×600.

recipients of two nuclear DNA (nDNA) genomes from the mother and the father, and two mtDNA genomes from the mother and the donor. Because little is understood about the maintenance of mitochondrial heteroplasmy and its nuclear regulation during human development, the effects of potential mixing of two mitochondrial populations are still being debated (Robertson, 1999).

Because of the empirical success of cytoplasmic transplantation techniques, and since there is no appropriate animal model which represents poor embryo development of this type, we are subjecting all aspects of ooplasmic transfer to detailed scrutiny, particularly mitochondrial inheritance, function and the occurrence of mtDNA mutations.

Recently, we have attempted to determine if active mitochondria are transferred from donor oocytes to recipient oocytes during ooplasmic donation; and if these mitochondria persist in the resulting preimplantation embryos. Confocal fluorescent imaging was used to investigate mitochondria after ooplasmic transplantation (Barritt *et al.*, 2001). Donor oocytes were stained with MitoTracker Orange CMTMRos (Molecular Probes, Eugene, OR, USA) to label functionally active mitochondria. Ooplasmic transfer was performed by injection of ~5–15% of the donor oocyte volume, using MitoTracker Orange CMTMRos-labelled donor oocytes and unlabelled recipient oocytes. Imaging was performed at 10 min and at 24 and 48 h after injection. Observations 10 min after injection showed recipient oocytes containing MitoTracker Orange CMTMRos-labelled donor mitochondria in a small, localized region (Figure 2a). Observation at 24 h after injection showed some diffusion of the labelled mitochondria throughout the recipient cytoplasm (Figure 2b). Observations at 48 h after injection showed segregation of

**Table II.** Incidence of detectable mtDNA heteroplasmy in non-viable oocytes and embryos, amniocytes, placenta and fetal cord blood

| Tissue | Samples analysed | Samples with mtDNA heteroplasmy |
|---|---|---|
| Oocytes and embryos | 30 | 17 |
| Amniocytes | 7 | 2 |
| Placenta | 12 | 2 |
| Fetal cord blood | 12 | 2 |

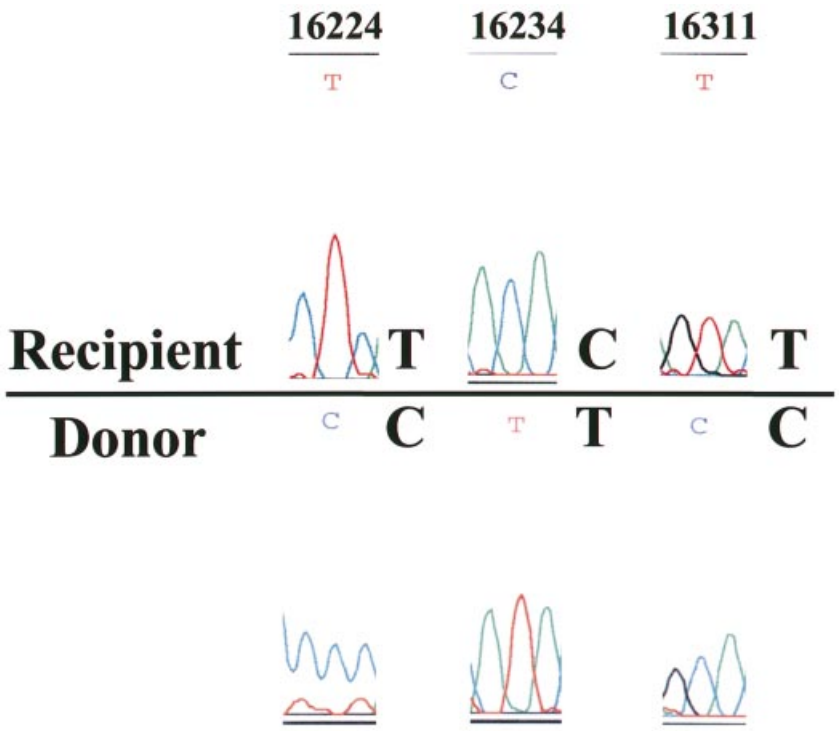


**Figure 3.** Automated DNA chromatographs comparing mitochondrial DNA (mtDNA) sequences from recipient and donor blood at base-pair positions 16 224, 16 234 and 16 311.

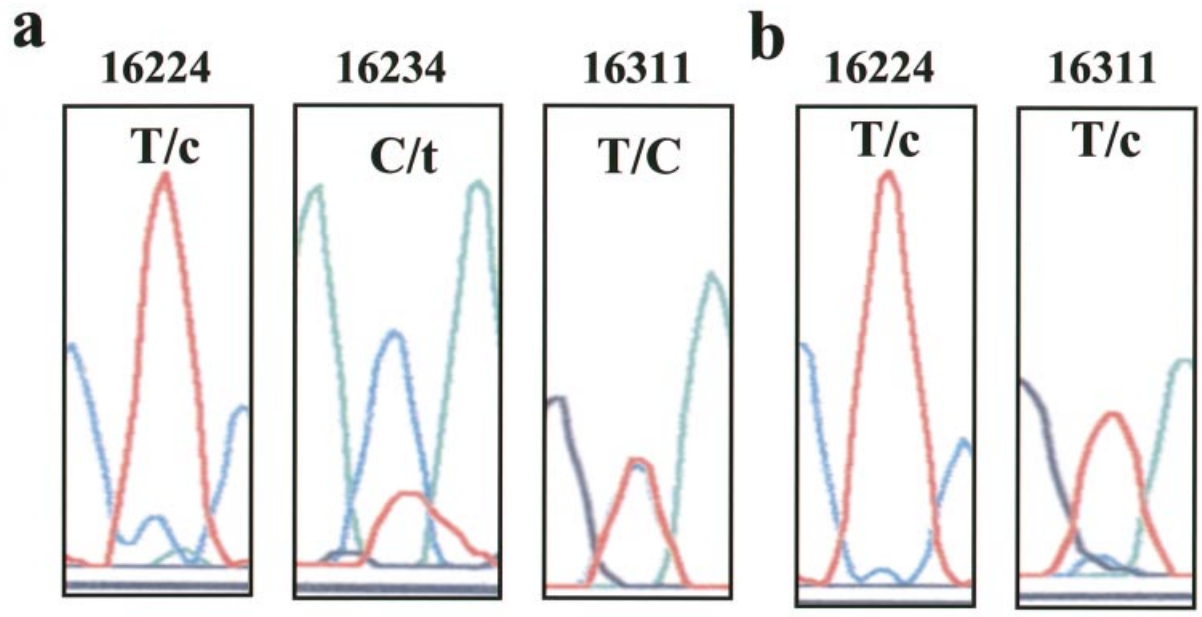


**Figure 4.** Automated DNA chromatographs comparing mtDNA sequences from hypervariable regions of (**a**) placenta and (**b**) fetal cord blood. Upper case nucleotides C and T represent the predominant base pair; lower case nucleotides c and t represent minor DNA sequences.

labelled mitochondria to some, but not all, blastomeres and cellular fragments. These results indicated that ooplasm extracted from donor oocytes contains active mitochondria, which are transferred to recipient oocytes by ooplasmic donation. Thus, the recipient oocyte contains a heteroplasmic population of mitochondria immediately after ooplasmic transfer. The nuclear regulation of heteroplasmic mitochondria in the cytoplasm is currently under investigation, as well as the potential transfer of other organelles and proteins.

We have also studied mtDNA sequences in tissues of clinical cases resulting from ooplasmic transfer (Brenner *et al.*, 2000; Barritt *et al.*, 2001). The hypervariable region of the mtDNA was

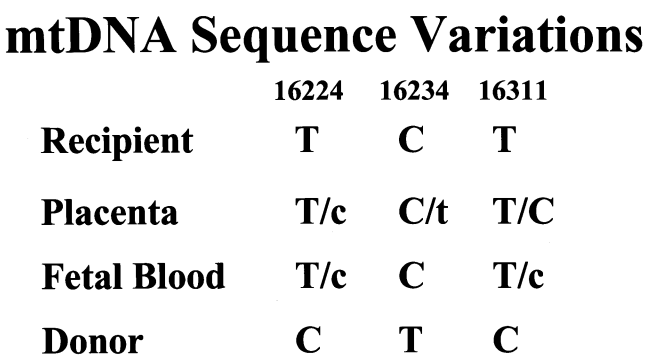


**Figure 5.** Summary of mtDNA sequence variations at base-pair positions 16 224, 16 234 and 16 311 in donor, recipient, fetal cord blood and placenta. Upper case nucleotides C and T represent the predominant base pair; lower case nucleotides c and t represent minor DNA sequences.

amplified and sequenced from both donor and recipient bloods, resulting in mtDNA fingerprints. The status of mtDNA heteroplasmy was then determined in non-viable single oocytes and embryos from clinical ooplasmic transplantation cases. In addition, products of conception from pregnancies achieved—including amniocytes, placenta and fetal cord blood—were analysed for heteroplasmy. In addition to the maternal mtDNA that was present, a small proportion of donor mtDNA was detected in some samples with the following frequencies: oocytes and embryos ($n = 17/30$), amniocentesis ($n = 2/7$), placentae ($n = 2/12$) and fetal cord blood samples ($n = 2/12$) (Table II). Mitochondrial DNA sequencing chromatograms demonstrate these findings (Figures 3, 4 and 5). Nuclear DNA fingerprinting of the chromosomal DNA showed that there was no inherited chromosomal DNA from the donor; and that maternal chromosomal DNA was inherited only from the recipient in both placenta and fetal cord blood of the babies. These findings show that ooplasmic transfer can alter the normal inheritance of mtDNA, resulting in sustained heteroplasmy representing both donor and recipient mtDNA; however, it does not alter the nuclear DNA inheritance patterns. These results also suggest that the heteroplasmic mitochondrial population persists and may be replicated during fetal development. It has been demonstrated that the mitochondrial genome is not replicated from the mature oocyte stage until the blastocyst stage during mammalian embryo preimplantation development (Piko and Taylor *et al*., 1987). Based on examination of the results obtained so far, we believe there is no reason to consider the minimal proportion of detected hypervariable mtDNA heteroplasmy as harmful, because it is known to occur naturally in normal individuals (Howell *et al*., 1992; Bendall and Sykes, 1995; Ivanov *et al*., 1996; Wilson *et al*., 1997). Detrimental mitochondrial heteroplasmy, resulting in mitochondrial diseases, occurs when mitochondrial populations contain rearranged mtDNA, with decreased functionality, in a mixed population with normal mitochondria (Brown and Wallace, 1994). Continued investigations are ongoing to explore the quantity of donor mtDNA in these tissues and children as they grow older.

Finally, although it is possible that the transfer of donor mitochondria is providing a benefit to the recipient oocyte, no evaluation of mitochondrial transfer was reported in other studies (Huang *et al*., 1999; Lanzendorf *et al*., 1999.

Other investigations into what factors may be involved with the success of ooplasmic transfer have been attempted (Van Blerkom *et al*., 1995, 1998). It has been demonstrated that reduced ATP content in human oocytes does not affect meiotic maturation, because it occurs over a wide range of ATP content. However, a higher potential for continued embryogenesis and implantation in the human is associated with embryos developing from oocytes with ATP contents of $\geq 2$ pmol/oocyte (Van Blerkom *et al*., 1995). A mouse model in which centrifuged donor mitochondria-enriched ooplasm is injected, showed that the ATP content of recipient oocytes 36 h after injection was increased by ~14% (Van Blerkom *et al*., 1998). This investigation did not determine if the potential of these injected mouse oocytes changed in association with embryogenesis or implantation. It was first reported that nuclear transplantation using germinal vesicle-stage oocytes had the capacity to mature and develop, thus providing a model to investigate nuclear/cytoplasmic interactions of mitochondria as well as imprinting mechanisms (Kono *et al*., 1996). Recently, a mouse model to investigate mitochondrial dysfunction has been developed by isolating cybrids containing mtDNA-defective mutants and introducing them into fertilized oocytes. This will provide a system to study how mutant mtDNAs are transmitted and distributed in differentiating tissues (Inoue *et al*., 2000). Further studies will be required to determine the role of mitochondria transferred during cytoplasmic transfer.

## Future outlook

Considering the experimental nature of these cytoplasmic investigations, and the theoretical concerns raised by some, a conservative approach must be maintained. Babies have been born after the transfer of fresh and frozen ooplasm and fresh zygote cytoplasm (Cohen *et al*., 1998; Huang *et al*., 1999; Lanzendorf *et al*., 1999). At present, there is insufficient evidence to demonstrate that any of these techniques is effective by itself. Some improvement in embryonic development was apparent in a few patients, and the promising pregnancy rates may be indicative of some underlying mechanism.

Cellular components that might benefit development include the pool of stored messenger RNAs and proteins that may regulate oocyte function and embryo development, as well as mitochondria and other organelles. Our studies demonstrate that donor mitochondria function normally after synchronous transfer, and are passed forward to blastomeres. The likely outcome of this mitochondrial 'boost' is the replication and incorporation of donor mitochondria into the inner cell mass and any resulting fetal tissues. At least two babies have been shown to contain the mtDNA from both the recipient and donor, but mtDNA heteroplasmy is not always evident. Only 50% of the non-viable oocytes, embryos and tissues had detectable mtDNA heteroplasmy. It is possible that a more sensitive molecular assay would detect a greater proportion, but it may also be true that the donor mitochondria persist and replicate in the recipient cytoplasm only under certain circumstances. When present, however, the two detected populations of mtDNA can persist at least until birth. Whether this mitochondrial alteration will continue into the germline of offspring resulting from these procedures remains to be seen. Additionally, we continue to investigate the percentage

of donor mitochondria passed to any children through ooplasmic transplantation using other molecular techniques.

During synchronous ooplasmic transfer, donor mitochondria become mixed with recipient mitochondria of the oocyte. This may be because the donor and recipient mitochondria are indistinguishable except at the mtDNA level. However, procedures that result in a mixture of asynchronous cytoplasm, may actually lead to donor mitochondria being recognized as foreign, similar to sperm mitochondria, and thus being eliminated. However, mtDNA testing has not been reported in asynchronous transfers.

In a totally asynchronous nuclear transfer-derived cloned sheep, somatic cell mitochondria were not found in the offspring (Evans *et al*., 1999). An alternative technique, using injection of naked somatic cell nuclei has now resulted in cloned mice (Wakayama *et al*., 1998; Wakayama and Yanagimachi, 1999). These predominantly mitochondria-free injections demonstrate, at least in the mouse, that 'foreign' mitochondria can survive for several generations (T.Wakayama, personal communication). Calves cloned from embryonic cells are often heteroplasmic (Hiendleder *et al*., 1999), also indicating some compatibility of oocyte and embryo mitochondria.

The general application of these advanced techniques by human IVF laboratories is most likely years away. Serious problems remain to be overcome, including synchronizing donor and recipient cycles, cryopreserving donor oocytes, and/or the asynchronous transplantation of cytoplasm between embryos and oocytes.

Similar micromanipulation techniques could also be extended to address other issues in human reproduction. One approach would be to circumvent the transmission of mitochondrial disease. A variety of serious diseases are associated with specific mtDNA mutations (Brown and Wallace, 1994), and such diseases may be transmitted maternally through mitochondria present in the oocyte. Cytoplasmic donation adds a small amount of cytoplasm to the recipient oocyte, estimated to be only 5–15% of the volume. Such a proportional increase in normally functioning mitochondria may overcome a potential energy production deficiency. However, to overcome heteroplasmy for a common mitochondrial mutation, essentially the entire oocyte cytoplasm would have to be exchanged. This may be accomplished through germinal vesicle or nuclear transplantation (Zhang *et al*., 1999). It is possible that a negligible amount of mitochondria would be transferred with a germinal vesicle-karyoblast; however, nuclear transplantation would replace the majority of defective mitochondria, by transferring the nucleus to an enucleated oocyte with normal mitochondria. Although the use of these advanced micromanipulation techniques is still years away from clinical practice, and the potential interactions between the nuclear and the mitochondrial genomes are still being determined, these methods could overcome the transmission of mitochondrial disease.

Experiments investigating the use of cytoplasts for gene therapy of mitochondrial diseases have been performed previously (Kagawa and Hayashi, 1997). These methods transfer normal mtDNA into mutant cells by cytoplast fusion, and the cybrids formed can complement the defective mtDNA correctly and safely. This study demonstrated the compatibility of different mtDNA genomes, and that creating heteroplasmy within single somatic cells, could overcome mtDNA defects. The cybrid formation by cytoplast fusion may be applied to oocytes or blastomeres during early preimplantation development to overcome the transfer of mitochondrial diseases in the future.

Another issue involving a mixture of different nuclear and cytoplasmic constituents would be the creation of artificial gametes. The haploidization and formation of artificial gametes by fusion of oocytes and non-germline cells will create gametes with potential for use in reproduction. However, these new artificial gametes will have a nuclear genetic component from a somatic cell and a mitochondrial component from another person's germline cell. The interactions between these two genomes may not behave in the same way as is associated with normal gamete production. Currently, we are investigating specific genes that are associated with poor embryonic development seen in the clinical IVF laboratory. As we determine the genes involved we will be able to clarify, and perhaps diagnose and treat, individual genetic developmental deficits (Cohen *et al.*, 1999).

Future development of subcellular diagnostic tools for assessing specific mRNAs in ooplasm is underway in our laboratory (Steuerwald *et al.*, 2000). This new technique will allow the 'biopsy' of oocytes via small ooplasmic samples that can be used for the genetic analysis of mRNAs involved with oocyte health and developmental potential. The quantification of developmental gene transcripts in the oocyte may be correlated with chromosomal and/or metabolic anomalies. When we can test for, and identify, the specific cause of an oocytes' deficiency we will be able to design therapeutics targeted at each individual oocyte. The need for cytoplasmic donation will decrease when we can determine the causes of the developmental defects and overcome them with targeted therapeutics, though how these future techniques will be applied in general IVF laboratories remains to be seen. However, without further extensive research into the mechanisms involved we cannot recommend the widespread application of any of these techniques.

## Conclusions

Ooplasmic transfer has been performed in a very limited number of cases, and is still considered experimental. While the success rate with this procedure is above expectations, we cannot at this point in time recommend its widespread application. Anomalies present in oocytes or spermatozoa of patients with abnormally developing embryos may be expressed later during development in unexpected ways, particularly considering general improvements in culture and embryo survival that have been made since the advent of IVF. Ooplasmic transfer may be considered an extra step in a series of interventions that has been suggested during the past decades. The procedure may either emphasize inherent conditions or alter certain developmental processes. Continued investigation into which ooplasmic constituents are transferred—and how they might potentially benefit couples with previously poor embryo development—is still required.

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## Note added in proof

We have been notified that one of the children, the male of a male–female twin has been diagnosed with a Pervasive Developmental Disorder at 18 months, a spectrum of diagnosis that has an incidence of 1 in 500 children. This information will be given to patients and addressed with the Internal Review Board.

## References


Alikani, M., Adler, A., Reing, A.M. *et al*. (1992) Subzonal sperm insertion and the frequency of gamete fusion. *J. Assist. Reprod. Genet*., **9**, 97–101.

Barritt, J.A., Brenner, C.A., Willadsen, S. and Cohen, J. (2000) Spontaneous and artificial changes in human ooplasmic mitochondria. *Hum. Reprod.*, **15**, 207–217

Barritt, J.A., Brenner, C.A., Malter, H. and Cohen, J. (2001) Mitochondria in human offspring derived from ooplasmic transplantation. *Hum. Reprod.*, **16**, 513–516.

Bendall, K.E. and Sykes, B.C. (1995) Length heteroplasmy in the first hypervariable segment of the human mtDNA control region. *Am. J. Hum. Genet*., **57**, 248–256.

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

Brenner, C.A, Barritt, J.A., Willadsen, S.M. and Cohen, J. (2000) Mitochondrial DNA heteroplasmy after human ooplasmic transplantation. *Fertil. Steril.*, **74**, 573–578.

Brown, M.D. and Wallace, D.C. (1994) Molecular basis of mitochondrial disease. *J. Bioenergy Biomembr.*, **26**, 273–289.

Byrne, J., Warburton, B.J., Kline, J. *et al.* (1985) Morphology of early fetal deaths and their chromosomal characteristics. *Teratology*, **32**, 297–315.

Clegg, K.B. and Piko, L. (1983) Quantitative aspects of RNA synthesis and polyadenylation in 1-cell and 2-cell mouse embryos. *J. Embryol. Exp. Morphol*., **74**, 169–182.

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.

Cohen, J., Brenner, C., Warner, C. *et al.* (1999) Genetics of the fertilizing egg. In Jansen, R. and Mortimer, D. (eds), *Towards Reproductive Certainty*. Parthenon Publishing, New York, pp. 231–246.

Evans, M.J., Gurer C., Loike J.D. *et al*. (1999) Mitochondrial DNA genotypes in nuclear transfer-derived cloned sheep. *Nature Genet*., **23**, 90–93.

Flood, J.T., Chillic, C.F., Van Uem, J.F. *et al.* (1990) Ooplasmic transfusion: prophase germinal vesicle oocytes made developmentally competent by microinjection of metaphase-II egg cytoplasma. *Fertil. Steril*., **53**, 1049–1054.

Gook, D.A., Schiewe, M.C., Osborn, S.M. *et al.* (1995) Intracytoplasmic sperm injection and embryo development of human oocytes cryopreserved using 1,2-propanediol. *Hum. Reprod.*, **10**, 2637–2641.

Gravholt, C.H., Juul, S., Naeraa, R.W. and Hansen, J., (1997) Prenatal and postnatal prevalence of Turner syndrome. A registry-based study. *Ugeskr Laeger.* **159**, 3160–3166.

Hiendleder, S., Schmutz, S.M., Erhardt, G. *et al.* (1999) Transmitochondrial differences and varying levels of heteroplasmy in nuclear transfer cloned cattle. *Mol. Reprod. Dev.,* **4**, 24–31.

Howell, N., Halvorson, S., Kubacka, I. *et al.* (1992) Mitochondrial gene segregation in mammals: is the bottleneck always narrow? *Hum. Genet.*, **90**, 117–120.

Huang, C.-C., Cheng, T.-C., Chang H.-H. *et al.* (1999) Birth after the injection of sperm and the cytoplasm of tripronucleate zygotes into metaphase II oocytes in patients with repeated implantation failure after assisted fertilization procedures. *Fertil. Steril*., **74**, 702–706.

Inoue, K., Nakada, K., Ogura, A. *et al*. (2000) Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. *Nature Genet*., **26**, 176–181.

Ivanov, P.L., Wadhams, M.J., Roby, R.K. *et al*. (1996) Mitochondrial DNA sequence heteroplasmy in the Grand Duke of Russia Georgij Romanov establishes the authenticity of the remains of Tsar Nicholas II. *Nature Genet*., **4**, 417–420.

Kagawa, Y. and Hayashi, J.I. (1997) Gene therapy of mitochondrial diseases using human cytoplasts. *Gene Ther*., **4**, 6–10.

Kola, I., Kirby, C., Shaw, J. *et al.* (1988) Vitrification of mouse oocytes results in aneuploid zygotes and malformed fetuses. *Teratology*, **38**, 467–474.

Kono, T., Obata, Y., Yoshimzu, T. *et al*. (1996) Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. *Nature Genet.*, **13**, 91–94.

Lanzendorf, S., Mayer, J., Toner, J. *et al.* (1999) Pregnancy following transfer of ooplasm from cryopreserved-thawed donor oocytes in to recipient oocytes. *Fertil. Steril*., **71**, 575–577.

Levron, J., Willadsen, S., Bertoli, M. *et al.* (1996) The development of mouse zygotes after fusion with synchronous and asynchronous cytoplasm. *Hum. Reprod.*, **11**, 1287–1292.

Levron, J., Willadsen, S.M., Schimmel, T. *et al.* (1998) Cryopreservation of activated mouse oocytes and zygote reconstitution after thaw. *Hum. Reprod.*, **13** (Suppl. 4), 109–116.

Mandelbaum, J., Belaisch-Allart, J., Junca, A.M. *et al.* (1998) Cryopreservation in human assisted reproduction is now routine for embryos but remains a research procedure for oocytes. *Hum. Reprod*., **13** (Suppl. 3), 161–177.

Muggleton-Harris, A., Whittingham, D.G. and Wilson, L. (1982) Cytoplasmic control of preimplantation development *in vitro* in the mouse. *Nature*, **299**, 460–462.

New York State Department of Health, USA (1999) Congenital Malformations Registry Annual Report: Statistical Summary of Children born in 1995 and diagnosed through 1997. New York State Department of Health, Section IV, 22 (www.health.state.ny.us/nysdoh/cur/1995/contents)

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.

Pratt, H.P.M. and Muggleton-Harris, A.L. (1988) Cycling cytoplasmic factors that promote mitosis in the cultured two-cell mouse embryo. *Development*, **104**, 115–120.

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

Simpson, J.L. (1990) Incidence and timing of pregnancy losses: Relevance to evaluating safety of early prenatal diagnosis. *Am J. Med. Genet.* **35**, 165–173.

Steuerwald, N., Cohen, J., Herrera, R.J. *et al.* (2000) Quantification of mRNA in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. *Mol. Hum. Reprod*., **6**, 448–453.

Strom, C., Ginsberg, N., Applebaum, M. *et al.* (1992) Analyses of 95 first trimester spontaneous abortions by chorionic villus sampling and karyotype. *J. Assist Reprod Genet*, **9**, 458–461.

Trounson, A. and Kirby, C. (1989) Problems in the cryopreservation of unfertilized eggs by slow cooling in dimethyl sulfoxide. *Fertil. Steril*., **52**, 778–786.

Tucker, M.J., Morton, P.C., Wright, G. *et al.* (1998) Clinical application of human egg cryopreservation. *Hum. Reprod*., **13**, 3156–3159.

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

Van Blerkom, J., Sinclair, J. and Davis, P. (1998) Mitochondrial transfer between oocytes: potential applications of mitochondrial donation and the issue of heteroplasmy. *Hum. Reprod.*, **13**, 2857–2868.

Wakayama, T. and Yanagimachi, R. (1999) Cloning of male mice from adult tail-tip cells. *Nature Genet.*, **22**, 127–128.

Wakayama, T., Perry, A.C., Zuccotti, M. *et al.* (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature*, **394**, 369–374.

Wilson, M.R., Polanskey, D., Replogle, J. *et al.* (1997) A family exhibiting heteroplasmy in the human mitochondrial DNA control region reveals both somatic mosaicism and pronounced segregation of mitotypes. *Hum. Genet.*, **100**, 167–171.

Zhang, J., Wang, C.W., Krey, L. *et al.* (1999) *In vitro* maturation of human preovulatory oocytes reconstructed by germinal vesicle transfer. *Fertil. Steril.*, **71**, 726–731.


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