Is Paternal Mitochondrial DNA Transferred to the Offspring Following Intracytoplasmic Sperm Injection?

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During intracytoplasmic sperm injection (ICSI) the whole sperm, including head, midpiece and tail, is injected into the middle area of the oocyte. To find out what happens to the sperm mitochondria after ICSI, we checked the first six children born after ICSI treatment for occurrence of paternal mitochondrial DNA (mtDNA). The difference between maternal and paternal mtDNA in the investigated couples in our study was confined to single-base pair substitutions and we had to rely on restriction enzyme cleavage to differentiate between the mitochondrial genomes of the parents. With this kind of assay we were able to reach a sensitivity of about 0.2% for the paternal mtDNA. However, as uneven partition between tissues of heteroplasmic mtDNA is expected to occur, it would not be unlikely that an enrichment to 0.2% would occur in a given tissue if paternal mtDNA was transmitted by the ICSI procedure. We did not detect this level in the blood in any of the six children.

KEY WORDS: in vitro fertilization; intracytoplasmic sperm injection; paternal inheritance; mitochondrial DNA.

INTRODUCTION

In attempts to overcome severe male subfertility, microsurgical fertilization techniques have been developed and clinically applied during the last 5 years. The most successful, but also the most invasive, among these techniques is intracytoplasmic sperm injection (ICSI) (1). Present knowledge about the process of fertilization of oocytes in vivo is based predominantly on extrapolation of data from different types of in vitro fertilization studies. However, one study utilizing

preembryos for morphological examination which had been flushed out of the fallopian tube in connection with an operative procedure has also been reported (2). Whether or not the whole midpiece and the sperm tail generally enters through the oolema and can move freely in the cytoplasm or remains in close connection with the oolema is thus partly an unsolved question. The human mitochondrion contains its own genome and has an independent replication, transcription, and translation system. When ICSI is applied the whole spermatozoon including head, midpiece, and tail is injected and deposited in the center of the oocyte. The question thus arises whether this procedure may introduce an increased risk for inheritance of paternal mitochondrial DNA. Since the ICSI technique was introduced at our IVF unit in March 1993, the first children were born in early 1994 and the first five deliveries, resulting in six healthy children, were selected for this investigation.

MATERIALS AND METHODS

Patients. The five couples all had relatively long-standing infertility which was attributed predominantly to male infertility. Conventional IVF had failed in three of the couples; the sperm quality was so poor that conventional IVF never was an option in two of the couples. The women were all regular in their cycles and were judged fertile on the basis of a standard infertility workup.

Following down regulation by daily administration of a gonadotropin releasing hormone (GnRH) analogue (Suprecur) for 3 weeks, starting on the first day of the cycle, purified urinary follicle stimulating hormone (FSH; Metrodin HP; Serono, Italy) was given in a daily subcutaneous injection (150–225 IU/day). The controlled ovarian hyperstimulation (COH) was moni-

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tored by repeated sonographic investigations combined with analyses of serum estradiol. When the leading follicle had reached a diameter of 18 mm, a single injection of human chorionic gonadotropin (hCG; Profasi; Serono, Italy) was given subcutaneously at a dose of 10,000 IU.

Forty-eight hours after the hCG injection retrieval of oocytes was performed by use of transvaginal ultrasound-guided follicular puncture. The oocytes were evaluated for maturity and all metaphase II oocytes injected (3). Forty-six to forty-eight hours following ICSI the embryos were replaced by standard transcervical procedures.

Analysis of mtDNA. Preparation of DNA from lymphocytes was performed as described earlier (4). The displacement loop (D-loop) is the most polymorphic region of the human mtDNA genome so, to differentiate between maternal and paternal mtDNA in each family, a 1558-bp PCR fragment of the mitochondrial D-loop was amplified. The standard PCR protocol used forward oligonucleotide primer (FONP)98 [nucleotides (nt) 15,791–15,810] and backward oligonucleotide primer (BONP)79 (nt 761–780). Numbering of nucleotides was according to the Cambridge human mtDNA sequence (5). The D-loop fragment was sequenced by automatic sequencing (6). With the primers FONP38 (nt 1–20) and BONP24 (nt 181–200), informative polymorphisms were identified in each family.

An identified polymorphism which created a new restriction enzyme cleavage site for restriction enzyme *ApaII* or *MnII* was chosen to investigate the occurrence of paternal mtDNA in the offspring. A PCR technique with the primer FONP38 and primer BONP24 was used to obtain a 200-bp PCR fragment of the D-loop. The last cycle of PCR was performed after the addition of γ -³²P-radiolabeled primer BONP24. About 0.1 μ g of radiolabeled PCR fragment was digested with 20 U of *ApaII* or 20U *MnII* overnight. The resulting fragments were separated by electrophoresis in 8% polyacrylamide gels. Scanning was performed with the Molecular Dynamics PhosphorImager and the results were analyzed by ImageQuant software using the "area of rectangles" method as described previously (7). The sensitivity of the assays of paternal mtDNA was determined by serial dilutions of paternal lymphocyte DNA in maternal lymphocyte DNA in each family. Paternal lymphocyte DNA (0.00, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1 ng) was mixed with maternal DNA (100 ng) and analyzed as above together with samples from the offspring. Various lengths of exposure of the phosphor

screen were used, to get a maximal pixel value for the fragment of maternal mtDNA.

RESULTS

The first six babies in this series were investigated together with their parents to address the question whether paternal mitochondrial DNA is transmitted to the offspring. The obstetrical data on the babies are summarized in Table I.

Informative polymorphisms with substitutions of single nucleotides were identified in all families. Substitutions which changed restriction enzyme cleavage patterns were used to differentiate between paternal and maternal mtDNA. In cases I and V a polymorphism at position 152, where the normal C was found to be substituted by a T in the other parent, was identified. This nucleotide substitution creates a new cleavage site for the restriction enzyme *MnII*, which was used for the analyses. The ability to detect minute amounts of paternal mtDNA in predominantly maternal mtDNA was tested by the addition of different amounts of paternal DNA to maternal DNA (Figs. 1A and B). In the family of case I the mother carries the polymorphism, which enabled cleavage by *MnII*. In this family the addition of 0.3 ng of paternal DNA to 100 ng of maternal DNA was needed to get a response, which differed significantly from the background. In the family of case V the mtDNA with the cleavage site was found in the father. This gave a lower background signal and enabled detection of paternal mtDNA at a level of 0.2%.

In the families of cases II, III, and IV a polymorphic site was found in nucleotide 73. In the fathers a G substituted for an A at this position, which created a cleavage site for the restriction enzyme *ApaII*. The detection limit of this assay was at the level of 0.2% of paternal mtDNA (Fig. 1C).

No signal above background which corresponded to paternal mtDNA was found in any of the children, i.e., the occurrence of paternal mtDNA was found to be <0.3% in case I and <0.2% in cases II–V.

DISCUSSION

Transmission of paternal mtDNA to the six investigated children who were products of in vitro fertilization by the ICSI technique was not found in the present study. It has been demonstrated convincingly that the mitochondrial genome is inherited exclusively from

Table I. Obstetrical Data on the Babies^a

Patient No.	Maternal age	GA (wk)	BW (g)	(% dev)	Length (cm)	Sex	Mode of delivery	AS	Malformation
1	35	39+1	2965	(-13)	47	m	Vaginal	9,10	No
2	29	37+3	2675	(-13)	46	f	Vaginal	9,10	No
3	31	39+4	3880	(+11)	51	f	Vaginal (VE)	9,10	VSD
4	31	35+0	1450	(-44)	42	f	CS	9,10	No
5I	33	36+2	2215	(-22)	47	f	Vaginal	9,10	No
5II	33	36+2	2635	(-8)	49	m	Vaginal breech	7,10	No

^a GA, gestational age; BW, birth weight; AS, Apgar score; VE, vacuum extraction; CS, cesarean section; VSD, ventricular septum defect. There were four singletons and one pair of twins (5 I and 5 II). All singletons underwent early amniocentesis with normal karyotypes. One pregnancy (patient 4) was complicated with severe preeclampsia and intrauterine growth retardation and required emergency cesarean section at a gestational age of 35 weeks. The baby weighed 1450 g, had a full Apgar score, stayed 25 days at the neonatal ward, and showed normal neonatal development. The other pregnancies were normal, without any complications. All babies were examined by an experienced pediatrician. One baby (patient 3) had a VSD without any clinical symptoms. It was defined as a minor malformation because of verified spontaneous close. The other babies appeared normal and healthy.

the mother (8–10). Recently paternal transmission of the mtDNA has been shown to occur in interspecific crossings (11), but accumulated data indicate that intra-specific mtDNA derived from sperm or other sources is efficiently eliminated at an early stage of embryonic development after normal in vivo fertilization and in different experimental situations including, e.g., injection of testes and liver mitochondria into zygotes of mice. However, the fate of the paternal mtDNA after the ICSI procedure, when the whole sperm including

midpiece and tail is placed in the center of the ovum without any prior contact with the zona pellucida or oolemma, has not been investigated but differences in basic cellular processes may well occur. It has, for instance, been shown that ICSI differs from natural fertilization in terms of a changed calcium flux and delayed cortical reaction (12). The present investigation was done as a first attempt to address the issue if the ICSI procedure would interfere with the normal mechanism for recognition and elimination of sperm

Fig. 1. Standard curve for detection of paternal lymphocyte DNA in maternal lymphocyte DNA by the serial dilution method in each family. To differentiate between maternal and paternal mtDNA in each family, a 200-bp PCR fragment of the mitochondrial D-loop was amplified. The last cycle PCR was performed after addition of γ -³²P-radiolabeled primer. The resulting fragments after cleavage of restriction enzyme were separated by electrophoresis in an 8% polyacrylamide gels. Scanning was performed with a Molecular Dynamics PhosphorImager (see text). (A) Standard curve in the family of case I. The mother carries the C152T polymorphism, which created a new restriction enzyme cleavage site for restriction enzyme *MnII*; <0.3% of paternal mtDNA was detected in the serial dilution. (B) Standard curve in which a C152T polymorphism was detected in the father of the family of case V and cleaved with restriction enzyme *MnII*; <0.2% of paternal mtDNA was detected in the serial dilution. (C) Standard curve in which an A73G polymorphism was detected in the fathers of the families of cases II, III, and IV. This polymorphism created a new restriction enzyme cleavage site for restriction enzyme *ApalI*; <0.2% of paternal mtDNA was detected in the serial dilution.

mitochondria and the sperm mtDNA, resulting in transmission of paternal mtDNA to the offspring. The sperm mitochondria seems to be recognized and eliminated from the fertilized egg at the late pronucleus phase in normal intraspecific matings of mice (11). However, in interspecific crossings Kaneda *et al.* were able to detect transmission of paternal mtDNA in 25 of 45 neonates. They were also able to show that the recognition was dependent on a nuclear-encoded factor and not dependent on species differences in the mtDNA genome. These experiments address several points that deserve to be commented on in relation to the ICSI procedure and possible transmission of paternal mtDNA. Of interest is the documentation of an uneven distribution between different tissues of paternal mtDNA in the interspecific crossings. If paternal mitochondria had an equal ability to survive and replicate during development, this would mean that the paternal mtDNA would be enriched in some tissue and diluted in others. It has been estimated that the human ovum contains about 100,000 mtDNA copies (13). The number of mtDNA copies in ejaculated mature spermatozoa has been estimated to be about 100 (14). The ratio of sperm-to-ovum mtDNA would thus amount to about 0.1%. The difference between maternal and paternal mtDNA in the investigated couples in our study was confined to single-base pair substitutions and we had to rely on restriction enzyme cleavage to differentiate between the mitochondrial genomes of the parents. With this kind of assay we were able to reach a sensitivity of about 0.2% for the paternal mtDNA. However, as uneven partition between tissues of heteroplasmic mtDNA is expected to occur, it would not be unlikely that an enrichment to 0.2% would occur in a given tissue if paternal mtDNA were transmitted by the ICSI procedure. We did not detect this level in blood in any of the six children.

Since more than 500 children have been born so far as a result of our ICSI program, there is naturally a possibility to expand the study group. However, we feel that a more interesting group to investigate in this connection is couples in whom epididymal and testicular spermatozoa (30 children born in Göteborg) have been utilized, since the number of mitochondria in this type of spermatozoa is higher (15). Another group of interest for the future might be children born after utilization of spermatids for fertilization since the spermatids contain an even higher number of mitochondria (15). In conclusion, it must, however, be emphasized that the presence of paternal mtDNA per se does not lead to defects or diseases in the offspring, at least not in a short-term perspective. On the other

hand, the mere suspicion that foreign DNA is permanently introduced into the genome after fertilization with the ICSI technique deserves further attention.

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