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Evaluation of Parental Mitochondrial Inheritance in Neonates Born after Intracytoplasmic Sperm Injection

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Summary

Intracytoplasmic sperm injection (ICSI) is now used when severe male-factor infertility has been documented. Since defective mitochondrial functions may result in male hypofertility, it is of prime importance to evaluate the risk of paternal transmission of an mtDNA defect to neonates. DNA samples from the blood of 21 infertile couples and their 27 neonates born after ICSI were studied. The highly polymorphic mtDNA D-loop region was analyzed by four PCR-based approaches. With denaturing gradient gel electrophoresis (DGGE), which allows 2% of a minor mtDNA species to be detected, the 27 newborns had a DGGE pattern identical to that of their mother but different from that of their father. Heteroplasmy documented in several parents and children supported an exclusive maternal inheritance of mtDNA. The parental origin of the children's mtDNA molecules also was studied by more-sensitive assays: restriction-endonuclease analysis (REA) of $\alpha[^{32}P]$-radiolabeled PCR products; paternal-specific PCR assay; and depletion of maternal mtDNA, followed by REA. We did not detect paternal mtDNA in nine neonates, with a sensitivity level of 0.01% in five children, 0.1% in two children, and 1% in two children. The estimated ratio of sperm-to-oocyte mtDNA molecules in humans is 0.1%–1.5%. Thus, we conclude that, in these families, the ICSI procedure performed with mature spermatozoa did not alter the uniparental pattern of inheritance of mtDNA.

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Introduction

Among different in vitro–fertilization (IVF) techniques, intracytoplasmic sperm injection (ICSI) is now proposed to infertile couples when severe male-factor infertility has been documented (Van Steirteghem et al. 1993, 1995, 1998). It is noteworthy that this technique, which consists of the injection of a single spermatozoon into oocytes, introduces the sperm midpiece with paternal mitochondria into the ooplasm, thereby raising the possibility of paternal contribution to mitochondrial inheritance. Indeed, it is classically assumed that, in mammals, the mitochondrial genome, in contrast to the nuclear genome, has an exclusively maternal mode of inheritance. Several hypotheses have been advanced to explain such a phenomenon (Smith and Alcivar 1993). In mammalian germ cells, the ratio between paternal and maternal mtDNA molecules has a range of 1:1,000–15:1,000 (Birky 1983; Chen et al. 1995; Manfredi et al. 1997). This can explain how the different mammalian offspring scored as not having the male's mtDNA (Hutchison et al. 1974; Giles et al. 1980; Gyllensten et al. 1985) may have it in amounts undetectable by the method used. However, by means of PCR, uniparental mtDNA transmission has also been documented in intraspecific hybrids of the *Mus musculus* mouse strain (Kaneda et al. 1995). By contrast, paternally inherited mtDNA molecules have been detected in mice resulting from interspecific crosses (Gyllensten et al. 1991; Kaneda et al. 1995; Shitara et al. 1998). These latter data suggest that, because of the large genetic differences between the two mouse strains used in the study, the mtDNA could escape mechanisms that inactivate paternal mitochondria. In addition, it has been shown that, although paternal mitochondria can be found in the fertilized oocytes, they are selectively degraded through a selective silencing process that occurs early in development (Szollosi 1965; Hiraoka and Hirao 1988; Birky 1995). However, none of these studies was performed in human fertilized oocytes, in which the mechanism of exclusive

maternal origin of mitochondria and mtDNA remains unclear.

Since the nature of the exclusion mechanism in egg cytoplasm is unknown, it is crucial to test whether this exclusion still exists after ICSI, a procedure that bypasses not only the female reproductive tract and corona radiata but also sperm capacitation, acrosome reaction, zona pellucida penetration, and membrane fusion between gametes. This technique, therefore, raises the question of a possible contribution of paternal mtDNA to the zygote. In addition, since this IVF procedure is proposed to patients with severe male-factor infertility, the etiology of this heterogeneous condition is important to consider. Although the genetic forms of hypofertility are still insufficiently documented, several nuclear genes have been found to be involved in this condition (Ma et al. 1993; Chandley and Cooke 1994; Reijo et al. 1995, 1996; Blendy et al. 1996; Nantel et al. 1996; Tapanainen et al. 1997), and, importantly, several lines of evidence suggest that a defective mitochondrial function could result in male hypofertility (Folgero et al. 1993; Kao et al. 1995; St. John et al. 1997). One cannot, therefore, exclude the possibility that male patients enrolled in an ICSI procedure could transmit their mutated mtDNA to the progeny (Lestienne et al. 1997; St. John et al. 1997). To test this hypothesis, we looked at mitochondrial inheritance in ICSI families, taking advantage of a highly polymorphic mtDNA sequence called the "D-loop region."

Subjects and Methods

Subjects

We studied 27 neonates born after ICSI, using mature spermatozoa from 21 infertile couples. The semen analysis was abnormal in 16 cases, and female-factor infertility was present in 5 women. In three couples, both a female- and a male-infertility factor were identified. Parental informed consent for blood samplings was obtained before ICSI.

DNA Extraction and Preparation of Standards

DNA was isolated from peripheral-blood samples obtained from each individual, according to standard techniques. To evaluate the sensitivity of detection of a biparental origin of the mtDNA, in each family, the paternal DNA sample was serially diluted, each dilution being subsequently mixed with the maternal DNA sample. A set of 12 standards with definite paternal-to-maternal DNA ratios (i.e., 1:2, 1:5, 1:10, 1:25, 1:50, 1:100, 1:250, 1:500, 1:1,000, 1:2,500, 1:5,000, and 1:10,000) was generated. Extreme caution was observed, to prevent introduction of previously amplified products into the children's DNAs.

Denaturing Gradient Gel Electrophoresis (DGGE) and Direct Sequencing of the PCR-Amplified D-Loop Region

All DNA samples were amplified by means of a classic PCR protocol, in a 100-μl reaction mixture, by use of two primers bracketing 625 bp of the highly polymorphic mtDNA D-loop region. The primer set consists of LOP1 (sense, 5′-TTAAACTATTCTCTGTTCTTTCAT-3′) and GCLOP2 (antisense, 5′-CGCCCGGCCCGACCCCCGCGCGTCCGGCGCCCGTCCGGCTCCAGCGTCTCGCA-3′); the first 33 nucleotides were added at the 5′ end, to create a high-temperature-melting domain, by use of MELT software (Lerman and Silverstein 1987). These primers, whose sequences corresponded to nucleotide positions (nt) 16009–160032 and nt 89–108, according to the nomenclature of Anderson (Anderson et al. 1981), generated a 702-bp DNA fragment. Within each family, PCR products, which were obtained from DNA samples from the parents and their children amplified in the same run, were analyzed on the same gel. A mix in a 1:1 ratio of paternal:maternal PCR products was subjected to a cycle of melting and reassociation, to generate heteroduplexes, in order to improve the sensitivity of the DGGE assay by anticipation of the gel localization of such possible heteroduplexes in the children's DNA. Aliquots of amplified DNA samples and of PCR mixes were electrophoresed at 80 V for 20 h in a 6.5% polyacrylamide gel with a linearly increasing gradient from 25% denaturing to 75% denaturing (100% denaturant = 7 M urea and 40% formamide) (Attree et al. 1989). The gel was stained in ethidium bromide and was visualized through UV transillumination. This DGGE procedure was applied to amplified DNA samples from the 27 ICSI newborns and their parents.

The double-stranded DNA products generated for DGGE analysis were subjected to direct sequencing, by use of an ABI 377 automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division). The complete sequence of the amplified region was determined from both strands in all cases, except in the presence of heteroplasmic length variations. Such heteroplasmies were revealed by blurred sequencing patterns that occurred from the same site in opposite directions after 5′ or 3′ labeling.

To characterize the nucleotide variations underlying the heteroplasmic DGGE patterns that did not result from heteroplasmic length variations, DNA was removed from each DGGE band, was reamplified with the same LOP1-GCLOP2 primers, and subsequently was analyzed by means of DGGE and direct sequencing. The resulting DGGE patterns, which were either homoplasmic or heteroplasmic, with four species generated in the same proportion (i.e., two homoduplexes and two het-

eroduplexes), were always in agreement with the sequencing data.

The Cambridge human mtDNA light-strand sequence was used as the reference sequence (Anderson et al. 1981). When heteroplasmy was documented, the inter-individual comparison of mtDNA variations within each hypofertile couple was assessed by taking into account the major mtDNA sequence.

Three PCR-Based Approaches to Detect Low Amounts of Paternal mtDNA in Large Amounts of Maternal mtDNA

Restriction-endonuclease analysis (REA) of $\alpha[^{32}P]$-radiolabeled PCR products.—Within each hypofertile couple, paternal and maternal restriction maps of the LOP1-GCLOP2 sequence were compared with each other, to select enzymes that recognized additional cutting sites in the paternal sequence. In families 1, 2, 4–7, 13, and 16, the LOP1-GCLOP2 PCR products were generated in the presence of $\alpha[^{32}P]$-dCTP. The DNA samples from the mothers and their children were amplified, together with dilution standards. The radiolabeled products were digested with the appropriate restriction enzyme (*Taq*I in family 1, *Hae*III in family 2, *Mse*I in families 4 and 16, *Hph*I in family 5, and *Mnl*I in families 6, 7, and 13) and were electrophoresed in a 6% denaturing polyacrylamide sequencing gel. The gel was dried and was exposed to a Phosphorimager screen for 6 h. Results were analyzed by use of a Storm 840 Phosphorimager (Molecular Dynamics). Collected data of each migration lane were analyzed with Image Quant software: Phosphorimager counts were plotted on a graph (*y*-axis) for each paternal-specific band for which the gel location was accurately determined (*x*-axis). The absence of a paternal-specific peak in the restriction products of maternal PCR samples was always documented. The restriction products of PCR samples obtained from children were carefully examined, to detect the eventual presence of paternal-specific peak(s).

Paternal-specific PCR assay (PS-PCR) followed by REA.—To detect the presence of extremely low amounts of paternal mtDNA in large amounts of maternal DNA, we also designed primers that would specifically amplify the paternal mtDNA sequence; the resulting products were subsequently identified by REA. For each family, several primers were tested for their ability to promote preferential or specific amplification of the paternal sequence, under different experimental conditions (i.e., annealing temperature, $MgCl_2$ concentration, and type of *Taq* polymerase). Sense and antisense primers were designed to be used with GCLOP2 and LOP1, respectively. To study mitochondrial inheritance in neonate(s) in a given ICSI family, we selected primer sets that allowed

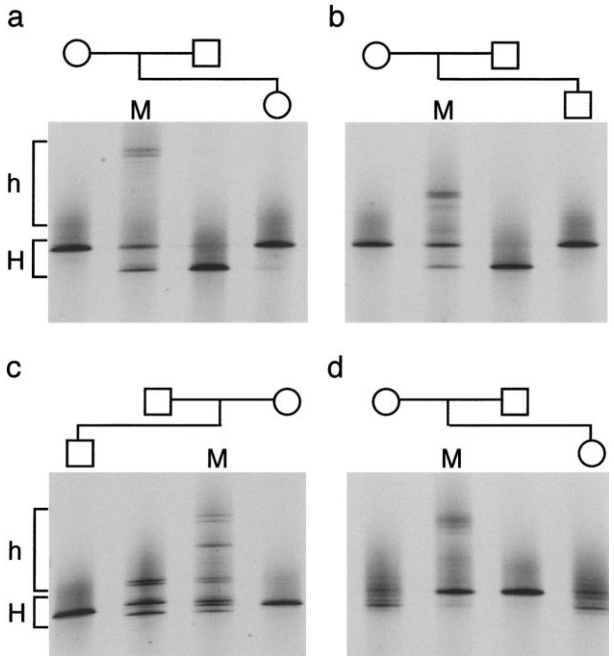

Figure 1 DGGE of mtDNA D-loop region amplified by PCR. Four representative DGGE patterns, of those in the 21 investigated families, are shown (*a–d*) H = homoduplexes; h = heteroduplexes. In *a* and *b*, all the individuals are homoplasmic; in *c*, only the father has heteroplasmy; in *d*, the mother and her daughter have a heteroplasmic pattern. In all experiments, a mix of paternal and maternal DNAs (lanes M) is analyzed in the same conditions, to anticipate the heteroduplex location in the gel.

the amplification of the paternal DNA mixed in a 1:1,000 ratio with the maternal DNA. The experimental conditions for such a sensitive PCR assay were successfully set up in two families: in family 1, the antisense primer (5′-ACTATGTACTGTTAAGGGTG-3′, paternal-specific hybridization sites are underlined), corresponding to nt 16311–16292, was used at 55°C with 1.5 mM $MgCl_2$ and standard Perkin-Elmer *Taq* polymerase; in family 3, the sense primer (5′-ACCTACCCACCCTCAACAGT-3′), corresponding to nt 16285–16304, was used at 61°C with 3 mM $MgCl_2$ and Gold *Taq* polymerase (Perkin-Elmer).

Depletion of maternal mtDNA, followed by REA.—In a few families, the paternal and maternal restriction-map differences of the D-loop–amplified products were such that one or several restriction enzymes did recognize the maternal DNA sequence only. DNA samples from both parents and their children, as well as from dilution standards, were submitted to digestion using the relevant maternal-specific restriction enzyme; the resulting products were subsequently submitted to a classic LOP1-GCLOP2 amplification prior to digestion with a paternal-specific restriction enzyme. The restriction products were analyzed by electrophoresis on nonden-

Table 1

Nucleotide-Sequence Variations Identified in the D-Loop Region of mtDNA from 42 ICSI Parents

		Nucleotide Sequence at[a]																									
	DGGE[b]	16067	16069	16079	16093	16114	16126	16129	16145	16147	16162	16171	16172	16179	16182	16183	16189	16192	16193	16193.1	16207	16209	16219	16221	16223	16224	16227
Cambridge		C	C	C	T	C	T	G	G	C	A	A	T	C	A	A	T	C	C	–	A	T	A	C	C	T	A
Family and parent:																											
1:																											
Mother	H	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	G	.	.	.	.
2:																											
Mother	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3:																											
Mother	H	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4:																											
Mother	h	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	hlv		.	.	.	.	.	.	.
Father	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5:																											
Mother	H	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
6:																											
Mother	h	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	C	.	hlv		.	.	.	.	T	.	G
Father	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7:																											
Mother	h	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	C	.	hlv		.	.	.	.	T	.	.
Father	h	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	hlv		.	.	.	.	.	.	.
8:																											
Mother	H	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
Father	H	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
9:																											
Mother	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10:																											
Mother	h	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
Father	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
11:																											
Mother	h	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	.	T	.	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
12:																											
Mother	H	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
Father	h	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
13:																											
Mother	h	.	.	.	.	.	.	.	.	.	.	.	.	.	–	–	C	.	hlv		.	.	.	.	.	.	.
Father	H	.	.	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	C	.	C	.	.	.	.	.
14:																											
Mother	H	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	G	.	.	.	T	.	.
Father	H	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
15:																											
Mother	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
16:																											
Mother	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
17:																											
Mother	H	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	h	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
18:																											
Mother	H	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
19:																											
Mother	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	.	.	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.
20:																											
Mother	h	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	C	.	hlv		.	.	.	.	.	.	.
Father	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
21:																											
Mother	H	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.
Father	H	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

[a] Dots indicate identity with the Cambridge sequence; dashes (–) indicate a deletion; hlv = heteroplasmic length variation of the homopolymeric C tract located at nt 16182–16193. For the mothers in families 10 and 11 and the fathers in families 12 and 17, who display heteroplasmic nucleotide substitutions, the major sequence is given.

[b] H = homoplasmic DGGE pattern; h = heteroplasmic DGGE pattern.

Nucleotide Sequence at																															
16231	16249	16248	16256	16261	16263	16270	16274	16278	16291	16292	16294	16296	16298	16304	16311	16320	16355	16356	16359	16362	16399	16422	16438	16482	16519	16526	72	73
T	T	C	C	C	T	C	G	C	C	C	C	C	T	T	T	C	C	T	T	T	A	T	G	A	T	G	T	A
.	C	.	.	.	.	.	.	T	.	T	T	T	.	.	.	.	.	.	.	.	.	C	A	.	C	.	.	G
.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
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.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	C	.	.	.	.	.	.	.	.	.	C	.	.	G
.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	C	.
.	.	.	.	.	.	.	.	.	.	.	.	T	T	C	.	.	.	.	.	.	.	.	.	.	C	.	.	G
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	G
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	C	.	.	G
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	G
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G
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.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	G
.	.	.	T	.	.	T	.	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	G
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.
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.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	.	.	.	.	.	C	.	.	G
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aturing polyacrylamide gels. By means of this approach, maternal- and paternal-specific restriction enzymes were used to investigate families 1, 2, 4, and 5: in family 1, *Sac*I and *Taq*I identified the maternal and paternal D-loop, respectively; the maternal- and paternal-specific restriction enzymes used in families 2, 4, and 5 were *Apa*LI and *Hae*III, *Apa*LI and *Mse*I, and *Apa*LI and *Hin*fI, respectively.

Results

Evaluation of Mitochondrial Inheritance, by Means of DGGE Analysis

To study the mitochondrial inheritance in the ICSI context, we first estimated the sensitivity of the DGGE procedure in the detection of heteroplasmy, by studying the major part of the highly polymorphic D-loop region of the human mtDNA. We took advantage of the analysis of two DNAs that display a different homoplasmic DGGE pattern, in order to determine the threshold of detection of a minor mtDNA species. By mixing the two DNA samples in different ratios prior to amplification, we observed that this technique allows the detection of two mtDNA molecular species, with the minor one present in a 2:100 ratio (data not shown).

The D-loop polymorphic region was subsequently amplified in all families. Within each couple, the mother always had a DGGE pattern different than that in the father. In all cases, this difference was confirmed by a systematic DGGE analysis of a mix of paternal and maternal DNAs: this always revealed the existence of heteroduplexes generated by single-stranded reassociation of the D-loop DNA–amplified fragments from each parent. These heteroduplexes, which mimic heteroplasmy (i.e., coexistence of two different mtDNA sequences), consist of a mismatched double-stranded DNA with one single strand from one parent associated with the complementary strand of the other parent. The D-loop region was simultaneously amplified in the 27 neonates born after ICSI. In each case, the DGGE pattern was compared with the two parental ones, as well as with the pattern obtained after the paternal and maternal DNAs had been mixed. As illustrated in figure 1, all the newborns exhibited a D-loop DGGE pattern with molecular species migrating at positions identical to those found in their mother. These results demonstrate the absence of paternal contribution in the mtDNA of the 27 newborns studied, with a sensitivity level of 2%.

As illustrated in figure 1*c* and *d*, the DGGE analysis also revealed that several parents did not display a homoplasmic pattern in the D-loop region. Indeed, 10 of

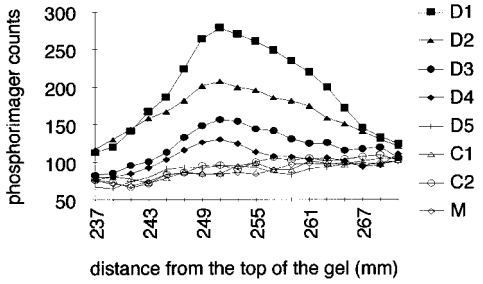

Figure 2 Quantification of paternal-specific mtDNA by means of REA of $\alpha[^{32}P]$-radiolabeled PCR products. One representative experiment is shown: analysis of the paternal-specific *Hae*III 61-bp restriction fragment of radiolabeled LOP1-GCLOP2 PCR products in family 2. Phosphorimager counts (*y*-axis) were recorded from each 2-mm portion of the migration lane containing the paternal-specific fragments (*x*-axis). PCR products were obtained by standard dilutions with different paternal:maternal DNA ratios—1:25 (D1), 1:50 (D2), 1:100 (D3), 1:250 (D4), and 1:500 (D5)—as well as from DNAs of the two children (C1 and C2) and their mother (M). The sensitivity level of detection of the paternal-specific band is of 0.2%–0.4 %. Similar results were observed for the paternal-specific *Hae*III 185-bp restriction fragment (data not shown).

Table 2

Sensitivity of Detection of Paternal mtDNA as Tested by Serial Dilutions of Paternal DNA in Maternal DNA—and Its Application to Study of mtDNA Inheritance in 27 Neonates Born after ICSI

Family (No. of Children)	Sensitivity of Detection of Paternal mtDNA (Method[a])	Origin of Parental mtDNA in Neonates
1 (2)	.01% (Depletion-REA/PS-PCR)	Maternal (>99.99%)
2 (2)	.01% (Depletion-REA)	Maternal (>99.99%)
3 (1)	.01% (PS-PCR)	Maternal (>99.99%)
4 (1)	.1% (Depletion-REA)	Maternal (>99.9%)
5 (1)	.1% (Depletion-REA)	Maternal (>99.9%)
6 (1)	1% ($\alpha[^{32}P]$-REA)	Maternal (>99%)
7 (1)	1% ($\alpha[^{32}P]$-REA)	Maternal (>99%)
8–21 (18)	2%[b]	Maternal (>98%)

[a] The method given is that which is most sensitive. Depletion-REA = depletion of maternal mtDNA, followed by REA using a paternal-specific enzyme; PS-PCR = PS-PCR followed by REA; $\alpha[^{32}P]$-REA = REA of $\alpha[^{32}P]$-radiolabeled PCR products.

[b] Assessed by either DGGE or $\alpha[^{32}P]$-REA.

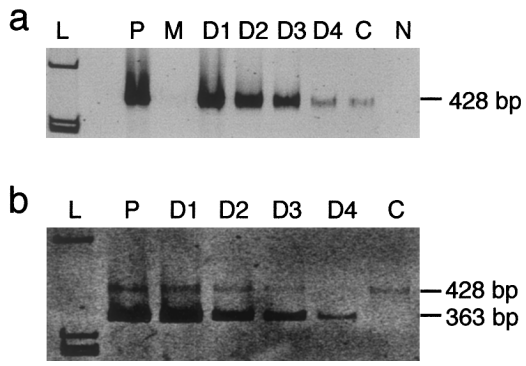

Figure 3 Quantification of paternal-specific mtDNA by means of PS-PCR followed by REA. One representative experiment is shown: analysis of the paternal-specific *Sph*I 363-bp restriction fragment in family 3. The mtDNA PCR products generated with a paternal-specific primer were analyzed by electrophoresis both before (panel *a*) and after (panel *b*) enzymatic digestion by *Sph*I, an enzyme that specifically recognized the paternal sequence. After digestion, the 428-bp fragment generated a 363-bp fragment, in the presence of paternal mtDNA only. The DNA templates analyzed in each lane originated from the father (lanes P), the mother (lanes M), and their child (lanes C), as well as from four standard dilutions with definite paternal:maternal DNA ratios—1:10 (D1), 1:100 (D2), 1:1,000 (D3), and 1:10,000 (D4). L = Φx/*Hae*III size marker. Note the absence of amplification of the maternal DNA with this paternal-specific primer, whereas all other templates including the child's DNA gave rise to a 428-bp amplified product; however, the paternal-specific 363-bp fragment was absent from the PCR product generated from the child's DNA. This emphasizes the importance of assessment of the paternal specificity of PCR products by means of a second method after preferential amplification of the paternal mtDNA.

42 individuals—that is, 7 mothers (those in families 4, 6, 7, 10, 11, 13, and 20) and 3 fathers (those in families 7, 12, and 17)—contained a mixed mtDNA population (i.e., heteroplasmy), as documented by the presence of heteroduplexes. Of the seven heteroplasmic mothers, five (those in families 4, 6, 7, 13, and 20) transmitted their different mtDNA species in equal proportion to their children. In one case (the mother in family 10), the proportion of the major maternal mtDNA species was increased in the newborn; in the remaining case (the mother in family 11), the major maternal species alone was found in the newborn's DNA. Taken together, these results demonstrate both the existence of heteroplasmy in several parents (males and females) and the exclusive maternal inheritance of the mixed DNA population, the level of heteroplasmy being either maintained or lessened in transmission from the mother to the newborn.

Characterization of mtDNA Sequence Differences within Each Hypofertile Couple

To design highly sensitive molecular approaches applicable to the detection of extremely low amounts of paternal mtDNA mixed with large amounts of maternal mtDNA, a major part (625 bp) of the D-loop region, corresponding to nt 16033–88 (according to the Cambridge coordinates), was sequenced in the 42 parents. Within all couples, paternal and maternal mtDNAs displayed several sequence differences (table 1), which were in agreement with the documented DGGE patterns. Overall, the mean pairwise mtDNA difference within couples consisted of six sequence variations. The same mean pairwise difference was observed among all individuals when the latter were considered at random. A total of 56 different sequence variations (48 transitions, 5 transversions, and 3 short insertions or deletions) were identified; among them, 9 were displayed by at least five individuals: 16519T→C, 73A→G, and 16311T→C in 26, 23, and 8 individuals, respectively; 16093T→C and 16189T→C in 6 individuals; and 16126T→C, 16172T→C, 16224T→C, and 16304T→C in 5 parents. A total of 36 distinct mtDNA haplotypes were defined, 4 of which were shared by at least two individuals (the 16304T→C variation in both the mother in family 9 and the father in family 16; the association of 16256C→T, 16270C→T, 16399A→G, and 73A→G in both the mother in family 2 and the father in family 10; the 16519T→C sequence in the father in families 2, 4, and 20; and the association of 16093T→C, 16224T→C, 16311T→C, 16519T→C, and 73A→G in both the mother in families 10 and 12 and the father in family 15).

Sequence analyses also determined the nature of the heteroplasmic substitutions underlying the complex DGGE banding patterns observed in 10 parents. The heteroplasmic banding pattern documented both in one father (in family 7) and in five mothers (in families 4, 6, 7, 13, and 20) resulted from the previously described length instability of a homopolymeric tract of cytosines induced by a T→C transition at nt 16189 (Bendall and Sykes 1995) (table 1). The four remaining heteroplasmies were due to the following nucleotide substitutions: 16192C→T, 16145G→A, 16311T→C, and 16360C→T in

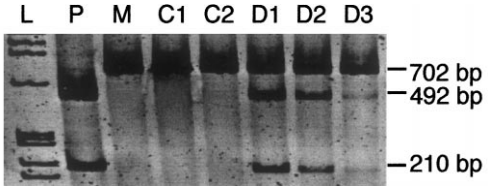

Figure 4 Quantification—by means of depletion of maternal mtDNA, followed by REA—of paternal-specific mtDNA. One representative experiment is shown: analysis of the paternal-specific *Taq*I 492- and 210-bp restriction fragments of PCR products generated from DNA templates that first have been digested with *Sca*I, which recognized the maternal D-loop sequence only (family 1). The DNA templates analyzed in each lane originated from the father (lane P), the mother (lane M), and their children (lanes C1 and C2), as well as from three standard dilutions with definite paternal:maternal DNA ratios—1:100 (D1), 1:1,000 (D2), and 1:10,000 (D3); L = Φx/*Hae*III size marker.

the father in family 17, the mother in family 11, the father in family 12, and the mother in family 10, respectively. The Cambridge sequence was the major molecular species at nt 16145 and nt 16360, whereas it represented the minor sequence at nt 16192 and nt 16311.

Evaluation of Mitochondrial Inheritance, by Means of Molecular Assays Based on the Characterization of Parental Sequence Divergences

We took advantage of the mtDNA sequence differences identified within each couple, to further study the parental origin of the children's mtDNA molecules, using three different molecular assays (table 2). REA of $\alpha[^{32}P]$-radiolabeled PCR products encompassing the major part of the D-loop region was first performed to investigate mitochondrial inheritance in eight families. This procedure, which was applied to the study of dilution standards, allowed paternal mtDNA to be specifically detected with a sensitivity level of 2% in four families (families 1, 5, 13, and 16), a sensitivity level of 1% in three families (families 4, 6, and 7), and a sensitivity level of 0.4% in one family (family 2) (fig. 2). By means of this procedure, an exclusive maternal origin of the mtDNA was documented in the children born to these parents.

To study mitochondrial inheritance, we also designed two other approaches based on the characterization of parental sequence divergences: (1) a PS-PCR whose specificity was verified by REA and (2) the depletion of maternal mtDNA, followed by REA. The first assay allowed the paternal mtDNA to be detected with a sensitivity level of 0.01% in two families (families 1 and 3; see fig. 3). The REA of PCR-amplified products derived from D-loop templates enriched in paternal mtDNA (depletion of maternal DNA) allowed paternal mtDNA to be detected with a sensitivity of 0.1% in two families (families 4 and 5) and 0.01% in two families (families 1 and 2) (fig. 4). In the same assays, paternal mtDNA was undetectable in all the neonates born to these families.

Discussion

Strikingly, IVF techniques are developing much more rapidly than our understanding of the underlying causes of hypofertility. This has been the case especially for ICSI, which is now largely accepted, by many clinicians, as the method of choice after failure of conventional IVF, especially to circumvent male hypofertility. Given its high efficiency, this technique is now even often proposed as the first therapeutic option and in less restrictive indications, such as extremely impaired semen characteristics. High pregnancy rates result from the bypassing of all natural steps involved in fertilization. However, each of these steps may play an important role in the physiological control of reproduction. It is therefore necessary to evaluate the potential risks that this new reproductive procedure has for neonates and for the human species.

As a first step in the assessment of the possible side effects of ICSI, we studied mtDNA inheritance in this situation. This is especially relevant in this case, given the relationship documented between male hypofertility and mitochondrial dysfunction (Cummins et al. 1994; Kao et al. 1995, 1998; Lestienne et al. 1997). Analyzing the mitochondrial D-loop region by means of DGGE, we investigated mtDNA transmission from 42 parents to their children born after ICSI. Within each couple, the two parents always displayed DGGE patterns different from one another. Using this approach, we demonstrated the absence of paternal mtDNA contribution in the 27 neonates conceived by ICSI and born to these 21 hypofertile couples.

However, the DGGE technique, which can detect a paternal contribution of $\geqslant 2\%$, may be considered not sensitive enough to allow accurate evaluation of parental mitochondrial inheritance in those neonates. Indeed, it has been estimated that, in the human species, each oocyte contains ~100,000 molecules of mtDNA (Chen et al. 1995), whereas the number of mitochondrial genomes per sperm is much lower, the estimates ranging from 1,500 (Manfredi et al. 1997) to 100 (Birky 1983). To further evaluate mtDNA inheritance in neonates born after ICSI, it was therefore necessary to set up more-sensitive assays that are able to detect low amounts of paternal mtDNA in large amounts of maternal mtDNA, with a sensitivity level of 1.5%–0.1%. To this end, we first characterized the polymorphic nucleotide variations of the major part of the D-loop region in all 42 parents and designed three sequence-specific assays (i.e., REA of $\alpha[^{32}P]$-radiolabeled PCR products; PS-PCR in which parental specificity was verified by REA; and depletion of maternal mtDNA, followed by REA) to accurately differentiate the two parental mtDNA species in a set of serial dilutions of paternal DNA in large amounts of maternal DNA. Following this procedure, we demonstrated the absence of paternal mtDNA in nine neonates born after ICSI, with an assay that detects fewer than 1 paternal mtDNA molecule in 100 maternal mtDNA molecules; in two of these children, the sensitivity level was 0.1%, whereas it was 0.01% in five neonates. We therefore conclude that, in these families, the ICSI technique performed with mature spermatozoa did not alter the uniparental pattern of inheritance of mtDNA, a result in keeping with a recent study reporting the absence of paternal mtDNA in six children born after ICSI, a study that had a sensitivity level of ~0.2% (Houshmand et al. 1997). However, although this was clearly demonstrated in mtDNA from blood, the possible existence

of a biparental mtDNA population in tissues that have not yet been explored cannot be ruled out. With regard to the remaining ICSI families, although numerous experimental conditions were tested, it was extremely difficult to dramatically increase the sensitivity of these PCR assays, without altering the specificity of amplification. This was even the case for experiments performed with paternal-specific primers (data not shown), a result in keeping with other studies based on the use of mismatched primers (Huang et al. 1992). In those neonates, the absence of paternal mtDNA was therefore demonstrated with a sensitivity level of 2%. Overall, these data, which argue against a major role of the bypassed steps in the exclusion mechanism of paternal mitochondria, suggest the harmlessness, from the mitochondrial transmission point of view, of ICSI performed with mature spermatozoa.

Our study also revealed that 10 of the 42 ICSI parents were heteroplasmic in the nt 16033–88 region. This was also observed in 21 fertile control couples (data not shown), the frequency of heteroplasmic individuals reaching 24% in both populations. The characterization of the nucleotide sequence underlying such heteroplasmies in the ICSI population revealed the existence of both (*a*) length heteroplasmies in a homopolymeric tract and (*b*) heteroplasmic nucleotide substitutions. Length heteroplasmies in homopolymeric tracts have been described in three regions of the D-loop: at nt 310 (Hauswirth and Clayton 1985), nt 568 (Torroni et al. 1994; Sternberg et al. 1998), and nt 16189 (Bendall and Sykes 1995); the latter site, which belongs to the region explored in the present study, was involved in the heteroplasmic length variations documented in 6 (15%) of the 42 ICSI parents; this frequency of heteroplasmic length variations was identical to that reported among Europeans (Bendall and Sykes 1995). Heteroplasmic nucleotide substitutions were also documented in the D-loop region of ~10% of the 42 ICSI parents: the heteroplasmic T→C substitution at nt 16311 has previously been reported by others (Comas et al. 1995), whereas the heteroplasmic substitutions at nt 16192, nt 16145, and nt 16360 are new. The transmission pattern of these parental heteroplasmies to offspring was also in agreement with an exclusively maternal inheritance of mtDNA in the ICSI families.

One should keep in mind that the ICSI technique, which is classically proposed to couples with male-factor infertility, can favor the transmission of nuclear defects that are responsible for hypofertility (In't Veld et al. 1997). This is particularly the case both for obstructive azoospermia that are due to congenital absence of the vas deferens in relation with CFTR-gene mutations (Chillon et al. 1995; Costes et al. 1995; Lissens and Liebaers 1997) and for nonobstructive azoospermia that are due to microdeletions involving the different *AZF* regions of the long arm of the Y chromosome (Vogt 1998). In addition, it is important to notice that, although paternal mtDNA is not transmitted through the ICSI procedure, mtDNA abnormalities can be transmitted through a nuclear-gene defect (Luft 1994; Wallace 1994; Kaukonen et al. 1996; Zeviani et al. 1998). In this context, the existence of mtDNA deletions associated with male-factor hypofertility is important to consider (Kao et al. 1995, 1998; Lestienne et al. 1997; St. John et al. 1997).

Finally, if the ICSI procedure uses immature gametes (Fishel et al. 1995; Tesarik et al. 1995; Tesarik and Mendoza 1996; Antinori et al. 1997; Barak et al. 1998), then it also bypasses both sperm maturation in the epididymis and even spermatogenesis within the testis. Under these conditions, it is important to evaluate not only mtDNA inheritance but also the long-term potential implications, such as modifications in the genomic imprinting process, that this IVF technique has for normal development—and even for life expectancy.

Acknowledgments

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