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Increased sperm mitochondrial DNA content in male infertility

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BACKGROUND: There is increasing evidence that mitochondrial DNA (mtDNA) anomalies in sperm may lead to infertility. Point mutations, deletions and the presence of a specific mtDNA haplogroup have been associated with poor sperm quality, but little attention has been paid to the role of mtDNA content. METHODS: Using density gradient separation and swim-up methods, we selected motile sperm from 32 normal and 35 abnormal sperm samples. The mtDNA/β-globin gene ratio was determined by real-time quantitative PCR. RESULTS: The average mtDNA/β-globin ratio of sperm collected from 100% density layers was 1.4 for normal sperm, 6.1 for sperm samples presenting at least one abnormal criterion [among the three criteria established by World Health Organization (1999), i.e. sperm count, motility and morphology], and 9.1 for sperm samples presenting two or more of these abnormal criteria. These differences are very highly significant (*P* < 0.0001). The mtDNA numbers were also much greater in sperm collected from the 40% density gradient layers (mean: 17.1, *P* < 0.001), known to contain the most abnormal sperm of the sperm samples, than in those collected from the 100% layers known to contain sperm with the best fertilizing ability. CONCLUSION: Our results showed significant mtDNA amplification in sperm collected from abnormal sperm samples.

Key words: male infertility/mitochondrial DNA/real-time PCR/sperm

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Introduction

Mitochondrial DNA (mtDNA), which is maternally transmitted, is a circular double-stranded DNA molecule composed of 16.5 kilobases. mtDNA codes for 13 essential subunits of the respiratory chain complexes that provide the main ATP supply of the cell (Anderson *et al*., 1981). The number of mitochondria per cell type is highly variable depending on the cell's energetic demand, with some mitochondria carrying up to 11 copies of mtDNA, whereas others may have none (Cavelier *et al*., 2000). Most mammalian somatic cells contain between 10^2 and 10^4 copies of mtDNA. Human oocytes contain an average of 200 000 mtDNA copies and we have shown that the increase of mtDNA copy numbers in these cells is correlated with their fertilizability (Reynier *et al*., 2001). Although oogenesis is associated with a strong amplification of mtDNA copy numbers, spermatogenesis is associated with a drastic reduction in mtDNA content, to about a tenth of its initial value, occurring mainly during spermiogenesis (Hecht *et al*., 1984). This reduction mainly occurs when the rounded spermatids take on an elongated form, accompanied by the loss of the residual bodies. The residual bodies, containing most of the spermatid cytoplasm, are then phagocytosed by Sertoli cells. At the molecular level, the reduction of mtDNA content is due to the down-regulation of the nuclear encoded mitochondrial transcription Factor A (tfam), which is the main factor controlling mtDNA copy number (Larsson *et al*., 1997). This sharp reduction in mtDNA content, together with the action of a specific ubiquitination-mediated mechanism of paternal mitochondrion destruction in the early embryo (Sutovsky *et al*., 1999), explains the absence of transmission of paternal mtDNA. Mature mammalian sperm are known to contain ~22–75 mitochondria, which form a tight helix around the flagellar basis of the midpiece, providing the ATP necessary for flagellar propulsion (Bahr and Engler, 1970; St John *et al*., 2000a). The few reports available on mtDNA quantification in mammalian sperm present widely varying results. Thus, Hecht *et al*. (1984) found 50–75 mtDNA copies per mouse sperm using the semi-quantitative Southern blot method. Using the same method, Manfredi *et al*. (1997) estimated that each human sperm contained ~1500 molecules

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of mtDNA. Using real-time quantitative PCR, Shitara *et al.* (2000) found 10 copies of mtDNA per mouse sperm, and 150 copies of mtDNA per mouse spermatid.

During the past few years, some evidence of mitochondrial involvement in male infertility has been found. First, male infertility, associated with asthenozoospermia (Folgero *et al.*, 1993) or oligoasthenozoospermia (Lestienne *et al.*, 1997), has been reported in patients suffering from typical mtDNA diseases, involving point mutations or multiple deletions of mtDNA. Secondly, sperm have been shown to be particularly prone to develop deletions of mtDNA (Cummins, 1998; Cummins *et al.*, 1998; Reynier *et al.*, 1998; St John *et al.*, 2001; O'Connell *et al.*, 2002a,b). Some studies have shown that, in human sperm, these deletions are associated with a decline of motility and fertility (Kao *et al.*, 1995, 1998). Thirdly, a correlation has been found between the quality of the semen and the functionality of the respiratory chain in sperm mitochondria (Ruiz-Pesini *et al.*, 1998, 2000a; Hoshi *et al.*, 2002). Moreover, it has been shown that mtDNA point mutations, mtDNA single nucleotide polymorphisms and mtDNA haplogroups can greatly influence semen quality (Holyoake *et al.*, 1999, 2001; Ruiz-Pesini *et al.*, 2000b; Sutarno *et al.*, 2002). The high rate of deletions or substitutions observed in sperm could be due to impaired mitochondrial maintenance or result from the deleterious effects of oxidative stress. Recently, the nuclear encoded mitochondrial-specific DNA polymerase gamma (*POLG*) has been reported to be involved in male infertility (Rovio *et al.*, 2001). The absence of the most common allele of the *POLG* gene was found to be associated with a range of defects in sperm quality. The genotype of the nuclear encoded glutathione S-transferase M1, involved in reactive oxygen species detoxification, has also been associated with male infertility and mtDNA deletion (Chen *et al.*, 2002). Lastly, sperm treatment with extracellular ATP has been shown to induce a significant increase in the fertilizing potential of sperm, demonstrating the importance of the mitochondrial function in male fertility (Rossato *et al.*, 1999). In order to investigate the role of mtDNA in male infertility we have quantified sperm mtDNA by means of real-time PCR.

Materials and methods

Patients and sperm samples

The Ethics Committee of the University Hospital of Angers approved the plan of our study. We collected 67 semen samples from 67 patients recruited from couples who presented for semen analysis or assisted reproductive technology at the In Vitro Fecundation Laboratory of the University Hospital of Angers, France. These couples suffered from either male or female infertility, or infertility of unknown aetiology. Semen samples were produced by masturbation after 3–5 days of abstinence. After liquefaction of the semen samples at room temperature, a standard manual protocol of analysis was used according to the criteria established by the World Health Organization (WHO) (World Health Organization, 1999). Thirty-two samples were classified as normal ($\geqslant 20 \times 10^6$ sperm/ml; type 'a' motility: ⩾25% or type 'a + b' motility: ⩾50%; and normal morphology: ⩾30%), and 35 samples were considered abnormal (Table I). Of the 35 abnormal sperm samples, 14 had only one abnormal criterion (among the three criteria established by the WHO corresponding to oligozoospermia, asthenozoospermia and teratozoospermia), and the remainder had two (n = 8) or more (n = 13) of these abnormal criteria. In order to eliminate seminal plasma and separate sperm according to morphology and motility, 1 ml of each semen sample was centrifuged in discontinuous three-layer Puresperm (Nidacon, Gothenburg, Sweden) density gradients (40, 60 and 100%) at 300 g for 20 min. All the 67 100% fractions, and 31 of the 40% fractions were then washed in 5 ml of Spermrinse (IVF Scandinavian, Stockholm, Sweden) and centrifuged at 600 g for 10 min. The resultant pellets were then layered with 300 µl of IVF-20 medium (IVF Scandinavian). The preparations were incubated in an atmosphere of 5% CO_2 at 37°C for 2 h, to permit the sperm to swim up. A total of 250 µl of supernatant sample was taken up. Sperm counting was performed on Kova-slide cells and the concentration was adjusted, when necessary, to $<10^6$ sperm per ml with 0.9% NaCl. Samples (200 µl) of each dilution were then stored at –20°C until DNA was extracted.

Preparation of DNA

DNA was extracted from each sperm sample by means of the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations, except for the addition of dithiothreitol (1% DTT) in order to break the sperm nucleus disulphide bonds, and also to dissociate mitochondria from the mitochondrial sheath (Sutovsky *et al.*, 1997; Carter *et al.*, 2000). Briefly, DNA bound specifically to glass fibre following the combined action of a chaotropic agent (guanidine), a detergent (Triton X-100), the enzyme proteinase K, and the DTT. After washing, the silica-bound DNA was eluted with 200 µl of pre-warmed (72°C) elution buffer and preserved at 4°C.

mtDNA/β-globin real-time PCR quantification

The external standard used for mtDNA quantification was a 158 bp PCR product. The nucleotide positions of the primers on the light strand mtDNA (according to the Cambridge reference sequence)

Table I. Real–time PCR quantification of mtDNA/β-globin in 100% density gradient layers

Sample	No. of patients	Sperm type							Quantification
		O	A	T	OA	OT	AT	OAT	mtDNA/β-globin ratio average values (range)
Sperm category									
All	67	3	8	3	3	0	5	13	3.8 (0.3–28.0)
Normal	32	–	–	–	–	–	–	–	1.4 (0.3–4.2)
Abnormal	35	3	8	3	3	0	5	13	6.1 (0.5–28.0)
Abnormal (1 criterion)	14	3	8	3	–	–	–	–	1.5 (0.5–6.0)
Abnormal (⩾2 criteria)	21	–	–	–	3	0	5	13	9.1 (0.8–28.0)

O = oligozoospermia; A = asthenozoospermia; T = teratozoospermia.

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were: D41 (3254–3277) and D56 (3126–3147). PCR reactions were carried out under standard conditions with 100 ng of total DNA in a 50 µl volume: 1.5 mmol/l $MgCl_2$, 75 mmol/l Tris–HCl (pH 9 at 25°C), 20 mmol/l $(NH_4)_2SO_4$, 0.01% Tween 20, 50 pmol of each primer, 200 µmol/l of each dNTP and 2 IU of GoldStar DNA polymerase (Eurogentec, Seraing, Belgium). Each of 35 cycles consisted of a denaturation step of 30 s at 94°C, a hybridization step of 30 s at 58°C, and an extension step of 1 min at 72°C. The PCR product was phenol–chloroform purified from low melting point agarose and cloned using the Topo TA Cloning Kit (Invitogen, Life Technologies, Groningen, The Netherlands) into pCR 2.1-Topo® vector. The recombinant plasmid was purified using Qia Prep Spin Miniprep Kit (Qiagen, Courtaboeuf, France) and was quantified by spectrophotometry. Purification quality was checked by means of the 260/280 absorbance ratio, and the absence of residual bacterial DNA was checked by agarose gel electrophoresis. It was assumed that 1 µg of a 4066 bp product (vector 3908 bp and insert 158 bp) contained 2.2×10^{11} molecules. Serial dilutions were then made in order to assess several concentrations of a known number of templates. These serial dilutions were used as the external standard for real-time PCR. The serial dilutions were all stored at –20°C in single-use aliquots.

A Roche LightCycler was used to determine the mtDNA copy number using LightCycler-Faststart DNA master SYBR Green 1 kit (Roche). A total of 20 µl PCR reaction mixtures were prepared as follows: 1×buffer containing 4 mmol/l $MgCl_2$, 0.2 mmol/l dNTP, 0.5 µmol/l of both primers (D41 and R56), SYBR green I dye, 0.25 IU hot start Taq DNA polymerase and 10 µl of the extracted DNA or 10 µl of Standard with a known copy number. The reactions were performed as follows: initial denaturing at 95°C for 7 min and 40 cycles at 95°C for 1 s, 58°C for 5 s, and 72°C for 13 s. The SYBR green fluorescence was read at the end of each extension step (72°C). A melting curve (loss of fluorescence at a given temperature between 66°C and 94°C) was analysed in order to check the specificity of the PCR product. For each run, a standard curve (log of the initial template copy number on the abscissa, and the cycle number at the crossing point on the ordinates) was generated using five 10-fold serial dilutions (10–100 000 copies) of the external standard (Figure 1). This curve allowed the determination of the starting copy number of mtDNA in each sample. All samples were tested twice.

In order to determine the number of sperm in each sample, we quantified the β-globin gene by means of the LightCycler-Control Kit DNA (Roche) according to the manufacturer's recommendations. Briefly, a 110 bp fragment of the β-globin gene was amplified using human genomic DNA as a standard. PCR reaction mixtures (18 µl) were added to 2 µl of the extracted DNA or to 2 µl of Standard with a known β-globin copy number. The reactions were performed on the LightCycler as follows: initial denaturing at 95°C for 7 min and 40 cycles at 95°C for 1 s, 55°C for 5 s, and 72°C for 10 s. The SYBR green fluorescence was read at the end of each extension step (72°C). The melting curve analysis was systematically performed. For each run, a standard curve was generated using five 10-fold serial dilutions (1–10 000 copies) of the Standard. The standard curve generated by the LightCycler software (version 3.5) allowed the determination of the starting copy number of β-globin gene in each sample. All samples were tested twice.

The calculation of mtDNA/β-globin ratio was performed taking into account the volume of the extracted DNA used as a template for PCR amplification (10/200 µl for mtDNA PCR and 2/200 µl for β-globin PCR).

Statistical analysis

The various groups of sperm were compared using the non-parametric Mann–Whitney, Kruskal–Wallis and Wilcoxon *U*-tests and the Student's *t*-test with Systat software, version 8.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $P < 0.05$.

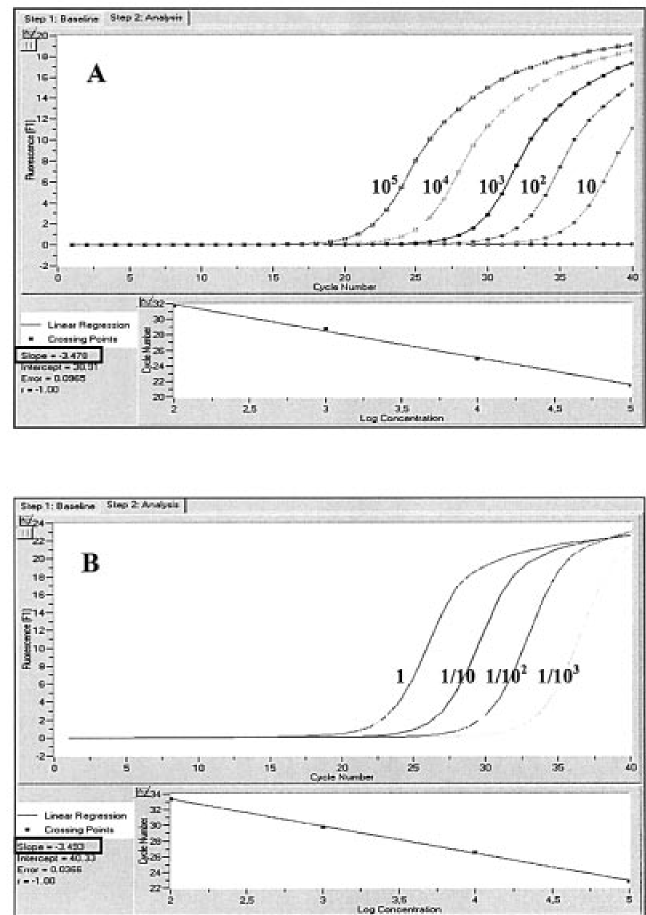

Figure 1. The PCR efficiency was almost the same for the standards as for the sperm extracts. (**A**) Standard curve used for mitochondrial (mt)DNA real-time PCR quantification. For each run, a standard curve was generated using five 10-fold serial dilutions (10–10^5 copies) of the target mtDNA standard. The slope of the standard curve was –3.48. (**B**) Serial dilutions of sperm extracts gave similar PCR efficiency (slope: –3.49).

Results

In order to compare the mtDNA content of normal and abnormal sperm, we eliminated round cells using a combined density gradient layer and a swim-up method capable of selecting only motile sperm. The absence of round cells in our sperm preparations was checked by light microscopy. The accurate quantification of mtDNA depends closely on the high recovery of mtDNA templates. We have shown that the DNA extraction technique used in the present study allows the recovery of >93% of the mtDNA (Reynier *et al.*, 2001). The correspondence between the results of the microscopic count of sperm (average: 83 220) and the β-globin gene quantification (average: 80 171) also demonstrated the efficiency of the mtDNA extraction (Student's test: P = not significant, and r = 0.85, P = 0.23). As shown by the linearity of the standard curve (Figure 1A), the mtDNA PCR efficiency was constant over the

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Table II. Intra-assay precision for mtDNA and β-globin quantification

	10 experiments: mtDNA copies		10 experiments: β-globin copies	
	1[a]	2[a]	3[a]	4[a]
	48 840	58 860	80 900	44 310
	52 320	57 680	82 470	37 260
	49 980	65 680	97 330	36 880
	46 700	55 240	89 860	37 220
	54 800	59 040	72 570	36 620
	53 100	61 000	88 510	39 390
	50 180	60 780	80 660	38 120
	50 120	56 280	80 000	36 010
	47 300	61 600	88 420	38 210
	54 920	64 360	75 490	42 710
Mean	50 826	60 052	83 621	38 673
SD	2886	3325	7399	2743
CV (%)	5.7	5.5	8.9	7.1

[a]Sperm sample.
CV = coefficient of variation.

Table III. Inter-assay precision for mtDNA and β-globin quantification

	5 experiments:mtDNA copies		5 experiments:β-globin copies	
	5[a]	6[a]	7[a]	8[a]
	124 600	136 600	153 200	60 950
	168 000	159 000	164 000	62 260
	165 200	141 600	129 000	52 650
	174 600	156 680	169 000	65 170
	172 140	150 760	162 700	68 800
Mean	160 908	148 928	155 580	61 966
SD	20 619	9625	15 920	6018
CV (%)	12.8	6.5	10.2	9.7

[a]Sperm sample.
CV = coefficient of variation.

concentration range studied (10–100 000 copies). Moreover, as shown by the standard curve obtained with three 10-fold dilutions of sperm extract which had the same slope as that obtained with standard dilutions (–3.49 and –3.48 respectively), the PCR efficiency was almost the same for the standards as for the sperm extracts (Figure 1B). The intra-assay precision of the mtDNA and β-globin quantification was assessed from 10 measurements of four different sperm DNA samples; the coefficients of variation (CV) were between 5.5 and 8.9% (Table II). The repeatability between runs (inter-assay) was assessed from five measurements of four different sperm DNA samples; the CV were between 6.5 and 12.8% (Table III).

The mtDNA/β-globin ratio, expressing the average copy number per haploid genome or per sperm, was determined for 98 sperm extracts (67 100% layers and 31 40% layers) obtained from the 67 ejaculates (Table I). The average ratio, 10.1 (0.3–70.4), varied considerably. The average mtDNA copy number per sperm was 3.8 in the 67 groups of sperm isolated from 100% density layers. The average ratio was 1.4 (0.3–4.2) in the group of 32 patients with normal sperm, compared with 6.1 (0.5–28.0) in the group of 35 patients showing at least one abnormal WHO criterion (Table I). The non-parametric Mann–Whitney *U*-test showed that this difference was highly significant ($P < 0.0001$) (Figure 2). The average mtDNA/β-globin ratio in the group of 14 patients with only one abnormal WHO criterion was 1.5 (0.5–6.0). This was not significantly different from the average ratio found in the normal group. The average mtDNA/β-globin ratio was 9.1 (0.8–28.0) in the group of 21 patients with two or more abnormal WHO criteria. This was significantly different from that of the normal group (1.4) on the Kruskal–Wallis *U*-test ($P < 0.0001$) (Figure 3). The mtDNA contents of two different density gradient fractions (40 and 100%) were also compared, two by two, for each of 31 patients without taking into account the initial sperm quality (Table IV). The Wilcoxon *U*-test showed that the mtDNA content was significantly higher ($P < 0.001$) (Figure 4) in the 40% layers (mean: 17.1; range: 0.8–70.4) than in the 100% layers (mean: 2.4; range 0.3–12.8).

Discussion

The determination of mtDNA/β-globin gene ratios using real-time quantitative PCR revealed that the average mtDNA copy number in motile human sperm was 10.1. This value is close to that found by Shitara *et al*. (2000) who, using a similar quantitative PCR technique, found an average of 10 mtDNA copies per mouse sperm. However, these results differ

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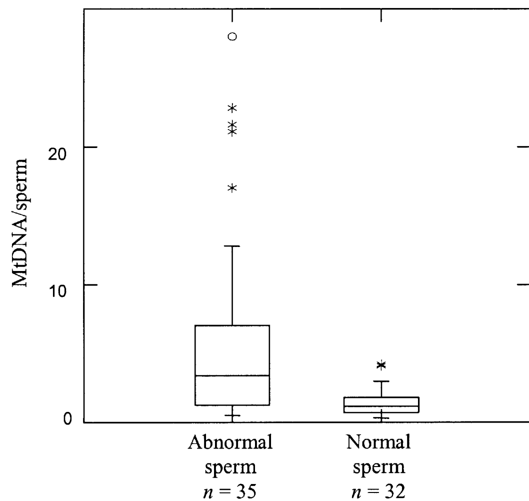

Figure 2. Comparison of mitochondrial (mt)DNA/β-globin ratio in the 100% layers for normal and abnormal sperm samples ($P < 0.0001$). Results are presented in box-and-whisker form: the box comprises 50% of the values, the line in the box indicates the median value, and the whiskers represent the distribution of values (mean ± 2 SD). °*Outliers.

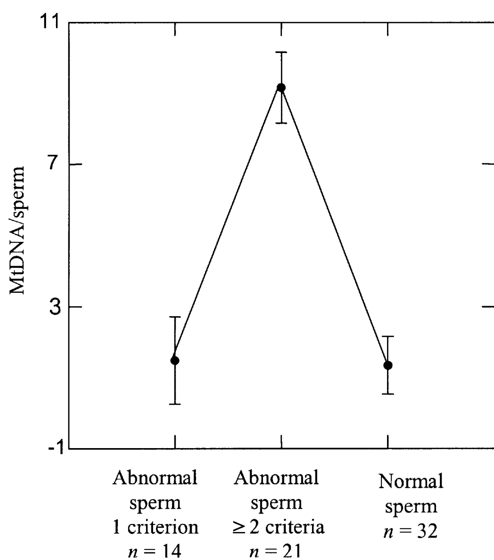

Figure 3. Comparison of the mtDNA/β-globin ratio in 100% layers between normal sperm samples, sperm samples with only one abnormal criterion and sperm samples with two or more abnormal OMS criteria, ($P < 0.0001$). Black points represent mean values; whiskers correspond to the SEM ($\sigma/\sqrt{n}$).

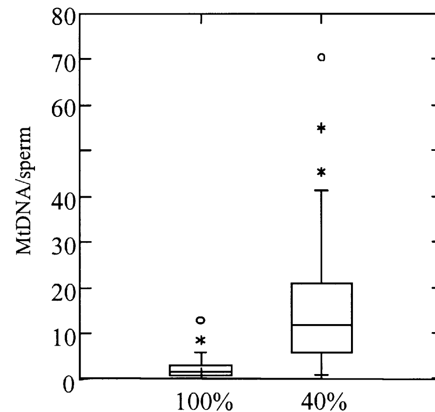

Figure 4. Comparison of the mtDNA/sperm ratio between the 100% Puresperm layers and the 40% Puresperm layers ($P < 0.001$). Results are presented in box-and-whisker form.

Table IV. Comparison of mtDNA/β-globin ratio between 40 and 100% density gradient layers (n = 31 patients)

Samples (layers)	mtDNA/β-globin ratio average values (range)
100%	2.4 (0.3–12.8)
40%	17.1 (0.8–70.4)

considerably from the values indicated by Hecht *et al*. (1984) who found 50–75 mtDNA copies per mouse sperm, and of Manfredi *et al*. (1997) who estimated that each human sperm contained ~1500 mtDNA copies. One explanation for these discordant results may lie in the use of the different methods of quantifying the mitochondrial genome. Indeed, the Southern blot method may lead to cross-hybridization with the large number of mitochondrial pseudogenes (~300) recently found in the nuclear genome (Mourier *et al*., 2001; Tourmen *et al*., 2002; Woischnik and Moraes, 2002). These nuclear pseudogenes share high levels of similarity with mtDNA and might have been erroneously recognized by the wide-ranging mtDNA probes such as those used in the Southern blot technique. In contrast, our PCR method was demonstrated to be mtDNA-specific since no false amplification of nuclear pseudogenes in cells without mtDNA was detected. Another explanation for the discrepancy of the various results reported might lie in the presence of round cells (mainly germinal cells and leukocytes) in the sperm preparations. Contamination by only a few of these cells, which have an mtDNA content exceeding that of sperm by a factor of >100, could easily lead to a serious overestimation of mtDNA content in sperm. In order to avoid this bias, we used a combination of density gradient centrifugation, which allowed partial elimination of round cells by retaining them at the 40–60% layer interface (Gandini *et al*., 1999), and of the swim-up technique. These combined techniques allowed us to obtain sperm samples completely free of contaminating round cells.

The motile sperm extracted from normal sperm samples were found to contain only 1.4 mtDNA molecules on average. This means that the majority of sperm mitochondria are almost totally devoid of mtDNA, and that many sperm probably do not contain any mtDNA at all. It has been shown that the paternal mtDNA, found in the early embryonic stages, is rapidly eliminated, and we suggest that the lack of mtDNA content in some sperm with the best fertilizing ability may also explain why the mitochondrial genome is not paternally transmitted. The functionality of the respiratory chain must therefore be temporally maintained in mature sperm until fertilization, despite the quasi-absence of mtDNA in their mitochondria. Indeed, it has been shown that mtRNA transcripts remain highly stable, and that the translation of mtRNA into subunits

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of the respiratory chain continues actively in the mitochondria of sperm, despite the complete absence of mtDNA replication (Rantanen and Larsson, 2000).

Point mutations, deletions or haplogroups of mtDNA could be involved in male infertility. In the present report we show that highly significant mtDNA amplification was found in abnormal sperm, highlighting the multiple implications of mitochondria in male infertility. First, the mtDNA content of motile sperm was found to be up to 28 times higher in sperm samples of poor quality than in normal sperm samples. Secondly, sperm collected from the 40% layers (which contain a majority of abnormal sperm) were found to have an mtDNA content up to 70-fold greater than that of sperm collected from the 100% layers (which contain sperm with the best fertilizing ability). This mtDNA amplification could have two main causes. One could be a feedback process operating to compensate low respiratory chain activity, thus leading to an increase of mtDNA. Indeed, such compensatory processes of increased mitochondrial biogenesis are frequently observed in mitochondrial pathology. Another cause of mtDNA amplification could be the abnormal differentiation and maturation of sperm in infertile patients. In fact, mtDNA amplification was more significant in sperm samples with at least two abnormal WHO criteria, suggesting a global disorder of spermatogenesis. We therefore postulate that insufficient mtDNA copy number reduction may occur when the maturation of sperm is perturbed.

In conclusion, our study shows that sperm mtDNA is strongly amplified in sperm samples from infertile patients presenting abnormal sperm characteristics. This raises the question about the risks involved in the use of a number of methods of assisted procreation, such as ICSI and round spermatid injection. These techniques involve the treatment of extreme cases of male infertility by means of sperm that may be abnormal, and immature sperm or sperm precursors (such as spermatids and spermatocytes) obtained from testicular biopsies, all of which have a higher mtDNA content than mature sperm. Furthermore it has been shown that oocytes from aged women or from women with ovarian deficiencies may have defective mtDNA genetic filters (St John *et al.*, 1997). Although the first studies on mtDNA inheritance after ICSI suggest that human embryos eliminate the mtDNA of the injected sperm (Danan *et al.*, 1999), one study has recently shown that abnormal paternal mtDNA transmission may not be uncommon when poor-quality gametes are used (St John *et al.*, 2000b). Thus, at present, it cannot be precluded that the intracytoplasmic injection of sperm with amplified mtDNA into defective oocytes may impair the mechanism of elimination of paternal mtDNA, thereby further jeopardizing embryonic development and mitochondrial inheritance.

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References

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F. *et al.* (1981) Sequence and organization of the human mitochondrial genome. *Nature*, **290**, 457–465.

Bahr, G.F. and Engler, W.F. (1970) Considerations of volume, mass, DNA, and arrangement of mitochondria in the midpiece of bull spermatozoa. *Exp. Cell Res.*, **60**, 338–340.

Carter, K.L., Robertson, B.C. and Kempenaers, B. (2000) A differential DNA extraction method for sperm on the perivitelline membrane of avian eggs. *Mol. Ecol.*, **9**, 2149–2154.

Cavelier, L., Johannisson, A. and Gyllensten, U. (2000) Analysis of mtDNA copy number and composition of single mitochondrial particles using flow cytometry and PCR. *Exp. Cell Res.*, **259**, 79–85.

Chen, S.S., Chang, L.S., Chen, H.W. and Wei, Y.H. (2002) Polymorphisms of glutathione S-transferase M1 and male infertility in Taiwanese patients with varicocele. *Hum. Reprod.*, **17**, 718–725.

Cummins, J. (1998) Mitochondrial DNA in mammalian reproduction. *Rev. Reprod.*, **3**, 172–182.

Cummins, J.M., Jequier, A.M., Martin, R., Mehmet, D. and Goldblatt, J. (1998) Semen levels of mitochondrial DNA deletions in men attending an infertility clinic do not correlate with phenotype. *Int. J. Androl.*, **21**, 47–52.

Danan, C., Sternberg, D., Van Steirteghem, A., Cazeneuve, C., Duquesnoy, P., Besmond, C., Goossens, M., Lissens, W. and Amselem, S. (1999) Evaluation of parental mitochondrial inheritance in neonates born after intracytoplasmic sperm injection. *Am. J. Hum. Genet.* **65**, 463–473.

Folgero, T., Bertheussen, K., Lindal, S., Torbergsen, T. and Oian, P. (1993) Mitochondrial disease and reduced sperm motility. *Hum. Reprod.*, **8**, 1863–1868.

Gandini, L., Lenzi, A., Lombardo, F., Pacifici, R. and Dondero, F. (1999) Immature germ cell separation using modified discontinuous Percoll gradient technique in human semen. *Hum. Reprod.*, **14**, 1022–1027.

Hecht, N.B., Liem, H., Kleene, K.C., Distel, R.J. and Ho, S.M. (1984) Maternal inheritance of the mouse mitochondrial genome is not mediated by a loss or gross alteration of the paternal mitochondrial DNA or by methylation of the oocyte mitochondrial DNA. *Dev. Biol.*, **102**, 452–461.

Holyoake, A.J., Sin, I.L., Benny, P.S. and Sin, F.Y.T. (1999) Association of a novel human mtDNA ATPase6 mutation with immature sperm cells. *Andrologia*, **31**, 339–345.

Holyoake, A.J., McHugh, P., Wu, M., O'Carroll, S., Benny. P., Sin, I.L. and Sin, F.Y. (2001) High incidence of single nucleotide substitutions in the mitochondrial genome is associated with poor semen parameters in men. *Int. J. Androl.*, **24**, 175–182.

Hoshi, K., Kasai, T., Ogawa, K., Mizuno, K., Nagai, S., Uchida, Y., Ohta, S., Fujie, M., Suzuki, K. and Hirata, S. (2002) Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. *Asian J. Androl.*, **4**, 97–103.

Kao, S.H., Chao, H.T. and Wei, Y.H. (1995) Mitochondrial deoxyribonucleic acid 4977-bp deletion is associated with diminished fertility and motility of human sperm. *Biol. Reprod.*, **52**, 729–736.

Kao, S.H., Chao, H.T. and Wei, Y.H. (1998) Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa. *Mol. Hum. Reprod.*, **4**, 657–666.

Larsson, N.G., Oldfors, A., Garman, J.D., Barsh, G.S. and Clayton, D.A. (1997) Down-regulation of mitochondrial transcription factor A during spermatogenesis in humans. *Hum. Mol. Genet.*, **6**, 185–191.

Lestienne, P., Reynier, P., Chretien, M.F., Penisson-Besnier, I., Malthiery, Y. and Rohmer, V. (1997) Oligoasthenospermia associated with multiple mitochondrial DNA rearrangements. *Mol. Hum. Reprod.*, **3**, 811–814.

Manfredi, G., Thyagarajan, D., Papadopoulou, L.C., Pallotti, F. and Schon, E.A. (1997) The fate of human sperm-derived mtDNA in somatic cells. *Am. J. Hum. Genet.*, **61**, 953–960.

Mourier, T., Hansen, A.J., Willerslev, E. and Arcander, P. (2001) The human genome project reveals a continuous transfer of large mitochondrial fragments to the nucleus. *Mol. Biol. Evol.*, **18**, 1833–1837.

O'Connell, M., McClure, N. and Lewis, S.E. (2002a) A comparison of mitochondrial and nuclear DNA status in testicular sperm from fertile men and those with obstructive azoospermia. *Hum. Reprod.*, **17**, 1571–1577.

O'Connell, M., McClure, N. and Lewis, S.E. (2002b) Mitochondrial DNA deletions and nuclear DNA fragmentation in testicular and epididymal human sperm. *Hum. Reprod.*, **17**, 1565–1570.

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Rantanen, A. and Larsson, N.G. (2000) Regulation of mitochondrial DNA copy number during spermatogenesis. *Hum. Reprod.*, **15** (Suppl. 2), 86–91.

Reynier, P., Chretien, M.F., Savagner, F., Larcher, G., Rohmer, V., Barriere, P. and Malthiery, Y. (1998) Long PCR analysis of human gamete mtDNA suggests defective mitochondrial maintenance in spermatozoa and supports the bottleneck theory for oocytes. *Biochem. Biophys. Res. Commun.*, **252**, 373–377.

Reynier, P., May-Panloup, P., Chretien, M.F., Morgan, C.J., Jean, M., Savagner, F., Barriere, P. and Malthiery, Y. (2001) Mitochondrial DNA content affects the fertilizability of human oocytes. *Mol. Hum. Reprod.*, **7**, 425–429.

Rossato, M., La Sala, G.B., Balasini, M., Taricco, F., Galeazzi, C., Ferlin, A. and Foresta, C. (1999) Sperm treatment with extracellular ATP increases fertilization rates in in-vitro fertilization for male factor infertility. *Hum. Reprod.*, **14**, 694–697.

Rovio, A.T., Marchington, D.R., Donat, S., Schuppe, H.C., Abel, J., Fritsche, E., Elliott, D.J., Laippala, P., Ahola, A.L., McNay, D. *et al*. (2001) Mutations at the mitochondrial DNA polymerase (POLG) locus associated with male infertility. *Nature Genet.*, **29**, 261–262.

Ruiz-Pesini, E., Diez, C., Lapena, A.C., Perez-Martos, A., Montoya, J., Alvarez, E., Arenas, J. and Lopez-Perez, M.J. (1998) Correlation of sperm motility with mitochondrial enzymatic activities. *Clin. Chem.*, **44**, 1616–1620.

Ruiz-Pesini, E., Lapena, A.C., Diez, C., Alvarez, E., Enriquez, J.A. and Lopez-Perez, M.J. (2000a) Seminal quality correlates with mitochondrial functionality. *Clin. Chim. Acta*, **300**, 97–105.

Ruiz-Pesini, E., Lapena, A.C., Diez-Sanchez, C., Perez-Martos, A., Montoya, J., Alvarez, E., Diaz, M., Urries, A., Montoro, L., Lopez-Perez, M.J., *et al*. (2000b) Human mtDNA haplogroups associated with high or reduced spermatozoa motility. *Am. J. Hum. Genet.*, **67**, 682–696.

Shitara, H., Kaneda, H., Sato, A., Inoue, K., Ogura, A., Yonekawa, H. and Hayashi, J.I. (2000) Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs throughout embryogenesis. *Genetics*, **156**, 1277–1284.

St John, J.C., Cooke, I. and Barrat, C. (1997) Mitochondrial mutations and male infertility. *Nat. Med.*, **3**, 124–125.

St John, J.C., Sakkas, D. and Barrat, C.L.R. (2000a) A role for mitochondrial DNA and sperm survival. *J. Androl.*, **21**, 189–199.

St John, J.C., Sakkas, D., Dimitriadi, K., Barnes, A., Maclin, V., Ramey, J., Barrat, C. and De Jonge, C. (2000b) Failure of elimination of paternal mitochondrial DNA in abnormal embryos. *Lancet*, **355**, 200.

St John, J.C., Jokhi, R.P. and Barratt, C.L. (2001) Men with oligo-asthenoteratospermia harbour higher numbers of multiple mitochondrial DNA deletions in their spermatozoa, but individual deletions are not indicative of overall aetiology. *Mol. Hum. Reprod.*, **7**, 103–111.

Sutarno, J.M., Cummins, J.M., Greeff, J. and Lymbery, A.J. (2002) Mitochondrial DNA polymorphisms and fertility in beef cattle. *Theriogenology*, **57**, 1603–1610.

Sutovsky, P., Tengowski, M.W., Navara, C.S., Zoran, S.S. and Schatten, G. (1997) Mitochondrial sheath movement and detachment in mammalian, but not nonmammalian, sperm induced by disulfite bond reduction. *Mol. Reprod. Dev.*, **47**, 79–86.

Sutovsky, P., Moreno, R.D., Ramalho-Santos, J., Dominko, T., Simerly, C. and Schatten, G. (1999) Ubiquitin tag for sperm mitochondria. *Nature*, **402**, 371–372.

Tourmen, Y., Baris, O., Dessens, P., Jacques, C., Malthièry, Y. and Reynier, P. (2002) Structure and chromosomal distribution of human mitochondrial pseudogenes. *Genomics*, **80**, 71–77.

Woischnik, M. and Moraes, C.T. (2002) Pattern of organization of human mitochondrial pseudogenes in the nuclear genome. *Genome Res.*, **12**, 885–893.

World Health Organization (1999) *WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction*. Cambridge University Press, Cambridge.

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