Can mitochondrial DNA mutations cause sperm dysfunction?

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Very low levels of somatic mitochondrial (mt)DNA deletions have been identified in the semen of infertile men. It has been suggested that these mutations cause infertility through an effect on sperm motility, but there has been no direct evidence to show that mutant mtDNA can affect sperm function. We have carried out semen analysis on a male harbouring the A3243G mtDNA mutation and show that high levels of mutant mtDNA strongly correlate with low sperm motility.

Key words: infertility/MELAS/mitochondrial encephalomyopathy/mitDNA/sperm

Introduction

Infertility affects 10–15% of couples. In 40% of cases, this is primarily due to the male and is related to sperm motility (asthenozoospermia) or sperm number (oligospermia) (Baker, 1994). Although sperm can survive by glycolysis, sperm motility is dependent upon oxidative metabolism (Ruiz-Pesini et al., 1998) and it has been suggested that abnormal mitochondrial function might cause male infertility (Cummins et al., 1994). Abnormalities of sperm structure and function have been identified in patients with mitochondrial disorders (Folgero et al., 1993), and there appears to be a relationship between mitochondrial (mt)DNA haplotype, respiratory chain function in sperm and asthenozoospermia (Ruiz-Pesini et al., 2000). Further evidence comes from a recent study reporting an association between a range of sperm quality defects, male infertility and a polymorphic variant in the CAG microsatellite repeat of the mitochondrial DNA polymerase γ gene (Rovio et al., 2001).

Low levels of mtDNA mutations have been identified in the semen of males seeking medical help for their infertility problem (Kao et al., 1995, 1998; Lestienne et al., 1997; St John et al., 2001). This raises the possibility that acquired mtDNA mutations might contribute to age-related sperm dysmotility, and the consequent decline in male reproductive capacity seen with advancing age (Cummins et al., 1994). Although this hypothesis is attractive, there has been no direct evidence to show that mutant mtDNA actually causes defective sperm motility, and not all studies have shown a clear relationship between male infertility and the proportion of mutant mtDNA in semen specimens (Cummins et al., 1998).

Inherited mtDNA mutations are a common cause of multisystem disease (DiMauro and Schon, 2001). Patients with mtDNA disease usually harbour a mixture of mutant and wild-type mtDNA in the same tissue (heteroplasmy). In-vitro and single cell studies have shown that individual cells must contain a high percentage level of mutant mtDNA before they express a respiratory chain defect (Larsson and Clayton, 1995), and the severity of the neurological clinical phenotype in patients with mtDNA disease correlates with the percentage of mutant mtDNA in clinically relevant tissues (Chinnery et al., 1997a; White et al., 1999). To explore the possible relationship between mutant mtDNA and sperm motility we studied two independent semen samples obtained from a male who had inherited the A3243G mtDNA from his mother [index case reported in Chinnery et al. (Chinnery et al., 1997b)]. We separated each semen sample into different fractions based upon sperm motility and measured the percentage of mutant mtDNA (mutation load) in each sperm fraction.

Materials and methods

Concentration, motility and sperm kinematics were determined on each sample using a 20 μm deep Microcell chamber and a Computer Assisted Sperm Analysis (CASA) system (Hobson Sperm Tracker, software version 7.01). For each analysis 200 sperm were studied, each for a minimum of 1 s, with a sampling frequency of 25 Hz. We were particularly interested in the percentage of sperm capable of fertilization (motility classes a and b), the curvilinear velocity (VCL) and the average path velocity (VAP) of sperm because these parameters reflect sperm oxidative metabolism (Ruiz-Pesini et al., 1998). Fractionation of the sperm was accomplished by self-migration into a discontinuous Percoll gradient. The 50, 60, 70 and 80% Percoll gradients were formed by mixing 5, 6, 7 and 8 ml of 100% isotonic Percoll stock solution with 5, 4, 3 and 2 ml of Earle’s balanced salt solution (EBSS). EBSS was prepared by mixing nine parts of water with one part of 10-fold concentrated EBSS (Gibco) and adding 0.3% bovine serum albumin (BSA, fraction V; Sigma), 25 mmol/l NaHCO₃ (Gibco), 0.5 mmol/l sodium pyruvate (Gibco) and 1% gentamycin (Gibco). The isotonic Percoll stock solution was prepared similarly, except that pure Percoll solution (Sigma) was used as diluent instead of water. The pH of these solutions after equilibration was 7.40 ± 0.5 and the osmotic pressure was 300–328 mOsm. The 80, 70, 60 and 50% Percoll solutions were layered carefully one on top of the other in this sequence (1 ml of each). Finally, 1 ml of sperm was placed on top of the column and the tube was incubated in a 5% CO₂ atmosphere at 37°C for 90 min. At the end of the incubation, 1 ml of each gradient layer was sequentially collected and washed with 5 ml EBSS before centrifugation at 300 g for 10 min. Sperm kinematics were assessed on each fraction before the extraction of genomic DNA. Mutation load was determined on each sperm fraction by last-cycle hot PCR as previously described (Chinnery et al., 1999).

A region including the mtDNA Leu (UUR) tRNA gene was amplified by
Figure 1. Sperm kinematics (a) and corresponding mtDNA analysis (b) of semen fractions isolated in a discontinuous Percoll gradient for a patient harbouring the A3243G mtDNA mutation. The corresponding Percoll fraction is shown between (a) and (b) in the box. (a) Computer-assisted sperm analysis was performed on the five semen fractions. The sperm were classified into four percentage groups: class a, fast progressively motile sperm; class b, progressively motile sperm; class c, non-progressively motile (twitching) sperm; and class d, immotile sperm. The motility of class a plus class b is, by convention, considered to represent the percentage of actively motile sperm that are capable of fertilization. Solid bars = motility a + b (%). Cross-hatch = curvilinear velocity, VCL (µm/s). Empty bars = average path velocity, VAP (µm/s). (b) Percentage level of the A3243G mtDNA mutation in each sperm fraction shown immediately below the corresponding Percoll fraction and kinematic analysis. –ve = DNA sample known not to contain the A3243G mutation.

Table 1. Semen analysis prior to Percoll gradient separation for the two independent unfractionated samples

<table>
<thead>
<tr>
<th>Semen parameters</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>WHO normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>2.9</td>
<td>3.2</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td>Sperm (×10⁹/ml)</td>
<td>12.37</td>
<td>16.50</td>
<td>20.00</td>
</tr>
<tr>
<td>Motility a+b (%)</td>
<td>22</td>
<td>16</td>
<td>&gt;50</td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>53.9</td>
<td>48.4</td>
<td>N/A</td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>34.7</td>
<td>29.2</td>
<td>N/A</td>
</tr>
<tr>
<td>Leukocytes (×10⁶/ml)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>≤1</td>
</tr>
</tbody>
</table>

WHO = World Health Organisation, 1999; VCL = curvilinear velocity; VAP = average path velocity; N/A = WHO reference range not available.

PCR and α32-P dCTP was added before the last cycle of the reaction. The presence of the A3243G mutation creates an additional HaeIII restriction site within the amplified fragment. The PCR products were therefore digested with the restriction endonuclease HaeIII (Roche) before separation on a 6% non-denaturing polyacrylamide gel. Mutation load was determined by phosphorimage analysis (Molecular Dynamics).

Results

The semen volume was within the normal range, but the concentration and motility were below normal for both samples (World Health Organization, 1999; Table I). Analysis after Percoll fractionation revealed a strong inverse correlation between sperm motility and A3243G mutation load in both samples (Figure 1). A higher mutation load was associated with lower sperm motility (Motility a + b, logarithmic relationship, R² = 92.7%, P = 0.009), a lower curvilinear velocity (VCL, R² = 90.5%, P = 0.013) and a lower average path velocity (VAP, R² = 89.8%, P = 0.014).

Discussion

Our observations provide direct evidence that a mtDNA mutation can cause sperm dysfunction. Reduced sperm motility has previously noted in a patient with mtDNA disease due to the A3243G mtDNA mutation (Folgero et al., 1993, 1995) and in two patients with a multiple mtDNA deletion disorder (Fadic et al., 1997; Lestienne et al., 1997), but asymptomatic carriers of the A3243G mutation may have normal sperm motility (Huang et al., 1994). MtDNA disease due to these mutations is relatively rare (Chinnery et al., 2000b), and we are not suggesting that it is a common cause of sperm dysmotility. Infertile adult males harbour a range of different mtDNA mutations acquired during life (Kao et al., 1995, 1998) and it is possible that different mutations may reach high levels within individual sperm progenitor cells, leading to dysfunctional sperm and consequent infertility (St John et al., 2001).

How can we explain the apparent linear relationship between sperm kinematics and the percentage level of mutant mtDNA? There are a number of possibilities. Each fraction of semen separated by the Percoll gradient contains a mixed population of sperm with different levels of motility. Higher concentrations of Percoll will enrich for highly motile sperm (with a corresponding low mutation load), but many motile sperm will also still remain in the lower concentrations of Percoll, effectively ‘diluting’ the level of the A3243G mutation at that level. An alternative explanation is that the threshold level for the A3243G mutation is much lower in vivo than in vitro. In cybrid cell lines, a biochemical defect was only present in those harbouring >90% A3243G mutation (Chomyn et al., 1992), but in-vivo studies using 31P-MRS have shown that very low levels of the A3243G mutation may cause a defect of mitochondrial energy metabolism (Chinnery et al., 2000a) and therefore lower levels of mutant mtDNA (~50%) may compromise sperm function in vivo. Only 5% of the sperm in the top Percoll fraction had motility adequate for fertilization, and the mean percentage level of mutant mtDNA in this fraction was 64% (Figure 1). It is worth noting, however, that an asymptomatic Chinese individual harboured 38% A3243G mutation in his semen and had normal sperm motility (Huang et al., 1994).

Normal semen contains a small number of leukocytes that may influence the amount of mutant mtDNA, and the patient studied here had very low levels (4%) of mutant mtDNA in his leukocytes (Chinnery et al., 1997b). However, the proportion of leukocytes to sperm in semen is extremely low (<1×10⁹/ml) and it is therefore very unlikely that the leukocyte mtDNA had a major effect on the semen mtDNA analysis reported here (sperm concentration 12.37 and 16.50×10⁶/ml).

The traditional view is that the level of pathogenic heteroplasmic mtDNA mutations tends to be low in rapidly dividing tissues and high in non-dividing tissues. Our observations of human sperm show that this need not necessarily be the case, and factors other than...
mitotic activity modulate cellular mutation load (Dubeau et al., 2000; Battersby and Shoubridge, 2001).

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