Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 322 (2004) 333–339
BBRC
www.elsevier.com/locate/ybbrc

Male infertility and mitochondrial DNA

Elena Carra[a], Donatella Sangiorgi[c], Francesco Gattuccio[b], Anna Maria Rinaldi[a,*]

[a] *Dipartimento di Biologia Cellulare e dello Sviluppo "A. Monroy" Università di Palermo Viale delle Scienze, Palermo, Italy*
[b] *Cattedra di Andrologia, Istituto Materno Infantile Università di Palermo, Palermo, Italy*
[c] *Dottorato di Ricerca in Biotecnologie Applicate alla Medicina della Riproduzione ed alla Medicina Perinatale, Palermo, Italy*

Received 13 July 2004

Abstract

The mitochondrial machinery plays a key role in the energy production and maintenance of spermatozoa motility. In this paper 200 idiopathic oligo-asthenozoospermic patients were classified on the basis of rapid progressive motility ("a") and sperm concentration. Mitochondrial enzymatic activity was studied and correlated to the viability of sperm cells. Mitochondrial DNA purified from both motile and non-motile sperm of the same individuals was amplificated using PCR. Results suggested that only motile sperm have organelles functional in oxygen consumption, unequivocally demonstrating that motility depends on the mitochondrial activity. Mitochondrial DNA of oligo-asthenozoospermic patients seemed to present some defects that made DNA unavailable for amplification.

Keywords: Idiopathic oligo-asthenozoospermia; Mitochondrial DNA; Male infertility

As recently summarised by Moore and Reijo-Pera [1], 10–15% of human couples are affected by infertility, and approximately 50% of these cases of infertility should be attributed to men. Sperm motility is one of the major determinants of male fertility.

Evidence supporting a role for genetics in human infertility is rare. In recent years, authors have localised on the azoospermic factor (AZF) region of the Y chromosome a cluster of genes DAZ (deleted in azoospermia) and RBM (RNA-binding motif), deleted in 10–15% of oligospermic and azoospermic men [2–11], supposed to be specific for testis and essentially transcribed in the germ cells.

It is generally accepted that mitochondria play a key role in the energy metabolism as they contain the enzymes of the oxidative phosphorylation system (OXPHOS), which satisfy the energetic needs of the cells. Mitochondrial DNA (mtDNA) codes for only few subunits of the OXPHOS enzymatic complexes [12]. Mutations of mitochondrial or nuclear DNA coding for subunits of mitochondrial machinery have been implicated in a variety of human diseases [13,14] especially in the organs with a high demand for respiratory energy, like skeletal muscle, heart, kidney, brain, liver, and germinal tissue [15–18]. Kao et al. [19] demonstrated that a 4977 bp mtDNA deletion was associated with diminished fertility and motility of human sperm and that the highest frequency of occurrence of the deletion coincided with reduced sperm motility. Cummins et al. [20], using the "common" deletion as an indicator of mtDNA damage, analysed total DNA from semen of normal, idiopathic, asthenozoospermic men, and patients affected by known genital pathologies (as congenital absence of vas deferens, germinal aplasia, and anatomic malformations). The authors found that although mitochondrial DNA deletions may be associated with primary testicular diseases, there is no significant correlation with semen quality. Ruiz-Pesini et al. [21] identified a specific mtDNA haplogroup (T) more

* Corresponding author Fax: +39 91 6577430.
E-mail address: rinaldi@unipa.it (A.M. Rinaldi).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.07.112

abundant in asthenozoospermic populations than the other haplogroup (H) more frequent in the non-asthenozoospermic. The authors also demonstrated specific differences in the activity of some OXPHOS enzymes, like cytochrome oxidase (COX) or NADH-dehydrogenase (NADH-DH) suggesting that the lower ATP production capacity of T haplogroup could be responsible for the differences in the motility of sperm. A strong effect of free radicals on mitochondrial inner membrane potential and mtDNA deletions has been suggested by St John et al. [22]. who have more recently demonstrated multiple mtDNA deletions associated with male infertility. The authors hypothesised that these deletions could be caused by free radicals inducing loss of DNA integrity and preferential replication of deleted mtDNA molecules during spermatogenesis [23].

In this paper mtDNA from idiopathic oligo-asthenozoospermic patients, with different sperm motility and sperm concentrations, has been studied by testing motile and non-motile sperm of the same individual. The enzymatic activity was investigated by incubating fresh semen samples with Mito-Traker Orange CM-H_2TMRos probe. The mitochondrial enzymatic activity was correlated to the viability of spermatic cells.

Materials and methods

Collection of samples. Semen was collected by masturbation under hygienic conditions after a period of 3/7 days of sexual abstinence in the Andrology Unit of the Istituto Materno Infantile. The Department is licensed by the Ethic Committee (Comitato Etico del Policlinico Universitario di Palermo-Sicilia). Seminograms after semen liquefaction were performed according to the World Health Organization (WHO) guidelines (WHO, [24,25]) within a period of 2 h, by the same biologist.

The variables taken into consideration were volume of ejaculate (ml), sperm concentration ($\times 10^6$/ml), forward motility (%), and morphology (% of atypical forms). The sperm cells were classified into four motility classes (a, b, c, or d) according to WHO: "a" rapid progressive motility, "b" slow or sluggish progressive motility, "c" non-progressive motility, and "d" no motility at all. Samples with spermatozoa agglutination, flagella disturbance, very high viscosity, and leukocytospermia were excluded.

Fluorescence microscopy. Vitality was tested by incubating 100 μl of fresh semen with "Live/Dead viability" reagents (Molecular Probes). The kit contains membrane-permeant SYBR 14 labels live sperm with green fluorescence, and membrane-impermeant propidium iodide which labels with red fluorescence nucleic acids of membrane-compromised sperm, as demonstrated in bovine [26], porcine, ovine, murine [27], goat, turkey [28], and human sperm [27,29].

Mitochondrial respiratory activity was tested by incubating for 5–10 min. One hundred microlitres of fresh semen with (0.2 pg f.c.) Mito-Tracker Orange CM-H_2TMRos (Molecular Probes), non-fluorescent reduced form of tetrametylrosamine, which does not fluoresce until it enters an actively respiring cell, where it is oxidised to the fluorescent mitochondrion-selective probe and then is sequestered in the mitochondria. Samples were analysed under the fluorescence microscope Olimpus BX 50 with a long pass optical filter set at 551 nm (Abs) and 576 nm (Em).

Preparation of motile and non-motile fractions. Ejaculates were diluted with three volume of PBS (2.7 mM KCl, 140 mM NaCl, 8.1 mM Na_2HPO_4, and KH_2HPO_4, pH 7.4). Non-motile cells were allowed to spontaneously sediment while motile sperms were collected from the supernatant and analysed under optical microscope.

After four washings with the same buffer, both fractions were pelleted and used for DNA purification. If the sediment contained a large number of leukocytes an additional purification step through glass-fibre filters was performed.

DNA purification. The pellets of total sperm or of motile and non-motile fractions were resuspended in lysis buffer (500 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% SDS, and 10 mM dithiothreitol) and incubated with 100 mg/ml Proteinase K (Sigma) for 15 h at 55 °C. Debris were removed by centrifuging at 4000 rpm for 1 min at room temperature (5417 R Eppendorf centrifuge).

The supernatant was extracted four times with phenol saturated with 100 mM Tris, pH.8.0, phenol: chloroform (1:1, v/v), and chloroform. DNA was precipitated with 100% ethanol at −20 °C for 3 h. The DNA was collected by centrifugation and suspended in MilliQ sterile water.

PCR amplification. PCR amplifications was conducted individually on DNA extracted from motile and non-motile fractions of sperm samples. The reaction was carried out in 30 μl volumes in PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, and 1.5 mM $MgCl_2$), containing 0.3 U *Taq* polymerase (Roche Molecular Biochemical), 0.5 μM of primers, and 187.5 μM of each deoxynucleoside triphosphate (dNTP).

For detection of mtDNA molecules cycling conditions were performed according to T_m of each primer pair. The primers (map positions listed in the Table 2) were designed according to the published sequences of human mitochondrial DNA (Anderson et al. [30]). For detection of Y chromosomal STRs, PCR amplification was performed in a 25 μl reaction volume containing 2 mM $MgCl_2$ buffer, 1 U *Taq* polymerase (Roche Molecular Biochemical), 200 μM of each deoxynucleoside triphosphate (dNTP), and the primers DYS19, DYS391, and DYS392 [31].

The human Y chromosome azoospermia locus was investigated with the primers described by [8]. The negative results in the PCRs were confirmed by three PCR amplification cycles.

Results

Semen from 200 idiopathic oligo-asthenozoospermic patients (Table 1) and from 20 normospermic subjects as control were analysed.

Sperm viability

In order to investigate sperm viability fresh semen aliquots were incubated with "Live/Dead viability" kit. The cell-permeant nucleic acid stain, SYBR, revealed

Table 1
Sperm samples divided into groups according to the motility of the sperm [23,24] and controls

Groups	Percentage of "a" motility	No. of patients
1	0	28
2	0–5	36
3	5–10	38
4	10–15	30
5	15–20	27
6	20–35	41
		Total 200
Control	35–60	20

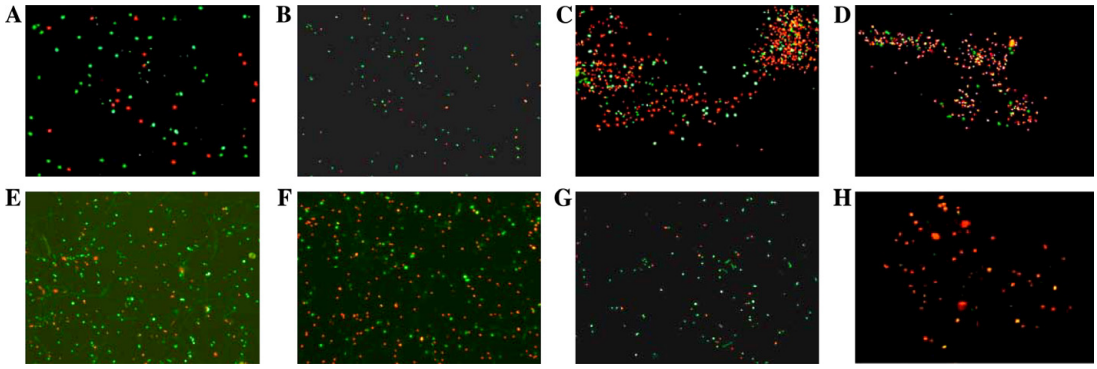

Fig. 1. Nemaspermic viability: fluorescence microscopic analysis of semen in vivo incubated with "Live/Dead viability" kit. Green: live sperm; red: membrane-compromised sperm. Ejaculates with different motility and sperm concentrations: (A) 20% "a" motility; (B) 15% motility and low nemaspermic concentration; (C) 10% motility and high nemaspermic concentration; and (D) 5% motility and high nemaspermic concentration. (E–G) 40% motility, high viscosity, and nemaspermic concentration. Observations 1 h (E), 2 h (F), and after ejaculation and diluted sample 3 h after ejaculation (G). Patient with 10% "a" motility observed three days after ejaculation (H) (10× magnification).

the presence of a live sperm with green fluorescing intact membranes. Dead sperms were labelled with propidium iodide and red fluoresce, as described in Materials and methods. Patient with 20% "a" motility and quite high nemaspermic concentration showed high percentage of alive green spermatozoa, and few dead red sperm cells (Fig. 1A). Patient with 15% "a" motility but lower nemaspermic concentration showed both dead (red) and viable (green) sperm cells (Fig. 1B), suggesting that live sperm cells are more abundant in the samples with higher cell concentrations. When the sperm motility, but not the concentration, decreased, the percentage of dead sperm cells increased (Figs. 1C and D). Sperm concentration and ejaculate viscosity seemed to seriously influence the sperm viability: patient with 40% of "a" motility and very high concentration (panel E) showed a severe decrease of viable sperm cells 2 h after ejaculation (panel F). The dilution with physiological solution restored the viability (observation after 3 h, panel G). Viability decreased during time: indeed, one or three days after ejaculation sperm lost motility and died, consequently the number of green fluorescent cells decreased and at the end the entire sperm cell population became red (panel H).

Sperm viability and mitochondrial respiratory activity

In order to correlate sperm viability with mitochondrial respiratory activity, we incubated fresh semen simultaneously with "Live/Dead viability" and Mito-Traker Orange CM-H_2TMRos reagents. Mito-Traker is the non-fluorescent reduced form of tetrametylrosamine, which does not fluoresce until it enters an actively respiring cell, where it is oxidised to the fluorescent mitochondrion-selective probe and then it is sequestered by mitochondria. Fig. 2 shows that in

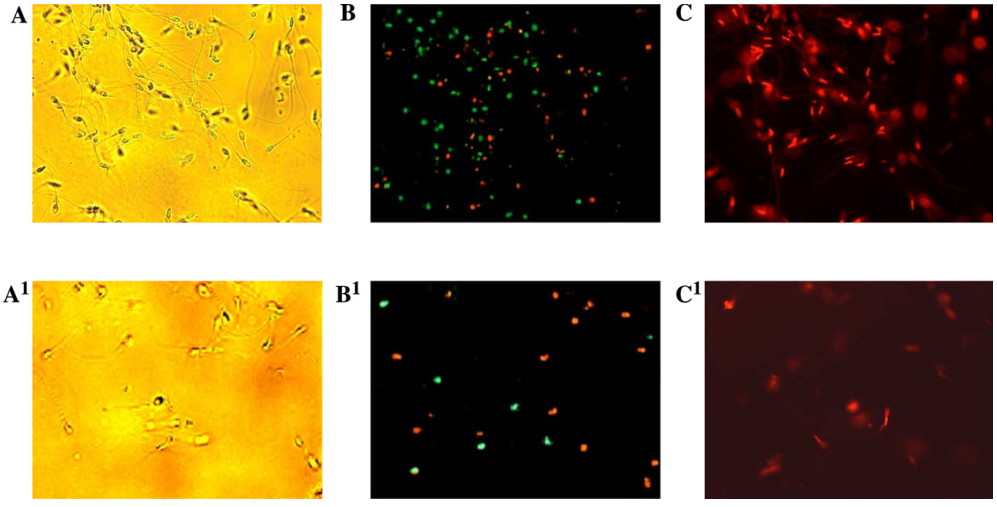

Fig. 2. Nemaspermic viability and mitochondrial respiratory activity tested by in vivo incubation with "Live/Dead viability" kit and Mito-Tracker Orange CM-H_2TMRos. Control with high motility and high sperm concentration (A); sperm viability of the same subject (B); and mitochondrial respiratory activity (C). Patient with 5% "a" motility and lower nemaspermic concentration (A^1); sperm viability of same patient (B^1); and mitochondrial respiratory activity (C^1) (20× magnification).

the control subject with high motility and high sperm concentration, motile sperms (green) (panel B) incorporated Mito-Traker into the mitochondria and hence became fluorescent (panel C). Vice versa, patient with 5% "a" motility and lower nemaspermic concentration showed few viable (green) and dead (red) spermatozoa (panel B^1), and, in addition, only the viable sperms fluoresced after incubation with Mito-Traker (panel C^1). These results suggested that mitochondrial enzymatic activity is necessary for motility, in that non-motile sperms have organelles non-functional in oxygen consumption.

Mitochondrial DNA

In order to study the possibility that defects of mitochondrial DNA could be responsible for sperm dysfunctions we purified DNA from the above-described semen samples and amplified the regions listed in the Table 2. Amplification of mitochondrial DNA was obtained in every region when DNA from controls was analysed. When DNA was prepared from oligo-asthenozoospermic patient with different motility we observed amplification of all the mitochondrial loci except for the region of ND5, which was amplified only in the DNA from samples with high sperm concentration. The sequencing of the PCR products tested the fidelity of amplification. In order to better understand these results, DNA was isolated from motile and non-motile fractions separated from the seminal samples of the same subjects. The products of predicted molecular mass were obtained when DNA from motile fractions of patients with different motility or of the controls was amplified. Surprisingly, no amplification inside the ND5 and ND6 and D-loop regions was obtained from the non-motile fraction of a large number of asthenozoospermic patients. The same DNA gave the expected products of the other mitochondrial and nuclear regions (Table 3). Oligospermic patients (below 20 million of sperm/ml) exhibited mtDNA defects. It is noteworthy that not all the patients in the same classification group presented mitochondrial DNA defects and the percentage of defects increased with a decrease of motility (Fig. 3).

In order to exclude the hypothetical presence of an inhibitor in the oligo-asthenozoospermic samples the following protocol was performed: cells were removed from the ejaculates by centrifugation; increasing aliquots of supernatants were added to a fresh ejaculate of control individuals. Results show that purified DNA gave the predicted amplification products as in the controls. On the other hand, the supernatant of controls added to the patient seminal samples did not change the PCR results. Possible DNA degradation by endonucleases was tested by purifying DNA from aliquots of controls or of patients, ejaculates processed immediately or stored at 4 °C for different times (7, 15, and 30 days) or at −20 °C (10, 20, and 30 days). The same PCR products were observed in all cases excluding the time and/or temperature dependent endogenous nuclease activation. Finally, we found that mtDNA defects were independent of time after ejaculation; in fact, semen of the 10 subjects analysed 6 months after ejaculation presented the same defects in the same regions shown soon after collection.

Table 2
Regions of mt-DNA and Y chromosome amplificated by PCR

Mitochondrial genome	Y chromosome
sub.ND1, NADH dehydrogenase	*DAZ*
m.p. 3.280–4.282	Yq regions (azo-oligospermia)
sub. ND5, ND6 NADH dehydrogenase	*RBM1*
m.p. 12.304–14.704	Yq regions (azo-oligospermia)
m.p. 8.201–13.650	
m.p. 13.176–13.729	
m.p. 13.640–14.700	
m.p. 8.358–13.573	
m.p. 7.048–13.650	
m.p. 8.358–15.042	
tRNA-Leu	*SPGY1*
m.p. 3.029–3.456	Yq regions (azo-oligospermia)
trna-Arg	*STR (CTAT/C)n*
m.p 9.774–10.273	DYS 19
	DYS 391
	DYS 392
D-loop	
m.p. 15–274	
m.p 145–389	
m.p. 15.971–16.259	
m.p. 16.140–16.420	

m.p.: map positions derived from the sequence of human mitochondrial DNA (Anderson et al. [29]).

Discussion

Defective sperm function has been identified as the greatest among the defined causes of male infertility. Some evidence suggests that one of the major determinants of male fertility is the mitochondrial machinery [32,33], which plays a key role in the energy production and maintenance of spermatozoa motility, hence motility is considered a good indicator of semen quality and male fertility. A direct and positive correlation between sperm motility and mitochondrial enzymatic activities has been biochemically demonstrated, suggesting that motility largely depends on energy production by mitochondria. Through the use of specific inhibitors of the mitochondrial I, III, and IV complexes, resulting in the progressive impairment of sperm motility and block

Table 3
Amplification of DNA purified from motile and non-motile fractions of the seminal samples of the same subjects with different percentages of "a" motility and normospermic controls

Amplification products								
Normal motility (%)	Nemaspermic immotile fraction				Nemaspermic motile fraction			
	ND1 ND2 tRNA Leu tRNA Arg regions	Inside the ND5 ND6 D-loop regions	Y-STR (CTA T/C)*n*	DAZ RBM1 SPGY1	ND1 ND2 tRNA Leu tRNA Arg regions	Inside the ND5 ND6 D-loop regions	Y-STR (CTA T/C)*n*	DAZ RBM1 SPGY1
0	+	–	+	+	+	+	+	+
5	+	–	+	+	+	+	+	+
10	+	–	+	+	+	+	+	+
15	+	–	+	+	+	+	+	+
20	+	–	+	+	+	+	+	+
35	+	+	+	+	+	+	+	+
Control	Positive amplification in every region							

(–), negative PCR; (+), positive PCR; negative results confirmed by three successive PCR amplifications, fidelity of positive results tested by sequencing the PCR products.

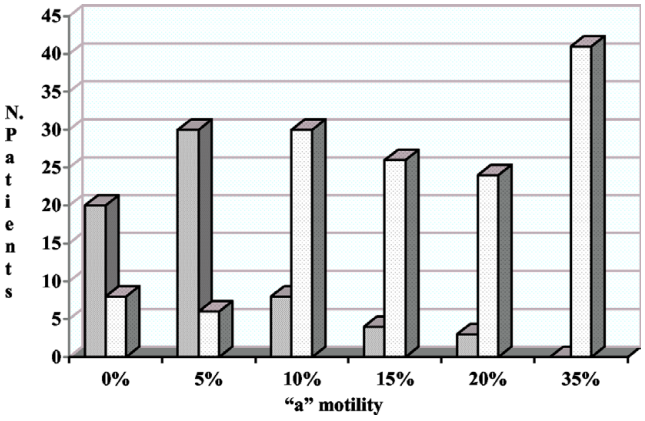

Fig. 3. Diagrams of mtDNA amplification of immotile fraction: ▨, negative amplification; ▤, positive amplification; in the ordinate the number of patients, in the abscissa the "a" motility percentage.

of flagella movements, the authors obtained interesting data suggesting a correlation between sperm motility and defects of mtDNA and nuclear DNA encoding subunits of OXPHOS complexes [18]. During maturation, spermatogonia develop a functional tail and mitochondria are rearranged in the midpiece in an 11–13 gyro helix (or individual spirals around the core), two mitochondria per gyro. Asthenozoospermia could potentially be caused by defects in the tail formation or by defects in the energy-producing machinery required to drive motility. It has been recently demonstrated that in some asthenozoospermic patients the sperm midpiece was significantly shorter than in normal subjects [34]. The molecular bases of these defects were studied many years ago with the purpose of investigating the aetiology and choosing an appropriate therapeutic strategy [35]. PCR amplifications of DNA have shown a substantially higher incidence of mtDNA deletions in infertile or subinfertile men as compared with unaffected individuals [19,20]. In the present paper 200 idiopathic oligo-asthenozoospermic patients were classified in 6 groups on the basis of "a" motility (rapid progressive motility) and sperm concentration. In order to test semen quality, sperm viability was analysed incubating in vivo semen aliquots with "Live/Dead viability" kit. Results suggested that a correlation between viability and the motility of sperm must exist: when the motility but not the concentration of sperm cells decreased, the percentage of dead sperm cells increased. Viable sperm cells were more abundant in the samples with higher "a" motility percentage (whatever sperm concentration is). In order to correlate sperm viability with mitochondrial respiratory activity, we simultaneously in vivo incubated semen with the "Live/Dead viability" kit and the Mito-Traker Orange CM-H_2TMRos reagents. The results showed that only motile sperm cells have organelles functional in oxygen consumption, unequivocally demonstrating that motility of spermatozoon is fully dependent on the functionality of the mitochondrial enzymatic activity. Amplification of mitochondrial DNA was obtained in every region when DNA from controls was analysed. When DNA was prepared from oligo-asthenozoospermic patients with different motility we observed amplification of every mitochondrial locus except for the region of ND5, which was amplified only in the DNA from samples with high sperm concentrations. Interestingly, when we separated and analysed both non-motile and the motile populations of sperm cells of the same subjects, only the non-motile fraction (neither the motile nor the controls) showed specific mitochondrial DNA defects. In addition, we observed that the motility decrease coincided with the increase of mtDNA defects, in fact, only patients with low sperm concentration (<20 million/ml) did not show amplification.

Oligozoospermic samples did not seem to possess an amplification inhibitor; on the other hand, the hypothesis of the activation of a specific endonuclease had been discarded. These defects were independent of time after ejaculation; in fact, semen of the 10 subjects analysed 6 months after ejaculation presented negative PCR in the same regions as when analysed soon after collection. The results suggested that mtDNA of oligo-asthenozoospermic patients could present some deletions or substitutions that make DNA unavailable for amplification. The fluorescence microscopic in vivo approach can be useful for classifying the quality of semen on the basis of vitality, life span of vitality, and relationship between vitality and concentration, motility and viability, and mitochondrial enzymatic activity.

Acknowledgments

The authors thank Dr. H.T. Jacobs for very clever and helpful criticism and suggestions; Prof. G. Giudice, I. Di Liegro, and F. Gianguzza for the critical reading of the manuscript. This work was supported by grants from MURST (60%), Dottorato di Ricerca in Biotecnologie Applicate alla Medicina della Riproduzione ed alla Medicina Perinatale, and E.C. (contract QLG1—CT—2001—00966 Mit EURO).

References

[1] F.L. Moore, R.A. Reijo-Pera, Male sperm motility dictated by mother's mtDNA, Am. J. Hum. Genet. 67 (2000) 543–548.
[2] R. Reijo, T. Lee, P. Salo, R. Alagappan, L.G. Brown, M. Rosenberg, et al., Diverse spermatogenic defect in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene, Nat. Genet. 10 (1995) 383–392.
[3] R. Reijo, R.K. Alagappan, P. Patrizio, D.C. Page, et al., Severe oligozoospermia resulting from deletions of azoospermia factor gene on Y chromosome, Lancet 347 (1996) 1290–1293.
[4] J.L. Pyor, M. Kent-First, A. Muallem, A.H. Van Bergen, W.E. Noltan, et al., Microdeletions in the Y chromosome of infertile men, N. Engl. J. Med. 336 (1997) 534–549.
[5] K. Ma, J. Inglis, A. Sharkey, W. Bickmore, R. Hill, E. Prosser, et al., A Y chromosome gene family with RNA-binding protein homology: candidates for the azoospermia factor AZF controlling human spermatogenesis, Cell 75 (1993) 1287–1295.
[6] A.C. Chandley, H.J. Cooke, Human male fertility Y-linked genes and spermatogenesis, Hum. Mol. Genet. 3 (1994) 1449–1452.
[7] D. Elliot, H. Cooke, The molecular genetics of male infertility, Bioassays 19 (1997) 801–809.
[8] P.H. Vogt, A. Edelmann, S. Kirsch, O. Henegariu, P. Hirschmann, F. Kiesewetter, et al., Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11, Hum. Mol. Genet. 5 (1996) 933–943.
[9] L. Tiepolo, O. Zuffardi, Localisation of factors controlling spermatogenesis in the non-fluorescent portion of the human Y chromosome long arm, Hum. Genet. 34 (1976) 119–124.
[10] K. Kobayashi, M. Kunihiko, H. Akiko, R. Komaki, K. Tomita, I. Matsushita, et al., PCR analysis of the Y chromosome long arm in azoospermic patients: evidence for a second locus required for spermatogenesis, Hum. Mol. Genet. 3 (1994) 1965–1967.
[11] M. Ruggiu, R. Speed, M. Taggart, S.J. McKay, F. Kilanowski, P. Saunders, et al., The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis, Nature 389 (1997) 73–77.
[12] A.A. Alcivar, L.E. Hake, C.F. Millette, J.M. Trasler, B.H. Norman, Mitochondrial gene expression in male germ cells of the mouse, Dev. Biol. 135 (1989) 263–271.
[13] T. Ozawa, Genetics and functional changes in mitochondria associated with aging, Physiol. Rev. 77 (1997) 425–456.
[14] G. Hofhaus, G. Attardi, Lack of assembly of the mitochondrial DNA-encoded subunits of the respiratory NADH dehydrogenase and loss of enzyme activity in human cell mutant lacking the mitochondrial ND4 gene product, EMBO J. 12 (1993) 3040–3048.
[15] D.C. Wallace, Disease of the mitochondrial DNA, Ann. Rev. Biochem. 61 (1992) 1175–1212.
[16] D.C. Wallace, Mitochondrial DNA variation in human evolution, degenerative diseases and aging, Am. J. Hum. Genet. 57 (1995) 201–223.
[17] J.M. Cummins, A.M. Jequier, R. Kan, Molecular biology of human male infertility: links with aging, mitochondrial genetics and oxidative stress? Mol. Reprod. Dev. 37 (1994) 345–362.
[18] E. Ruiz-Pesini, C. Diez, A.C. Lapena, A. Perez-Martos, J. Montoya, E. Alvarez, et al., Correlation of sperm motility with mitochondrial enzymatic activities, Clin. Chem. 44 (1998) 1616–1620.
[19] S.H. Kao, H.T. Chao, Y.H. Wci, Mitochondrial deoxyribonucleic acid 4977-bp deletion is associated with diminished fertility and motility of human sperm, Biol. Reprod. 52 (1995) 729–736.
[20] J.M. Cummins, A.M. Jequier, R. Martin, D. Mehemet, J. Goldblatt, Semen levels of mitochondrial DNA deletions in men attending an infertility clinic do not correlate with phenotype, Int. J. Androl. 21 (1998) 47–52.
[21] E. Ruiz-Pesini, A.C. Lapena, C. Diez-Sanchez, A. Perez-Martos, J. Montoya, E. Alvarez, et al., Human mtDNA haplogroups associated with high or reduced spermatozoa motility, Am. J. Hum. Genet. 67 (2000) 682–696.
[22] J.C. St John, D. Sakkas, C.L.R. Barrat, A role for mitochondrial DNA in sperm survival, J. Androl. 21 (2000) 189–199.
[23] J.C. St John, R.P. Jokhi, C.L.R. Barrat, Men with oligoasthenoteratozoospermia harbour higher numbers of multiple mitochondrial DNA deletions in their spermatozoa, but individual deletions are not indicative of overall aetiology, Mol. Hum. Reprod. 7 (2001) 103–111.
[24] WHO. World Health Organization Laboratory Manual for the examination of human semen and semen-cervical mucus interaction, Cambridge University Press, Cambridge, 1992.
[25] WHO. World Health Organization. Manual for the Standardised Investigation, Diagnosis and Management of the Infertile Male, Cambridge University Press, Cambridge, 2000.
[26] D.L. Garner, Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide, J. Androl. 15 (1994) 620–629.
[27] D.L. Garner, L.A. Johnson, Viability assessment of mammalian sperm using SYBR-14 and propidium iodide, Biol. Reprod. 53 (1995) 276–284.
[28] A.M. Donoghue, Viability assessment of turkey sperm using fluorescent staining and flow cytometry, Poultry Sci. 74 (1995).
[29] K. König, H. Liang, M.W. Berns, B.J. Tromberg, Cell damage by near-IR microbeams, Nature 377 (1995) 20.
[30] S. Anderson, A.T. Bankler, B.G. Barrell, M.H. de Bruijn, L. Coulson, J. Drouin, et al., Sequence and organization of the human mitochondrial genome, Nature 290 (1981) 457–465.
[31] M. Kayser, P. de Knijff, P. Dieltjes, M. Krawczak, M. Nagy, T. Zerjal, A. Pandya, C. Tyler-Smith, L. Roewer, Applications of microsatellite-based Y chromosome haplotyping, Electrophoresis 18 (1997) 1602–1607.
[32] J.C. St. Johns, I.D. Cooke, C.L. Barratt, Mitochondrial mutations and male infertility, Nat. Med. 3 (1997) 124–125.

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

[34] A.J. Mundy, T.A. Ryder, D.K. Edmonds, Asthenozoospermia and the human sperm mid-piece, Hum. Reprod. 10 (1995) 116–119.

[35] A. McLaren, Genetics and human reproduction, TIG 14 (1998) 427–431.