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ANALYSIS OF SPERM MOTILITY RELATED TO TRANSCRIPTIONAL ALTERATIONS OF MITOCONDRIAL GENES IN MALES AFFECTED BY INFERTILITY

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Infertility is a problem afflicting about 1/6 couples, and in 40% of cases this is primarily due to the male. Male infertility is a multifactorial pathology and it seems mainly related to sperm motility or sperm number. However, a diagnosis of infertility is frequently not followed by a precise explanation of its cause, reflecting our poor understanding of the spermatogenesis-related regulatory mechanisms and gene expression profiles. Therefore, this study was design to investigate the relative gene expression of a specific gene profile in ejaculate spermatozoa of men affected by infertility. This profile included 13 mitochondrial gene encoding subunits of respiratory chain and 7 nuclear sperm motility-related genes. We used values of progressive sperm motility (PR) to separate subjects affected by infertility into two groups, showing PR values higher (H group) or lower (L group) than the mean of the sample, and to classify fertile men (control group). We did not obtain a statistically significant difference in nuclear gene expression patterns in spermatozoa among these three groups. On the other hand, we observed an over-expression in 11/13 tested mitochondrial genes in the population of infertile males with altered sperm motility compared to the control group. This over-expression led us to speculate that there is an abnormal mRNA transcription of these 11 subunits, that impaired the normal energy supply ensuring sperm motility. Regarding the under-expression of 2/13 tested mitochondrial genes, we could assume that the spermatozoa mtDNA has accumulated mutations involving these two genes (CYB and ND4L). In conclusion, our results will provide useful information for the development of molecular diagnostic tools for clinical assessment of sperm health. However, further investigation into other sperm-related genes is needed to establish their roles in male fertility.

Infertility is a problem afflicting 10-15% of couples, and in 40% of cases this is primarily due

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to the male (1, 2). Male infertility was shown to be a complex and multifactorial pathology (3, 4) and the underlying physiologic and biochemical aspects remain largely to be understood. A diagnosis of infertility is frequently not followed by a precise explanation of its cause and the case is classified as idiopathic, reflecting our poor understanding of the basic process of spermatogenesis and the associated regulatory mechanisms (5).

Increased availability of technological tools to investigate the molecular basis underlying selected cases of male infertility contributed to focus attention on genetic determinants, including chromosomal abnormalities and single gene disorders (6-11, 1). Reduced sperm count (oligospermia) and reduced sperm motility (asthenozoospermia) are among the two most frequent signs of male infertility (12-17, 2). Insufficient availability of energy can be the cause of both reduced motility and loss of vitality; consequently, since mitochondria are responsible for constantly providing energy to the sperm in its travel through the cervix (18, 1), the attention of researchers has been dedicated to mitochondrial alterations and male infertility (13, 19, 2, 20, 21).

Mitochondrion is the principal site of oxidative phosphorylation of the cell and contains its own genetic system consisting of a single circular dsDNA molecule composed of 16569 bp (7). Mitochondrial DNA (mtDNA), which is maternally transmitted, codes 13 of the 67 polypeptides of the respiratory chain complexes that provide the main ATP supply of the cell (20). In particular, all mitochondrial complexes of the electron transport chain, with exception of complex II, are encoded in part by the mitochondrial genome, the remainder being encoded by the nucleus (22, 13, 19).

Mature mammalian sperm are known to contain ~22-75 mitochondria, which form a tight helix around the flagellar basis of the mid-piece, providing the ATP necessary for flagellar propulsion (20). Although mutations affecting mtDNA have been reported and related to defects in energy production and abnormalities of sperm morphology (15; 19, 1, 2), several authors suggest that factors other than mutations are effectively responsible for defects underlying male infertility (2). Interestingly an excess of mtDNA and its transcripts seems to affect sperm motility and sperm counts (23), while maintenance of mtDNA in low copy number helps to maintain normal sperm function (24), suggesting that excessive transcriptional activity of mitochondrial genes may result in ROS-mediated damage to mtDNA and sperm degeneration.

In the light of the above considerations, this work was aimed at studying the relative gene expression levels of the 13 mitochondrial genes encoding the respiratory chain complexes subunits between normal (control group) and altered sperm motility (infertile group) male subjects. Transcription of six nuclear genes, previously related to infertility was also studied as control.

MATERIALS AND METHODS

Studied population

We collected 98 semen samples from 98 men (mean age 34.9±7.4, ages ranging 22 to 55) who referred to the Division of Andrology, Pisa University, Italy, from July to September 2010, for infertility analysis. Sperm samples were produced by masturbation after 3–4 days of sexual 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) in 2010. According with these criteria, the rate of progressive sperm motility (PR), not-progressive sperm motility (NP) and immotile sperm (IM) were tested (Fig. 1). Thus, values of PR were used to separate subjects affected by infertility into two groups showing PR values higher (H group) or lower (L group) than the mean of the sample (Fig. 1). These patients were also screened for known causes of infertility including chromosome anomalies and Y chromosome AZF deletions. The enrolled individuals showed altered sperm motility but had normal karyotype, clinical examination (no varicocele, infection or other reversible cause of infertility was found) and hormonal profile (FSH: 1.0–10.5 IU/L, LH: 0.7–8.0 IU/l, Testosterone: 3.0–10.0 ng/ml, Inhibine B: 80–400 pg/ml, Oestradiol: 22–49 ng/l, Prolactin: 2–15 ng/ml). Normal semen samples were obtained from 40 healthy men of proven fertility (mean age 33.6±6.8, ages ranging 24 to 52) according to 2010 WHO criteria. Control group normal sperm (C group) met the following criteria: > $15x10^6$ spermatozoa/ml with more than 58% active sperm, > 32% of PR, and < $1x10^6$ lymphocyte/ml per high power field (40X).

The nature of the study was explained to all the subjects who met the inclusion criteria and they were asked to indicate their acceptance by voluntarily signing an informed consent complying with the Helsinki

Declaration.

RNA extraction and purification

According to counts of spermatozoa, adequate aliquots of each sample were processed to extract total cellular RNA from $5x10^6$ live spermatozoa. Semen samples were then diluted 1/2 with sterile RNAse-free water, subjected to liquefaction at 37°C, and spermatozoa were selected and separated from somatic cells by centrifugation through a two-layer (45% and 90%) gradient of Percoll at 400 g for 20 min. The fraction in the 90% layer was washed in 2 ml of Tyrode and the tube was centrifuged at 400 g for 10 min. The pellet was collected with extreme care and was subjected to extraction of total RNA. Total RNA was extracted from spermatozoa using the Total RNA Purification kit (Norgen Biotek, Thorold, Canada) according to the manufacturer's instructions. The purified RNA samples were subjected to RNAase-free DNAse treatment (Fermentas Life Sciences, M-MEDICAL S.r.l, Milan, Italy).

Retro-transcription and Real Time qPCR

Identical volumes of each RNA sample were retro-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories S.r.l, Milan, Italy) and diluted to a final volume of 0.1 ml. Real Time qPCR was performed using the Maxima SybrGreen QPCR master mix (Fermentas Life Sciences, M-MEDICAL S.r.l, Milan, Italy), with 1 µl of cDNA as template, to amplify specific fragments of the 14 genes of mtDNA (including RNR1 as mitochondrial housekeeping gene) and of 8 additional nuclear genes relevant to mitochondrial function (TPX1, ODF2, AKAP4, LDHC, CLGN, THEG, TSGA2 and GAPDH as nuclear housekeeping gene). Relevant characteristics of primers used for PCR amplification are reported in Table I (Table I). Reactions were performed using the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The relative gene expression levels between L and H or L and C groups were compared by Student *t*-tests performed at a significance level of $P \leq 0.05$, using statistical analysis tools of the Microsoft Excel software.

RESULTS

The cohort of subjects affected by infertility was

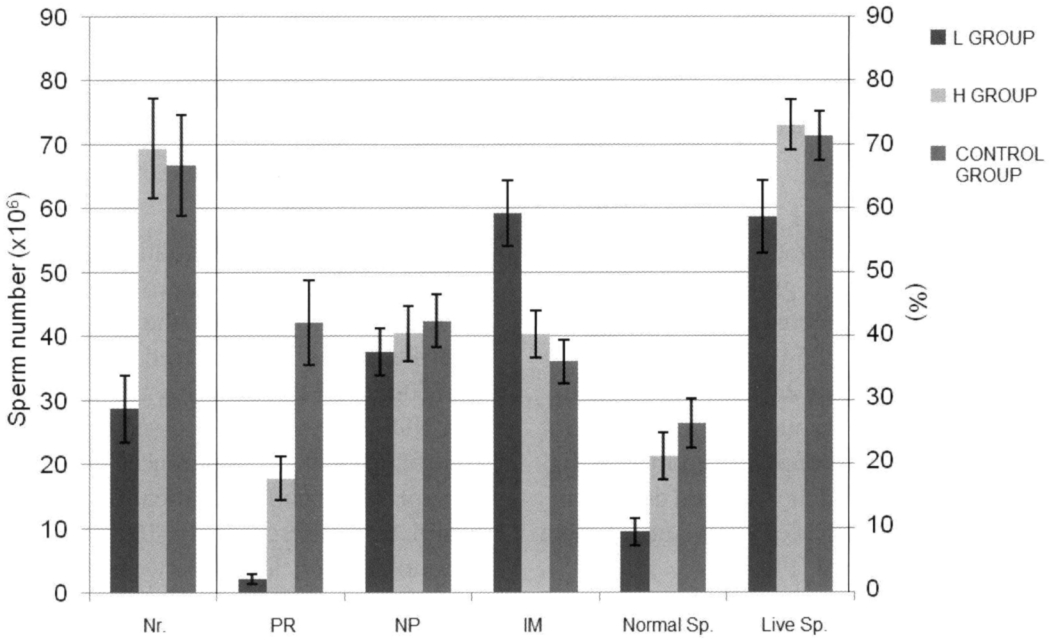

Fig. 1. *Results of semen analysis of both the infertility groups (L and H groups) and the healthy control subjects (control group). The subjects affected by infertility was divided into 2 groups basing on PR values being higher (H group) or lower (L group) than the mean of the cohort. The L group consisted of 44 subjects, the H group of 54 subjects and the control group of 40 subjects. The mean values and the standard deviations, indicated as histogram bars, for each studied label in all three groups are shown. Sperm number (Nr) are expressed as x 10^6/mL (Y-axis on the left), whereas the percentage (%) of the sperm with progressive motility (PR), sperm with non-progressive motility (NP), immotile sperm (IM), normal spermatozoa (Normal Sp) and live spermatozoa (Live Sp) are indicated (Y-axis on the right).*

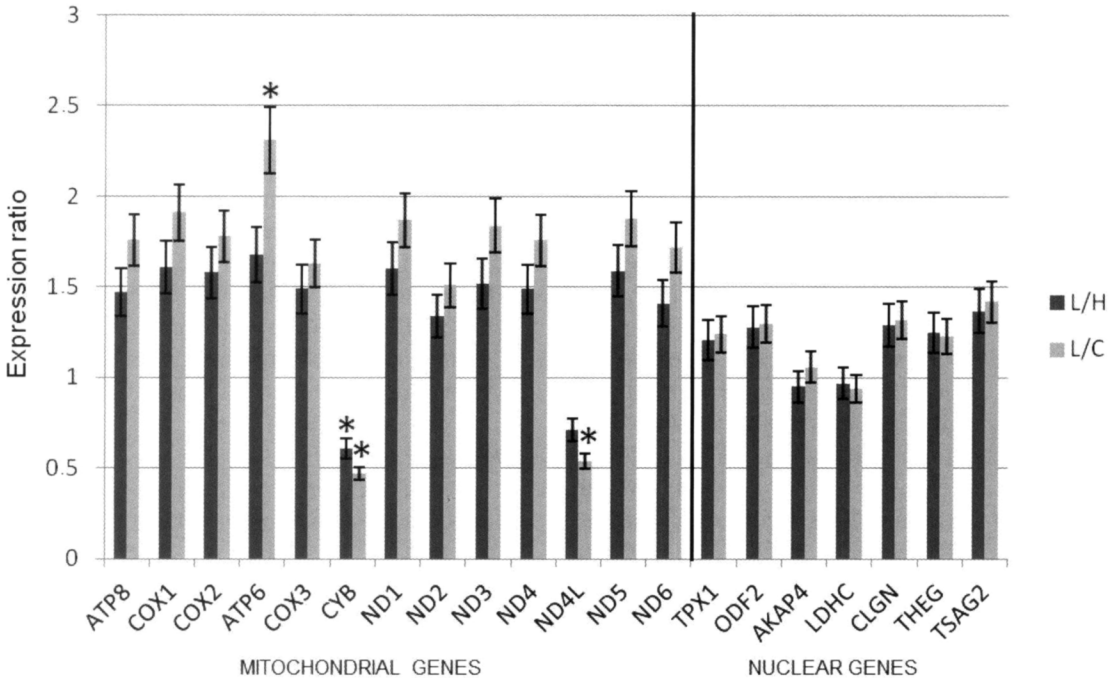

Fig. 2. *Results of transcriptional profiling expressed as fold expression ratios between L and H groups (L/H) or L and control groups (L/C). We studied the relative gene expression levels of the 13 mitochondrial genes encoding the respiratory chain complex subunits (listed in Table I) between normal and altered sperm motility male subjects. Transcription of six nuclear genes previously related to infertility (listed in Table I), was also studied as control. Results show that in the studied population of infertile males with altered sperm motility, most mitochondrial genes, with the exception of CYB and ND4L, are over-expressed compared to healthy controls, but these differences in expression level are not statistically significant ($p \geq 0.05$). The only statistical significant differences obtained ($p \leq 0.05$) are indicated by asterisks. On the other hand, no significant difference are observed in expression levels of the studied nuclear genes ($p \geq 0.05$).*

divided into 2 groups basing on PR values being higher (H group) or lower (L group) than the mean of the cohort. The L group consisted of 44 subjects and the H group consisted of 54 subjects.

Results of semen analysis of both the infertility cohort (divided in L and H group) and of the control healthy subjects cohort (C group) are reported in Fig. 1, where the mean values and the standard deviations, indicated as histograms bars, for each studied label in all three groups are shown (Fig. 1).

Results of transcriptional profiling for each gene observed are expressed as fold expression ratios between L and H groups (L/H) or L and C groups (L/C) (Fig. 2). We studied the relative gene expression levels of the 13 mitochondrial genes encoding the respiratory chain complexes subunits (listed in Table I) between normal and altered sperm motility male subjects. Transcription of six nuclear genes previously related to infertility (listed in Table I), was also studied as control.

Results show that in the studied population of infertile males with altered sperm motility, most mitochondrial genes, with the exception of CYB and ND4L, are over-expressed compared to healthy controls, but these differences in expression level are not statistically significant ($p \geq 0.05$). However, significant differences ($p \leq 0.05$) in mitochondrial gene expression levels between L/H were detected only for gene CYB and between L/C for genes ATP6, CYB and ND4L (Fig. 2). Moreover, no significant difference was observed in expression levels of the studied nuclear genes ($p \geq 0.05$). The significant differences of expression profiles were also evident when the infertile male group was divided in two sub-groups according to sperm motility: in fact, the H group showed expression profiles more similar to

Table I. *Relevant characteristics of primers used for qPCR reactions.*

Primer	Gene/product	Sequence	Tm (°C)	Amplified fragment (bp)
Mitochondrially encoded gene				
qRNR1mt FW	RNR1/ribosomal 12S RNA	CTAGAGGAGCCTGTTCTGTAATCGA	62.98	77
qRNR1mt RV		AAGATGGCGGTATATAGGCTGAGCA	62.98	
qND1mt FW	ND1/NADH-ubiquinone oxidoreductase chain 1 (complex I)	CTCATATGAAGTCACCCTAGCCATC	62.98	121
qND1mt RV		GGGTCATGATGGCAGGAGTAATC	62.43	
qND2mt FW	ND2/NADH-ubiquinone oxidoreductase chain 2 (complex I)	TCTCTCAATCTTATCCATCATAGCAGG	61.93	121
qND2mt RV		GTACGGTAGAACTTCTATTATTCATCC	61.93	
qND3mt FW	ND3/NADH-ubiquinone oxidoreductase chain 3 (complex I)	AACGGCTACATAGAAAAATCCACCCC	63.22	145
qND3mt RV		GTAGGGGTAAAAGGAGGGCAATTTCT	63.22	
qND4Lmt FW	ND4L/NADH-ubiquinone oxidoreductase chain 4L (complex I)	GGAATACTAGTATATCGCTCACACCTC	63.45	159
qND4Lmt RV		TTCGCAGGCGGCAAAGACTAGTAT	62.72	
qND4mt FW	ND4/NADH-ubiquinone oxidoreductase chain 4 (complex I)	TCACTCTCACTGCCCAAGAACTATCA	63.22	109
qND4mt RV		CATAAGTGGAGTCCGTAAAGAGGTATC	63.45	
qND5mt FW	ND5/NADH-ubiquinone oxidoreductase chain 5 (complex I)	TGCTCATCAGTTGATGATACGCCC	62.72	151
qND5mt RV		ATGCTAAGGCGAGGATGAAACCGA	62.72	
qND6mt FW	ND6/NADH-ubiquinone oxidoreductase chain 6 (complex I)	TCATACTCTTTCACCCACAGCACC	62.72	149
qND6mt RV		GGGGGAATGATGGTTGTCTTTGGA	62.72	
qCOX1mt FW	COX1/cytochrome c oxidase subunit I (complex IV)	GACGTAGACACACGAGCATATTTCAC	63.22	108
qCOX1mt RV		TTTCATATTGCTTCCGTGGAGTGTGG	63.22	
qCOX2mt FW	COX2/cytochrome c oxidase subunit II (complex IV)	CATAACAGACGAGGTCAACGATCC	62.72	141
qCOX2mt RV		TCGCCTGGTTCTAGGAATAATGGG	62.72	
qCOX3mt FW	COX3/cytochrome c oxidase III (complex IV)	GGAGTATCAATCACCTGAGCTCAC	62.72	131
qCOX3mt RV		GACTCGAAGTACTCTGAGGCTTGT	62.72	
qATP6mt FW	ATP6/ATP synthase 6 (complex V)	CCATACACAACACTAAAGGACGAACC	63.22	111
qATP6mt RV		TGGTTGGTGTAAATGAGTGAGGCAG	62.98	
qATP8mt FW	ATP8/ATP synthase 8 (complex V)	CCCCCATACTCCTTACACTATTCC	62.72	89
qATP8mt RV		TTTTATGGGCTTTGGTGAGGGAGG	62.72	
qCYBmt FW	CYTB/cytochrome b (complex III)	GCCTCAACCGCCTTTTCATCAATC	62.72	106
qCYBmt RV		GGCAGATAAAGAATATTGAGGCGCC	62.98	
Nuclearly encoded gene				
qGAPDH FW	GAPDH/glyceraldehyde-3-phosphate dehydrogenase	GAAGGTGAAGGTCGGAGTCAAC	62.12	133
qGAPDH RV		CCTGGAAGATGGTGATGGGATTTC	62.72	
qTPX1 FW	CRISP2/cysteine-rich secretory protein 2 or TPX1/testis-specific	ATGGAATGGAGCAGAGAGGTAACAAC	63.22	114
qTPX1 RV	protein 1	GAGATTCTCACCACATCTTGTACTGG	63.22	
qODF2 FW	ODF2/outer dense fiber of sperm tails isoform 2	ATGAAGGAGGAGAAGGACTTCACC	62.72	136
qODF2 RV		TATCCTTCAAGGCCATGACCTGCT	62.72	
qAKAP4 FW	AKAP4/A kinase (PRKA) anchor protein 4	TCTTCTCTGGTAATCCAGATGGCC	62.72	118
qAKAP4 RV		GGGGACTGATTCTCTCTTTGTTCC	62.72	
qLDHC FW	LDHC/lactate dehydrogenase C, transcript variant 1	GTGGTTGTAATCTAGACTCTGCCC	62.72	127
qLDHC RV		CCACTCCATAAGGGCACACTAGAA	62.72	
qCLGN FW	CLGN/calmegin	CCCTGATCCTAATGCTGAAAAACCTG	63.22	197
qCLGN RV		GACTCCAGATTCCCTGATAGTTAGG	62.98	
qTHEG FW	THEG/theg spermatid protein	TGGAATACAGAGCGTCGAGTCG	62.12	157
qTHEG RV		GCCTTCGGCTTTGACAACTGGA	62.12	
qTSGA2 FW	RSPH1/radial spoke head 1 homolog (Chlamydomonas)	ACGGCCATGGCGTATACTACTACA	62.72	127
qTSGA2 RV	or TSGA2/testis specific A2 homolog (mouse)	ACCCAGGTGCCAACATACTTACTG	62.72	

those of healthy controls ($p \leq 0.05$) as compared to the L group (data no shown).

DISCUSSION

An estimated, 1/6 couples experience difficulty in conceiving a child. Male-factor infertility affects 1/20 men in the general population (23) and causes about half the cases in which assisted reproductive techniques are recommended (25). Male infertility is a complex and multifactorial pathology (3, 4) and, although it seems mainly related to sperm motility or sperm number, a diagnosis of infertility is frequently not followed by a precise explanation of its cause, reflecting our poor understanding of the spermatogenesis-related regulatory mechanisms and gene expression profiles (5). However, several independent laboratories have demonstrated the presence of a complex repertoire of mRNAs in ejaculated human spermatozoa (25, 26), heralding a new era in the study of spermatogenesis. These recent findings raise the possibility of using spermatozoa mRNAs as a molecular tool in the clinical assessment of male infertility. To achieve this objective, a global profile of human germ cell-specific spermatogenesis-related gene expression patterns needs to be established (26). Therefore, this study was performed to investigate the relative gene expression of a specific gene profile in ejaculated spermatozoa of men affected by infertility and of fertile men. This profile included 13 mitochondrial gene encoding subunits of respiratory chain and 7 nuclear genes, represented by the *theg* spermatid protein (THEG) gene, the testis specific A2 homolog (TSGA2) gene and five sperm motility-related genes (Table I). These last genes include the A kinase anchor protein 4 (AKAP4), that plays a role in capacitation and the acrosome reaction; the calmegin (CLGN), that mediates the adhesion and interaction between the sperm and the egg; the outer dense fiber of sperm tails 2 isoform 1 (ODF2), the lactate dehydrogenase C, transcript variant 1 (LDHC) and the testis-specific protein 1 (TPX1), which are involved in the motility of sperm.

Regarding the seven studied nuclear genes, no significant difference was observed in their expression levels ($p > 0.05$) comparing the group of infertile men with the control group (fertile men). These data are in contrast with those obtained by Wang and colleagues, who found a significant decrease of expression levels in 2/5 sperm motility-related genes (TPX-1, LDHC) of subjects with motility-impaired sperm (26).

As mentioned above, sperm motility is believed to be one of the most important parameters in evaluating the fertilizing ability of ejaculated sperm. For example, Hirano reported that rapid sperm movement is important for the fertilizing capacity of human sperm (27). Amann and Bongso have shown correlations between the velocity of sperm movement or sperm motility and fertilization rates (28, 29). In this study, we used PR values to separate subjects affected by infertility into two groups showing PR values higher (H group) or lower (L group) than the mean of the sample, and to classify normal and impaired semen samples. However, we did not obtain a statistically significant difference in nuclear gene expression patterns in spermatozoa among these three groups. This result might indicate that the impaired motility could depend on different mitochondrial gene expression patterns. In fact, sperm motility is strictly dependent upon oxidative metabolism and it has been suggested that abnormal mitochondrial function might cause male infertility (2). Normally spermatogenesis is associated with a drastic reduction in mtDNA content, to about a tenth of its initial value, occurring mainly during spermiogenesis. Therefore, mature mammalian sperm contain ~22-75 mitochondria providing the ATP necessary for flagellar propulsion (20). Interestingly, an excess of mtDNA and its transcripts seems to affect sperm motility and sperm counts (23), while maintenance of mtDNA in low copy number helps to maintain normal sperm function (24), suggesting that excessive transcriptional activity of mitochondrial genes may result in ROS-mediated damage to mtDNA and sperm degeneration. In particular, May-Panloup and colleagues found highly significant mtDNA amplification in abnormal sperm, highlighting the multiple implications of mitochondria in male infertility (20). In our study we observed that 11/13 tested mitochondrial genes were over-expressed in the population of infertile males with altered sperm motility as compared to healthy controls ($p < 0.05$). The significant differences of expression profiles were evident also when the

infertile male group was divided in two sub-groups (L group and H group) according to sperm motility. In fact, the H group showed expression profiles more similar to those of healthy controls as compared to the L group ($p < 0.05$). Although we did not investigate the mtDNA copies number in abnormal sperm, the observed over-expression in 11/13 mtDNA genes encoding subunits of respiratory chain, led us to speculate that there is an abnormal mRNA transcription of these 11 subunits. This over-expression would most likely be started in the testis, where the great number of mitochondria, encoding for those mRNAs that will be included in the mature sperm, is concentrated. We could also speculate that the observed over-expression is due to an mtDNA amplification in mature spermatozoa, to compensate low respiratory chain activity. Indeed, such compensatory processes of increased mitochondrial biogenesis are frequently observed in mitochondrial pathology (20).

Finally, regarding the observed under-expression of the ND4L and CYB genes, we could assume that testicular spermatids, from which the mature sperm and its mitochondria originated, have accumulated mtDNA mutations involving the promoters or the coding sequences of these two genes, as St. John and colleagues have also observed (1).

In conclusion, we tried to construct a sperm motility-related gene expression profile by spermatozoa mRNA retro-transcription and Real Time PCR, focusing our attention on seven nuclear genes and 13 mitochondrial genes, which encode polypeptide subunits of the mitochondrial ATP-generating pathway. Our results will provide useful information for similar future studies and for the development of molecular diagnostic tools for clinical assessment of sperm health. However, further investigation into other genes in spermatozoa is needed to establish their roles in male fertility.

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REFERENCES

1. St John JC, Sakkas D, Barratt CL. A role for mitochondrial DNA and sperm survival. J Androl 2000; 21:189-99.
2. Spiropoulos J, Turnbull DM, Chinnery PF. Can mitochondrial DNA mutations cause sperm dysfunction? Mol Human Reprod 2002; 8:719-21.
3. Hauser R, Sokol R. Science linking environmental contaminant exposures with fertility and reproductive health impacts in the adult male. Fertil Steril 2008; 89:e59-65.
4. O'Flynn O'Brien KL, Varghese AC, Agarwal A. The genetic causes of male factor infertility: a review. Fertil Steril 2010; 93:1-12.
5. Gianotten J, Lombardi MP, Zwinderman AH, Lilford RJ, van der Veen F. Idiopathic impaired spermatogenesis: genetic epidemiology is unlikely to provide a short-cut to better understanding. Hum Reprod Update 2004; 10:533-9.
6. Ozawa T. Mechanism of somatic mitochondrial DNA mutations associated with age and diseases. Biochim Biophys Acta 1995; 1271:177-89.
7. Shoffner JM, Lott MT, Voljavec AS, Soueidan SA, Costigan DA, Wallace DC. Spontaneous Kearns-Sayre/chronic external ophthalmoplegia plus syndrome associated with a mitochondrial DNA deletion: a slip-replication model and metabolic therapy. Proc Natl Acad Sci USA 1989; 86:7952-6.
8. Shoffner JM, Wallace DC. Mitochondrial genetics: principles and practice. Am J Human Genet 1992; 51:1179-86.
9. Michikawa Y, Mazzucchelli F, Bresolin N, Scarlato G, Attardi G. Aging dependent large accumulation of point mutations in the human mtDNA control region for replication. Science 1999; 286:774-9.
10. Pesole G, Gissi C, DeChirico A, Saccone C. Nucleotide substitution rate of mammalian mitochondrial genomes. J Mol Evol 1999; 48:427-34.
11. Clayton DA. Transcription and replication of mitochondrial DNA. Human Reprod 2000; 15(S):11-7.
12. Gavella M, Lipovac V. NADH-dependent oxidoreductase (diaphorase) activity and isozyme pattern of sperm in infertile men. Arch Androl 1992; 28:135-41.
13. Cummins JM, Jequier AM, Kan R. Molecular biology of human male infertility: links with aging, mitochondrial genetics, and oxidative stress? Mol

Reprod Dev 1994; 37:345-62.
14. Kao S, Chao HT, Wei YH. Mitochondrial deoxyribonucliec acid 4977bp deletion is associated with diminished fertility and motility of human sperm. Biol Reprod 1995; 52:729-36.
15. Ankel-Simons F, Cummins JM. Misconception about mitochondria and mammalian fertilization: implications for theories on human evolution. Proc Natl Acad Sci USA 1996; 93:3859-63.
16. Lestienne P, Reynier P, Chretien MF, Penisson-Besnier I, Malthiery Y, Rohmer V. Oligoasthenospermia associated with multiple mitochondrial DNA rearrangements. Mol Human Reprod 1997; 3:811-14.
17. Ruiz-Pesini E, Lapena AC, Diez-Sanchez C, et al. Human mtDNA haplogroups associated with high or reduced spermatozoa motility. Am J Human Genet 2000; 67:682-96.
18. Folgero T, Bertheussen K, Lindal S, Torbergsen T, Oian P. Mitochondrial disease and reduced sperm motility. Human Reprod 1993; 8:1863-8.
19. Cummins J. Mitochondrial DNA in mammalian reproduction. Rev Reprod 1998; 3:172-82.
20. May-Panloup P, Chretien MF, Savagner F, Vasseur C, Jean M, Malthiery Y, Reynier P. Increased sperm mitochondrial DNA content in male infertility. Human Reprod 2003; 18:550-6.
21. Thangaraj K, Joshi MB, Reddy AG, Rasalkar AA, Singh L. Sperm mitochondrial mutations as a cause of low sperm motility. J Androl 2003; 24:388-92.
22. Anderson S, Bankier AT, Barrell BG, et al. Sequence and organisation of the human mitochondrial genome. Nature 1981; 290:457-65.
23. Tremellen K. Oxidative stress and male infertility - a clinical perspective. Human Reprod Update 2008; 14:243-58.
24. Wai T, Ao A, Zhang X, Cyr D, Dufort D, Shoubridge EA. The role of mitochondrial DNA copy number in mammalian fertility. Biol Reprod 2010; 83:52-62.
25. Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. Lancet 2002; 360:772-7.
26. Wang H, Zhou Z, Xu M, Li J, Xiao J, Xu ZY, Sha J. A spermatogenesis-related gene expression profile in human spermatozoa and its potential clinical applications. J Mol Med (Berl) 2004; 82:317-24.
27. Hirano Y, Shibahara H, Obara H, Suzuki T, Takamizawa S, Yamaguchi C, Tsunoda H, Sato I. Relationships between sperm motility characteristics assessed by the computer-aided sperm analysis (CASA) and fertilization rates *in vitro*. J Assist Reprod Genet 2001; 18:213-18.
28. Amann RP Can the fertility potential of a seminal sample be predicted accurately? J Androl 1989; 10:89-98.
29. Bongso TA, Ng SC, Mok H, Lim MN, Teo HL, Wong PC, Ratnam SS. Effect of sperm motility on human in vitro fertilization. Arch Androl 1989; 22:185-90.