Reproduction Advance Publication first posted on 20 September 2016 as Manuscript REP-16-0342

RESEARCH ARTICLES

ID REP-16-0342.R2

Sperm of patients with severe asthenozoospermia show biochemical, molecular, and genomic alterations

Bonanno Oriana[1], Romeo Giulietta[2], Asero Paola[1], Pezzino Franca Maria[2], Castiglione Roberto[1], Burrello Nunziatina[1], Sidoti Giuseppe[3], Frajese Giovanni Vanni[4], Vicari Enzo[1], D'Agata Rosario[1,5]

[1]Section of Endocrinology, Andrology and Internal Medicine, Department of Medical and Pediatric Sciences, University of Catania, Catania - Italy; [2]Section of Clinic Pathology and Molecular Oncology, Department of Biomedical Sciences, University of Catania, Catania - Italy; [3]Division of Internal Medicine, Garibaldi Hospital Catania - Italy; [4]Endocrinology, Department of Sport Medicine, University of Rome Foro Italico, Rome - Italy. [5]Emeritus Professor of Endocrinology and Andrology at the University of Catania - Italy

Running head: Sperm alterations in asthenozoospermic patients

Key words: asthenozoospermia, sperm abnormalities, reactive oxygen species, male infertility.

For submission to *Reproduction*

Reprint request: Prof. Enzo Vicari, U.O.C. Andrologia - Endocrinologia, Department of Medical and Experimental Medicine, University of Catania, Catania - Italy, via S. Sofia, 78 - Catania - Italy. Email: vicarienzo@email.it

SUMMARY

Severely low sperm motility is a frequent cause of infertility. This pathological condition is multifactorial. However, mechanisms underlying the development of this condition are not completely understood. Single abnormalities have been reported in sperms of patients with asthenozoospermia. In the present study, we characterized, in 22 normozoospermic men and in 37 patients with asthenozoospermia, biochemical, molecular, and genomic abnormalities that frequently occur in sperm of patients with asthenozoospermia. We evaluated a panel of sperm biomarkers that may affect the motility and fertilizing ability of sperm of patients with severe asthenozoospermia. Since reactive oxygen species (ROS) production is involved in the pathogenesis of such sperm abnormalities, we determined the association between ROS production and sperm abnormalities. High percentage of patients with severe asthenozoospermia showed increased basal and stimulated ROS production. Moreover, these patients showed increased mitochondrial DNA (mtDNA) copy number but decreased mtDNA integrity and they were associated with elevated ROS levels. Furthermore, mitochondrial membrane potential was also significantly decreased and again associated with high ROS production in these patients. However, the rate of nuclear DNA fragmentation was increased only in less than one-fifth of these patients. An important cohort of these patients showed multiple identical biochemical, molecular, and genomic abnormalities, which are typical manifestations of oxidative stress. The most frequent association was found in patients with high ROS levels, increased mtDNA copy number and decreased integrity, and low MMP. A smaller cohort of the aforementioned patients also showed nDNA fragmentation. Therefore, patients with asthezoospermia likely present reduced fertilizing potential because of such composed abnormalities.

INTRODUCTION

Low sperm motility (asthenozoospermia) alone or in combination with other sperm abnormalities is a frequent cause of infertility. Common causes of asthenozoospermia are genital tract infections, varicocele, sperm antibody (ASA), metabolic diseases and tail anatomic abnormalities. Most patients with asthenozoospermia are idiopathic. Cellular energy for sperm motility and propulsion is produced through oxidative phosphorylation, in the mitochondria, a major producer of ATP, through the electron transport chain (Bahr & Engler 1970, St John *et al*. 2000). Treatment of sperm with extracellular ATP significantly increases their fertilization potential (Rossato *et al.* 1999). This complex biochemical and molecular mechanism is genetically controlled by mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) (Bruijn *et al.* 1981). Thus, in addition to other factors (Calogero *et al*. 1998, Narisawa *et al.* 2002), genomic integrity of mtDNA and nDNA plays an important role in maintaining good sperm motility. The mitochondria regulate also cell apoptosis by releasing several apoptotic factors (Susin *et al*. 1999). Many infertile men have fragmented nDNA (Varum *et al*. 2007).

Several studies support the importance of the mitochondria in maintaining sperm quality and motility; in fact asthenozoospermia (Folgero *et al.* 1993) and oligoasthenozoospermia (Lestienne *et al.* 1997) have been reported in patients with typical mitochondrial diseases characterized by point mutations or multiple deletions in mtDNA. Various large deletions in and fragmentation of mtDNA have been observed in sperm with poor sperm quality (Kao S *et al.* 1995, Kao *et al.* 1998, Song & Lewis 2008). Moreover, comparison of several polymorphic regions in mtDNA has shown an association between mtDNA haplogroup and asthenozoospermia (Ruiz – Pesini *et al*. 2000). Additional studies indicate that sperm in abnormal semen samples show quantitative alterations in mtDNA and that sperm of infertile men show increased mtDNA content or copy number (May-

Panloup *et al*. 2003, Song & Lewis 2008). Diez-Sanchez et al. (2003) showed a clear difference in mtDNA copy number between progressively motile and non-progressively motile sperm. These qualitative and quantitative alterations in abnormal sperm may be because of impaired mitochondrial maintenance or oxidative stress-induced deleterious effects on mtDNA (May-Painloup *et al.* 2003, Shamsi *et al*. 2009, Venkatesh *et al.* 2009). Since nuclear and mitochondrial gene products are dependent on each other, nDNA fragmentation may be a more reliable predictor of impaired sperm motility (Muratori *et al.* 2000). Causes of nDNA damage are not completely understood. However, several studies suggest that increased intracellular or extracellular reactive oxygen species (ROS) (Aitken & Curry 2011) and the consequent oxidative stress play a key role in inducing nDNA damage. Sperm contain several ROS substrates such as unsaturated fatty acids, DNA, and proteins and possess limited endogenous antioxidant capacity (Alvarez *et al.* 1987, Aitken *et al.* 1989). Therefore, sperm are highly susceptible to oxidative damage, which in turn affects mtDNA and nDNA (Aitken & De Juliis 2010). In addition, loss of mitochondrial membrane potential (MMP) and subsequent decrease in energy production may decrease sperm motility (Marchetti *et al.* 2004), which are often associated with elevated ROS levels (Wang *et al.* 2003).

Sperm can be affected by endogenous ROS production or by ROS formed in leukocytes present in semen (Whittington & Ford 1999). Use of recently developed probes against mitochondria-produced ROS has shown that mitochondria are the main source of ROS in sperm (Koppers *et al.* 2008, Aitken *et al.* 2012). Once initiated, ROS production becomes a self-perpetuating peroxidation mechanism (Aitken *et al.* 2012) by generating peroxyl and lipid radicals that perpetuate the chain reaction of lipid peroxidation, a process which is very harmful to sperm (Alvarez *et al.* 1987, Aitken & Curry 2011), at biochemical and molecular levels (Agarwal & Allamanemi 2004) because it damages different

substrates, including permanent damage of the axoneme (de Lamirande & Gagnon 1992, Hughes *et al*. 2009).

Therefore the present study evaluated a panel of sperm biomarkers that exert detrimental effects on sperm motility in men with severe asthenozoospermia and determined the association of ROS overproduction to these biofunctional sperm alterations. To this end, we performed biochemical, genomic, and molecular analyses of sperm collected from patients with high initial percentage of non-progressive motile sperm.

MATERIAL AND METHODS

Chemicals and reagents

All chemicals used in this study were purchased from Sigma (Milano, Italy), unless otherwise specified. Percoll was purchased from Codisan (Milano, Italy), and 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and dimethyl sulfoxide (DMSO) were purchased from Bouty (Milano, Italy). 12-myristate, 13-acetate phorbol ester (PMA) was purchased from VWR International (Milano, Italy), and 5,5'.6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) dye was purchased from Space Import-Export (Milano, Italy). Annexin V, PI, LPN DNA-Prep Reagent (L DNA-Pr), and Mebstain Apoptosis (Meb-Ap) Kit were purchased from Beckman Coulter (Milano, Italy). DNA isolation kit was purchased from Qiagen (Milano, Italy), and TOPO TA Cloning (TOPOT-Cl) Kit and AccuPrime Pfx (AcP-Pfx) DNA polymerase were purchased from Invitrogen (Milano, Italy). iQ SYBR Green Kit was from Thermofisher (Milano, Italy).

Preparation of human sperm

This study included 37 men recruited from couples who underwent semen analysis at the Andrology Centre of Catania University (EAA Andrology Centre) as a part of their fertility evaluation. As the study was intended to be performed in patients with a high percentage sperm with low motility, semen samples were collected from patients whose sperm showed arbitrary progressive motility (a + b ≤20%) and non-progressive motility (c >50%). Patients included in the study were diagnosed with idiopathic asthenozoospermia, clinical palpable varicocele, inflammation of the accessory sex glands, or as overweight following physical examination and history taking. Smokers, patients with known exposure to toxic chemicals, alcohol intake and/or drug abuse,

systemic diseases, and recent hormonal treatment were criteria of exclusion. In addition, the study included 22 healthy men with normal sperm parameters (according to the *WHO 2010 guidelines*) whose fertility status was unknown and who volunteered to participate in the study. Exclusion criteria for this group were cigarette smoking, history of cryptorchidism and varicocele, known exposure to toxic chemicals, and presence of genital inflammation. This study was approved by the Institutional Research Review Board of the University of Catania Medical School, all subjects provided written informed consent. Semen samples from men in both the study groups were collected in sterile plastic jars through masturbation after 3–5 days of abstinence. Routine semen analysis was performed within 1 h after ejaculation by using a light microscope to determine conventional sperm parameters (WHO 2010). An aliquot of the semen sample was used for evaluating ROS production. The remaining semen sample was purified by performing Percoll density gradient centrifugation and was used for molecular and genomic analyses.

Purification of human spermatozoa was achieved using a 2-step discontinuous Percoll gradient (90% / 45%) obtained by diluting isotonic Percoll (90 ml Percoll supplemented with 10 ml of 10x Ham's F10 (WHO 2010) solution, 370 µl sodium lactate syrup, 3 mg sodium pyruvate, 210 mg sodium hydrogen carbonate, and 100 mg polyvinyl alcohol) with HEPES-buffered Biggers, Whitten, and Whittingham medium (BWW) (Biggers *et al*. 1971), according to Mitchell et al. (2011). Next, up to 3 ml liquefied semen was layered on top of each gradient and was centrifuged at 500xg for 30 min. Sperm pellet obtained from the base of the high-density fraction of the gradient was recovered, washed with 3 ml BWW and pelleted by centrifugation at 600xg for 10 min.

The final pellet was suspended in a low volume of BWW and was examined under a light microscope. Generally no round cells were found. However samples containing round cells or > 15% immotile sperms(d) were discarded.

Measurement of ROS production

Aliquots of 5-10x10^6 sperm were washed with two volumes BWW and were centrifuged at 300xg for 5 min. Seminal plasma was discarded. ROS production was measured by performing a chemiluminescence assay, as described previously (D'Agata *et al.* 1990). Briefly, 5 µl luminol, which was stored as a 20 mM stock solution in DMSO, and 8 µl horseradish peroxidase (1550 IU/ml in PBS), which was added to sensitize the assay (Krausz *et al.* 1992), were added to 500 µl of the washed sperm suspension as probes. Next, the sperm suspension was diluted with 500 µl BWW, and basal and stimulated ROS production was determined by measuring chemiluminescence with Bioluminate LB 9500 T luminometer (Berthold Technologies, Bad Wildbad, Germany) in an integrated mode for 10 min. Results are expressed as the number of photons counted per minute (cpm)/10 × 10^6 sperms.

Basal chemiluminescent signal (basal ROS) was monitored at 37°C until its stabilization (approximately 5 -10 min). After the system returned to baseline, the sperm suspension in the lumivial was stimulated with 2 µl formyl-leucyl-phenylalanine (FMLP), a polymorphonuclear leukocyte-specific chemiluminescent probe (Krausz *et al*. 1992, Krausz *et al.* 1994), and was monitored for additional 7 min to determine the magnitude of peak obtained. After the signal returned to baseline, 4 µl 10 µM/l PMA was added to the sperm suspension. PMA increases ROS production by stimulating kinase C in both leukocytes and sperm (Ford 1990), resulting in a sustained increase in the chemiluminescent signal (Krausz *at al.* 1994).

Flow cytometric analysis

Flow cytometric analysis was performed using EPICS XL (Beckman Coulter), as reported previously (Perdichizzi *et al*. 2007). In all, 10.000 events were measured for each

sample at a flow rate of 200-300 events/s and were analyzed using SYSTEM II™ Software, 3.0 Version (Coulter Electronics, Milan, Italy).

Determination of MMP

The lipophilic cationic fluorescent dye JC-1 was used to differentiate and label mitochondria with high and low membrane potential. Sperm with intact mitochondria show an intense red-orange fluorescence. In contrast, JC-1 treated sperm with low MMP form monomers that show green fluorescence (Troiano *et al.* 1998).

In the present study, MMP was determined by adjusting the density of the sperm suspension at 0.5-1×10^6 cells/ml with 500 µl phosphate buffer and by incubating the sperm with JC-1 in the dark at 37°C for 10-15 min. JC-1 was dissolved in DMSO to obtain 1 mg/ml stock solution. JC-1 (20 µg) was diluted in 480 µl PBS before adding it to the sperm suspension.

Annexin V/PI assay

PS externalization was determined by staining sperm with FITC-labeled annexin V and PI by using a commercial kit (Perdichizzi *et al.* 2007). Double staining allows the distinction of (a) viable sperm (sperm not stained with annexin V and PI), (b) sperm in the early stage of apoptosis (PS externalization) (sperm stained with annexin V but not with PI), (c) sperm in the late phase of apoptosis (sperm stained with annexin V and PI), and (d) necrotic sperm (sperm stained with PI but not with annexin V). Briefly, an aliquot of the semen sample containing 0.5 × 10^6 sperm/ml was resuspended in 500 µl binding buffer, was labeled with 1 µl annexin V-FITC and 5 µl PI, was incubated in the dark for 10-15 min, and was analyzed immediately. Signals were detected using FL-1 (FITC) and FL-3 (PI) detectors.

PI staining

The degree of chromatin compaction was evaluated using the sperm PI staining. (Perdichizzi *et al.* 2007).

Briefly, an aliquot containing approximately 1 × 10^6 sperm/ml was incubated with 100 µl lysing and permeabilizing reagent in the dark at room temperature. After 10 min, 500 µl L DNA-Pr (containing PI,RNAse type A, NaN salts, and stabilizer) was added to the sperm suspension, and the suspension was incubated in the dark for 30 min.

Sperm with normal chromatin packaging emitted low PI fluorescence because less amount of PI reached the DNA. In contrast, sperm containing endogenous nicks in DNA emitted high fluorescence.

TUNEL assay

The evaluation of fragmentation of DNA was obtained through the Tunnel assay as previously reported (Perdichizzi *et al.* 2007). Briefly, the assay was conducted on aliquots of about 1×10^6 washed sperm, which were labeled using the Meb-Ap Kit. To obtain a negative control, deoxynucleotidyl tansferase was omitted from the reaction mixture, and positive control was obtained by pretreating the sperm with 1 µg/ml deoxyribonuclease I, RNAse-free at 37 °C for 60 min before labeling.

Extraction of total DNA

DNA from the sperm samples of patients and controls was extracted using a DNA isolation kit, according to the manufacturer's instructions. Extracted DNA was quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Euroclone, Milano, Italy) in triplicate.

Long-range polymerase chain reaction for determining mtDNA integrity

Long-range polymerase chain reaction (long PCR) was performed to amplify approximately half of the mitochondrial genome (8.7 kb) by using AcP-Pfx DNA polymerase, which is inactive at ambient temperatures and is activated after initial denaturation to determine mtDNA integrity.

Long PCR was performed in a 50-µl reaction mixture containing 1 × buffer with dNTPs,

- forward primer (5'-AAGGATCCTCTAGAGCCCACTGTAAAG-3'),
- reverse primer (5'-TTGGATCCAGTGCATACCGCCAAAAG-3'),
- 2.5 U DNA polymerase,
- 200 ng sperm DNA.

Amplification conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 25–35 cycles of denaturing (95 °C for 15 s), annealing (62 °C for 1 min), and extension step at 68 °C for 9 min. PCR products obtained were visualized by electrophoresis on 0.8% agarose gels. DNA extracted from sperm treated with H_2O_2 for 1 h at 37 °C was used as control. Results of long PCR showed that DNA amplification decreased after H_2O_2 treatment.

Determination of mtDNA copy number

Quantitative PCR (qPCR) was performed to determine the relative copy number of mtDNA, which was calculated using the copy number ratio of mitochondrial gene encoding 16S rRNA to nuclear gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Song & Lewis 2008). To synthesize standard DNA, PCR was performed using 16S RNA primers under the following amplification conditions: the first cycle at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and

extension at 72 °C for 1 min; and final extension at 72 °C for 10 min. Before cloning, PCR products obtained were electrophoresed on 1% agarose gel, which produced a single 150-bp band. The 150-bp fragment was cloned into the PCR 2.1-TOPO vector by using the TOPOT-Cl Kit, was sequenced to confirm the accuracy of the inserted sequence, and was used as standard DNA for performing qPCR. Plasmid DNA obtained was quantified using NanoDrop 1000 spectrophotometer was diluted to obtain 1×10^2 to 1×10^8 copies/µl, and was stored in a -80°C in a freezer.

The amount of mtDNA and GAPDH was determined using 2 primer sets specific to the mitochondrial 16S rRNA gene and nuclear GAPDH. The mitochondrial amplification reaction was performed in duplicate with 16S rRNA (forward primer 5'-ACTTTGCAAGGAGAGCCAAA-3' and reverse primer 5'-TGGACAACCAGCTATCACCA-3'). Nuclear GAPDH was amplified using forward primer 5'-GGATGATGTTCTGGAAGAGCC-3' and reverse primer 5'-AACAGCCTCAAGATCATCAGC-3'. Primers were included in triplicate along with negative control samples and a range of standards. The qPCR was performed using ABI 7300 (Applied Biosystems, Milano, Italy) with iQ SYBR Green Kit, according to manufacturer's instructions.

The SYBR green dye binds to double-stranded DNA but not to single-stranded DNA and can be used for monitoring DNA amplification during qPCR (10 ng template; initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 30 s). Moreover, the dye emits bright fluorescence upon binding to DNA. Melting curve analysis was performed to verify the accuracy and specificity of amplification.

306 **Statistical analysis**

307 All the variables were initially tested using Kolmogorov–Smirnov test to determine
308 data normality. Data of normally distributed variables were expressed as mean ± SD and
309 those of non-normally distributed variables were expressed as median and 25-75
310 percentile. Groups were compared using unpaired Student's t-test and non-parametric test
311 (Mann–Whitney U test) for normal and non-normal distribution, respectively. Correlation
312 analysis between the study variables was performed using Spearman's non-parametric
313 test with untransformed values. For all the statistical tests, differences with $P < 0.05$ were
314 considered significant. Difference in deletion frequency was determined using χ^2 test.

315 All analyses were performed using SAS statistical software package version 9.1
316 (SAS Institute Inc., Cary, NC, USA).

317

318

319

320

321

322

323

324

325

326

327

328

329

330

RESULTS

Median age of controls was 33.3 years (range, 20.1-40.7 years), which was not significantly different from that of patients (26.4 years [range, 22.9-38.1]). As expected, all the parameters of sperm from patients were significantly lower than those of sperm from healthy controls. However, sperm with normal morphology were comparable. Moreover, ejaculates of patients showed high leukocyte infiltration ($P < 0.0001$; Table 1). In contrast, ejaculates of only 6 controls (27.2%) showed leukocyte contaminations (less than 1×10^6/ml).

Seminal ROS production

ROS production was measured using the total population of unfractionated cells to determine the overall oxidative status of the ejaculate. Basal ROS production (in 58.3% samples) and stimulated ROS production (in 70.8% and 83.3% samples treated with FMLP and PMA, respectively) were significantly higher ($P < 0.0001$) in patients than in controls (Fig.1). In all the patients with increased spontaneous, basal level of activity, FMLP- and PMA-stimulated ROS production was higher than the basal ROS production. Consistently, chemiluminescent signals after PMA stimulation were elevated significantly over those after FMLP stimulation ($P < 0.001$). Significant correlation was observed between basal and stimulated ROS production ($r = 0.44$, $P < 0.03$ and $r = 0.76$, $P < 0.00001$ for FMLP- and PMA-stimulated ROS, respectively) in semen samples of patients with asthenozoospermia. Furthermore, FMLP- ($r = 0.5$, $P < 0.01$) and PMA-stimulated ROS production ($r = 0.46$, $P < 0.02$) but not basal ROS production were correlated with leukocyte concentration in the semen samples. Moreover, strong correlation was observed between PMA- and FMLP-stimulated ROS production ($r = 0.89$, $P < 0.000002$), but in three cases production was stimulated by the addition of PMA only, with the response being lower than

that with FLMP. However, no correlation was observed between basal as well as stimulated ROS production and sperm parameters or between ROS production and mtDNA copy number or flow cytometric sperm variables, except DNA fragmentation.

Integrity of mtDNA in sperm of patients with asthenozoospermia and controls

Long PCR amplified an 8.7-kb fragment from the 16-kb mitochondrial genome in all the samples. This 8.7-kb fragment contains several genes encoding subunits of energetic complexes as well as the common deletion types 4.3 kb and 7.4 kb in sperm (Song & Lewis 2008). Fig. 2 shows the representative products of long PCR from 2 normozoospermic controls and 2 patients. The high intensity of the full-length band indicated the presence of normal intact mtDNA (Fig.2, lanes 1 and 2). In contrast, the low intensity of full-length mtDNA band indicated poor mtDNA integrity because of low amount of mtDNA, fragmentations and deletions. The results of long PCR showed differences in mtDNA integrity in sperm of controls and patients. Normozoospermic controls showed high intensity of full length band and did not contain deletions in mtDNA, indicating normal intact mtDNA. In contrast, 36 (97.2%) patients showed multiple deletions in mtDNA (Fig.2, lanes 3 and 4). This difference in the frequency of deletions in mtDNA between patients and controls was highly significant ($P < 0.0001$). Only 1 patient with asthenozoospermia had intact mtDNA; however, this patient showed high PS externalization and had abnormal chromatin compactness values (results not shown). Moreover, this patient had the highest mtDNA copy number and showed the highest PMA-stimulated ROS production. In addition, this patient showed low progressive sperm motility of 12%, with 1 million leukocytes in the ejaculate. Furthermore, abnormal mtDNA was associated with high ROS production in sperm samples of 83.4% patients.

Alterations in mtDNA copy number in sperm of patients and controls

The median mtDNA copy number in sperm of patients with asthenozoospermia was 14.8 (percentile, 5.4-29.68; range, 1-61), which was significantly higher than that in controls (median, 5.75 [percentile, 4.72-7.05; range, 1.1-10]; P <0.006). This increase in mtDNA copy number was observed in 45.8% patients. Moreover, patients with increased mtDNA copy number showed high ROS production.

Integrity of nDNA

Integrity of nDNA, which was measured using the TUNEL assay, was not significantly different between the 2 study populations (median, 2.4% [percentile, 1.4-3.2; range 0.5-4.0] vs. 2.0% [percentile, 0.9–5.0; range 0.6-34.6] in controls and patients, respectively; Table 1). In all, 16.6% patients showed very high rate of nDNA fragmentation (>10%). However, the rates of nDNA fragmentation in the remaining patients were within normal limits or between 4%–5.1%. Moreover, the rate of nDNA fragmentation was correlated with basal ROS production in patients (r =0.48, P <0.016).

MMP

The sperm of only 8 (21.6%) patients showed normal (high) MMP compared with that of controls (mean, 55.3% ±21.7 vs. 86.1% ±7.9; P <0.0001). Low MMP was associated with high ROS production in a high percentage (78.9%) of patients.

Other flow cytometric parameters

No significant differences were observed in percentage viability, PS externalization, late apoptosis, necrosis, or abnormal chromatin compactness between the sperm of the 2 study populations (Table 1). Interestingly, a negative correlation was observed between

alive sperm and PS externalization (r = -0.555, P <0.005), late apoptosis (r = -0.666, P <0.0003) and necrotic cell numbers (r = -0.446, P <0.028). In contrast, a positive correlation was observed between PS externalization and late apoptosis (r =0.555, P <0.0048).

Subpopulations of patients with multiple abnormalities in sperm

Next, we determined whether patients with asthenozoospermia showing abnormal ROS in their ejaculates had multiple identical ROS-associated abnormalities in their sperm. Almost all patients showing low MMP and high ROS production had deletions in the mtDNA of their sperm, and almost half of these patients (44.6%) showed increased mtDNA copy number (cohort a). Moreover, 12.6% of these patients also showed high rate of nDNA fragmentation (>10%) (cohort b).

DISCUSSION

The results of this study provide further evidence that severe asthenozoospermia is associated with various abnormalities in sperm. The sperm of patients with asthenozoospermia showed increased ROS production, mitochondrial and nuclear genomic alterations, and multiple molecular abnormalities. However, the frequency of abnormalities in these different nonconventional biofunctional sperm parameters varied in patients with asthenozoospermia (Fig. 3).

We found that ejaculates of 58.3%, 70.8%, and 83.3% patients, showed increased basal, FMLP-stimulated, and PMA-stimulated ROS production respectively, compared to controls. This result indicated that ROS was overproduced under basal condition irrespective of its source, i.e., leukocytes or sperm, which was consistent with the results of Whittington & Ford (1999). As from leukocyte specific FMLP agonist stimulation, leukocytes, were the main ROS producer in semen samples from a little more than two-thirds of the patients, as elsewhere reported (Krausz *et al.* 1992, Whittington & Ford 1999). However, the lack of correlation between ROS levels and sperm motility, a major target of ROS, is unclear. Moreover, the results of previous studies are inconsistent in this regard (Whittington *et al*. 1999; Kao *et al.* 2008). Although PMA is the most powerful stimulant for oxidant stimulation by human sperm (Krausz et al.1992), ROS production in patients after PMA can not be specifically compartmentalized to some extent to leukocytes or sperm, since the probe is a stimulus for both leukocytes and sperm. However, consistently PMA-stimulated ROS production were elevated significantly over those after FMLP, suggesting some amount of ROS production by sperm.

In the present study, we observed that almost 50% patients with severe asthenozoospermia showed significantly increased mtDNA copy number, which was consistent with the results of previous studies that non-progressively motile sperm show

increased mtDNA copy number (May-Panloup *et al*. 2003, Amaral *et al.* 2007, Song & Lewis 2008). Recent studies have shown a negative correlation between mtDNA copy number and sperm motility in men with varicocele; moreover, varicocele correction improves sperm motility and decreases mtDNA copy number in these patients (Gabriel *et al.* 2012). Furthermore, mtDNA copy number increases in men living in hypoxic conditions at high altitudes for 1 year compared with that in men living in plains (Luo *et al.* 2011). This increase in mtDNA copy number might be induced by elevated oxidative stress (Lee *et al.* 2000, Liu *et al.* 2003).

Our results showed decreased mtDNA integrity in almost all sperm samples from patients with asthenozoospermia. These mitochondrial genomic alterations are hallmarks of spermatogenetic dysfunction (Hecht & Liem 1984, May-Panloup *et al*. 2003, Song & Lewis 2008) and severely alter mitochondrial function in abnormal sperm. Genomic alterations were also observed in nDNA; however, the frequency of alterations in nDNA was lower than that in mtDNA. The rate of nDNA fragmentation was not significantly different between controls and patients with asthenozoospermia. However, a small percentage of patients with asthenozoospermia showed high rate of nDNA fragmentation (>10%). Furthermore, nDNA fragmentation was correlated with basal ROS production, which was consistent with what was previously reported (Aitken *et al.* 2010). This finding was also consistent with the notion that nDNA fragmentation is often associated with oxidative stress (De Iuliis *et al.* 2009). Oxidative stress or ROS production in the mitochondria induces breaks in nDNA (Wang *et al*. 2003, Aitken & De Iuliis 2010). However, mtDNA is more susceptible to the harmful effects of excess ROS production than nDNA (Yakes *et al.* 1997, Sawyer *et al.* 2003). This may be one of the reasons for the higher incidence of abnormalities in mtDNA than in nDNA in patients with asthenozoospermia. Thus, mitochondrial dysfunction may be involved in the pathogenesis

of asthenozoospermia in these men. Men with multiple mutations and large deletions in mtDNA showed severe phenotypic defect (Kao *et al.* 1995, St John *et al*. 1997, Salehi *et al*. 2006).

A significantly lower number of sperm of patients with severe asthenozoospermia had normal (high) MMP values as further expression of mitochondrial dysfunction. In fact, only one-fifth of patients with asthenozoospermia had normal MMP values. MMP is a good predictor of sperm quality. Such cell abnormality will result in less energy production for sperm function and motility. Sperm with high MMP values have intact acrosome, high fertilizing capacity, and normal motility and morphology. In contrast, sperm with low MMP values are of low quality and are associated with low IVF rates (Kasai *et al.* 2002, Marchetti *et al.* 2004). This is in turn correlated with ROS production (Wang *et al.* 2003). Dysruption of MMP may occur during early stages of apoptosis in germ cells (Erkkilä *et al.* 1999) and thus before the induction of nDNA damage in sperm.

Other flow cytometric parameters were not significantly different between patients with asthenozoospermia and normozoospermic controls. Sperm vitality is a good predictor of gamete quality. An inverse correlation was observed between sperm viability and signs of cell apoptosis, such as PS externalization, early and late stages of apoptosis, necrosis, and chromatin compactness. This has important clinical implications in that the selection of good quality sperm for ICSI conventionally focuses on mobile and consequently viable sperm. Furthermore, positive correlation was observed between PS externalization and late apoptosis, indicating that PS externalization promoted the entry of sperm into the senescence phase, followed by apoptosis and death.

These abnormalities in sperm of patients with severe asthenozoospermia are typical manifestations of ROS- induced damage and are based on a continuum of decreased sperm motility; decreased MMP; decreased mtDNA integrity; increased mtDNA copy

number; PS externalization; caspase activation; oxidative nDNA damage, including nDNA fragmentation; late apoptosis; and death. The final damaging consequences on sperm of such exposure depend on the capacity of sperm of these men to withstand oxidative stressor, possibly compounded by a compromised total antioxidant capacity in their seminal fluid (Pasqualotto *et al.* 2000, Kao *et al.* 2008). ROS-induced cellular damage also depends on whether ROS production is extracellular (leukocytes) or intracellular (sperm). Extracellular ROS production exerts less damage on nDNA (Henkel *et al*. 2005). In addition, time and site of ROS exposure, oxidants produced by morphologically poor sperm, and other round cells are also important determinants of the degree of severity of ROS-induced cellular damage (Henkel *et al.* 2005). So as the generation of these reactive free radicals overwhelms the defense system, this induces oxidative stress, which is characterized by a cascade of cellular damage (Aitken *et al.* 2010). This may be the reason why only a subpopulation of patients with asthenozoospermia showed increased nDNA fragmentation, which is caused by prolonged exposure to ROS (Aitken *et al*. 2010). Basal ROS status in the ejaculates of our patients correlates with nDNA damage observed, thus supporting the role of ROS in inducing nDNA alterations. However, these multiple abnormalities observed in sperm of patients with asthenozoospermia may also result from deranged spermatogenesis and then aborted apoptosis (Sakkas *et al*. 2003).

The general findings of this study are consistent with the hypothesis that sperm of patients with asthenozoospermia have several biochemical, molecular, genomic, and functional abnormalities, which may decrease their fertilization potential (Tesarik *et al.* 2002, Zidi-Iran I *et al.* 2016). This was also observed in patients with asthenozoospermia included in the present study. During follow-up, sperm of 2 patients in subgroup b could not lead to pregnancy after as many as 3 ICSI attempts. In contrast, sperm of 1 patient who had low MMP as the only abnormality led to pregnancy during the first ICSI attempt

(results not shown). We observed that patients with this pathological condition could be subgrouped according to the presence of only 1 or more cellular abnormalities, with high ROS production, increased mtDNA deletions and copy number, and reduced MMP being the most common associated abnormalities. Rate of nDNA fragmentation was high only in less than one-fifth of the patients with asthenozoospermia. As time elapses and the cause persists, the current picture might get worse in the absence of any treatment. No robust clinical implications can be drawn from the results of the present study because the study included limited number of patients. However, the present findings strengthen the association of high frequency biochemical and biofunctional sperm alterations in patients with severe asthenozoospermia, and emphasize evaluating male factor by sperm function tests to determine hidden anomalies which may better define the fertility status in vivo and in vitro.

To our knowledge, this is the first study to examine, all together, biochemical, functional, molecular, and genomic abnormalities in sperm of patients with asthenozoospermia. These abnormalities make up an evolutionary spectrum of progressive alterations in presence of oxidative free radical offense, whose final result will be either survival or senescence and then death, eventually going through several intermediate steps. Our results further indicate that sperm of infertile patients with severe asthenozoospermia who are candidates for ICSI may harbor cellular abnormalities, which may jeopardize oocyte fertilization and embryonic development depending on the severity of these abnormalities.

Authors' roles

R.D. designed the study, analyzed and interpreted the data, and drafted the manuscript. O.B., G.R., P.A., F.M.P., and N.B. performed some analyses. O.B. obtained the data for her Ph.D. thesis. E.V. performed clinical evaluation of patients and designed the study. R.C. and G.S. organized and revised the manuscript. F.G.V collaborated in the drafting of the manuscript. E.V. and R.D contributed equally in the study.

Declaration of interest

The authors declare that they do not have any conflicts of interest that could prejudice the impartiality of the reported research.

Funding

This research did not receive any grant from any funding agency in the public, commercial, or not-for-profit sector.

Acknowledgments

The authors acknowledge the active collaboration of technicians of the Andrology Center for helping in selecting asthenozoospermic samples.

REFERENCES

Agarwal A & Allamanemi SS 2004 Oxidant and antioxidant in human fertility. Middle East Fertility Society Journal **9** 187-197.

Aitken RJ, Clarkson JS & Fishel S 1989 Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biology of Reproduction **41** 183-197.

Aitken RJ & Curry BJ 2011 Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. Antioxidant Redox Signal **14** 367-381.

Aitken RJ & De Iuliis GN 2010 On the possible origins of DNA damage in human spermatozoa. Molecular Human Reproduction **16** 3-13.

Aitken RJ, De Iuliis GN, Finnie JM, Hedges A & McLachlan RI 2010 Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. Human Reproduction **25** 2415-2426**.**

Aitken RJ, Iones KT & Robertson SA 2012 Reactive oxygen species and sperm function-In sickness and in health. Journal of Andrology **33** 1096-1104.

Alvarez JG, Touchstone JC, Blasco L & Storey BT 1987 Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Journal of Andrology **8** 338-348.

Amaral A, Ramalho-Santos J & St John JC 2007 The expression of polymerase gamma and mitochondrial transcription factor A and the regulation of mitochondrial DNA content in mature human sperm. Human Reproduction **22** 1585-1596.

Bahr GF & Engler Wf 1970 Considerations of volume, mass, DNA, and arrangement of mitochondria in the midpiece of bull spermatozoa. Experimental Cell Research **60** 338-340.

606 **Biggers JD, Whitten WK, Whittingham DG** 1971 The culture of mouse embryos in
607 vitro. In Methods of mammalian embryology, pp 86-116. Ed JC Daniel. San Francisco,
608 CA:WH Freeman Press.
609 **Bruijn MH, Coulson AR, Drouin J, Eperon JC, Nierlich DP, Rpe BA & Sanger F**.
610 1981 Sequence and organization of the human mitochondrial genome. Nature **290** 457-
611 465.
612 **Calogero AE, Fishel S, Hall J, Ferrara E, Vicari E, Greeen S, Hunter A, Burrello**
613 **N, Thornton S & D'Agata R** 1998 Correlation between intracellular cAMP
614 content,kinematic parameters and hyperactivation of human spermatozoa after incubation
615 with pentoxifylline. Human Reproduction **13** 911-915.
616 **D'Agata R, Vicari E, Moncada ML, Sidoti G, Calogero AE, Fornito MC,**
617 **Minacapilli G, Mongioi A & Polosa P** 1990 Generation of reactive oxygen species in
618 subgroups of infertile men. International Journal of Andrology **13** (5) 344-351.
619 **De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A,**
620 **Nixon B & Aitken RJ** 2009 DNA damage in human spermatozoa is highly correlated with
621 efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a
622 marker of oxidative stress. Biology of Reproduction **81** 517-524.
623 **de Lamirande E & Gagnon C** 1992 reactive oxygen species and human
624 spermatozoa. Effects on the motility of intact spermatozoa and on sperm axonemes.
625 Journal of Andrology 13 368-378.
626 **Diez-Sanchez C, Ruiz-Pesini E, Lapena AC, Montoya J, Perez-Martos A,**
627 **Enriquez JA & Lopez-Perez MJ** 2003 Mitochondrial DNA content of human spermatozoa.
628 Biology of Reproduction **68** 180-185.
629 **Erkkilä K, Pentikäinen V, Wikström M, Parvinen M & Dunkel L** 1999 Partial
630 oxygen pressure and mitochondrial permeability transition affect germ cell apoptosis in the

631 human testis. Journal of Clinical Endocrinology and Metabolism **4** 4253-4259.

632 **Folgero T, Bertheussen K, Lindal S, Torbergsen T & Oian P** 1993 Mitochondrial
633 disease and reduced sperm motility. Human Reproduction **8** 1863-1868.

634 **Ford WCL** 1990 The role of oxygen free radicals in the pathology of human
635 spermatozoa: Implications for IVF. Clinical IVF forum. In: Clinical IVF Forum. Current
636 views in assisted reproduction, pp 123-139. Eds PL Matson and BA Libermann.
637 Manchester University Press.

638 **Gabriel MS, Chan SW, Alhathal N, Chen JZ & Zini A** 2012 Influence of
639 microsurgical varicocelectomy on human sperm mitochondrial DNA copy number: a pilot
640 study. Journal of Assisted Reproduction and Genetics**29** 759-764.

641 **Hecht NB & Liem H** 1984 Mitochondrial DNA is synthesized during meiosis and
642 spermiogenesis in the mouse. Experimental Cell Research **154** 293-298.

643 **Henkel R, Kierspel E, Stalf T, Mehnert C, Menkveld R, Tinneberg HR, Schill WB**
644 **& Kruger TF** 2005 Effect of reactive oxygen species produced by spermatozoa and
645 leukocytes on sperm functions in non-leukocytospermic patients. Fertility and Sterility **83**
646 635-642.

647 **Hughes LM, Griffith R, Carey A, Butler T, Donne SW, Beagley KW, Aitken RJ**
648 2009 The spermostatic and microbicidal actions of quinones and maleimides: toward a
649 dual-purpose contraceptive agent. Molecular Pharmacology **76** 113-124.

650 **Kao S, Chao HT & Wei YH** 1995 Mitochondrial deoxyribonucleic acid 4977-bp
651 deletion is associated with diminished fertility and motility of human sperm. Biology of
652 Reproduction **52** 729-736.

653 **Kao SH, Chao HT & Wei YH** 1998 Multiple deletions of mitochondrial DNA are
654 associated with the decline of motility and fertility of human spermatozoa. Molecular
655 Human Reproduction **4** 657-666.

Kao SH, Chao HT, Chen HW, Hwang TJS, Liao TL & Wei YH 2008 Increase of oxidative stress in human sperm with lower motility. Fertility and Sterility **89** 1183-1190.

Kasai T, Ogawa K, Mizuno K, Nagai S, Uchida Y, Ohta S, Fujie M, Suzuki K, Hirata S & Hoshi K 2002 Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. Asian Journal of Andrology **4** 97-103.

Koppers AJ, De Juliis GN, Finnie JM, McLaughlin EA & Aitken RJ 2008 Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. Journal Clinical Endocrinology and Metabolism **93** 3199-3207.

Krausz C, West K, Buckingham D & Aitken RJ 1992 Development of a technique for monitoring the contamination of human semen samples with leucocytes. Fertility and Sterility **57** 1317-1325.

Krausz C, Mills C, Rogers S, Tan SL & Aitken RJ 1994 Stimulation of oxidant generation by human sperm suspensions using phorbol esters and formyl peptides: relationships with motility and fertilization in vitro. Fertility andSterility**62** 599–605.

Lee HC, Yin PH, Lu CY, Chi CW & WeYH 2000 Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cell. Journal of Biochemistry **348**(pt2):425-432.

Lestienne P, Reynier P Chretien MF, Pennisson-Besnier I, Malthiery Y & Rohmer V 1997 Oligoasthenospermia associated with multiple mitochondrial DNA rearrangements. Molecular Human Reproduction **3** 811-814.

Liu CS, Tsai CS, Kuo CL, Chen HW, Lii CK, Ma YS & Wei YH 2003 Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes. Free Radical Research **37** 1307-1317.

Luo Y,Liao W, Chen Y, Cui J, Liu F, Jiang C, Gao W & Gao Y 2011 Altitude can alter the mtDNA copy number and DNA integrity in sperm, Journal of Assisted

Reproduction and Genetics **28** 951-956.

Marchetti C, Jouy N, Leroy-Martin B, Defossez A, Formstecher P, & Marchetti P 2004 Comparison of four fluorochromes for the detection of the inner mithocondrial membrane potential in human spermatozoa and their correlation with sperm motility. Human Reproduction **19** 2267-2276.

May-Panloup P, Chrètien MF, Savagner F, Vasseur C, Jean M, Malthièry Y & Reynier P 2003 Increased sperm mitochondrial content in male infertility. Human reproduction **18** 550- 556.

Mitchell LA, De Iuliis GN & Aitken RJ 2011 The Tunnel consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. International Journal of Andrology **34** 2-13.

Muratori M, Piomboni P, Baldi E, Filimberti E, Pecchioli P, Moretti E, Gambera L, Baccetti B, Biagiotti R, Forti G et al. 2000 Functional and ultrastructural features of DNA-fragmented human sperm Journal of Andrology 21903-912.

Narisawa S, Hecht NB, Goldberg E, Boatright KM, Reed JC & Millán JL 2002 Testis-specific cytochrome c-null mice produce functional sperm but undergo early testicular atrophy. Molecular and Cellular Biology **22** 5554-5562.

Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ & Agarwal A 2000 Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fertility and Sterility **73** 3 459-464.

Perdichizzi A, Nicoletti F, La Vignera S, Barone N, D'Agata R, Vicari E & Calogero AE 2007 Effects of tumor necrosis factor-α on human sperm motility and apoptosis. Journal of Clinical Immunology **27** 152-162.

Rossato M, La Sala GB, Balasini M, Taricco F, Galeazzi C, Ferlin A & Foresta C

1999 Sperm treatment with extracellular ATP increases fertilization rates in in-vitro fertilization for male factor infertility. Human Reproduction **14** 694-697.

Ruiz-Pesini E, Lapena AC, Diez-Sanchez C, Perez-Martos A, Montoya J, Alvarez E, Diaz M, Urries A, Montoro L, Lopez-Perez MJ et al. 2000 Human mtDNA haplogroups associated with high or reduced spermatozoa motility. American Journal of Human Genetics **67** 682-696.

Sakkas D, Seli E, Bizzaro D, Tarozzi N, Manicardi GC. 2003 Abnormal spermatozoa in the ejaculate :abortive apoptosis and faulty nuclear remodeling during spermatogenesis. Reproductive Biomedecine Online **7** 428-432.

Salehi MH, Houshmand M, Bidmeshkipour A, Shariat S & Panahi SS M 2006 Low sperm motility due to mitochondrial DNA multiple deletions. Journal of Chinese Clinical Medicine **9** 181-185.

Sawyer DE. Mercer BG, Wiklendt AM & Aitken RJ 2003 Quantitative analysis of gene-specific DNA damage in human spermatozoa. Mutation Research **529** 21-34.

Shamsi MB, Venkatesh S, Tanwar M, Sharma Rk, Dhawan A, Kumar R, Gupta NP, Malhotra N, Singh N, Mittal S et al. 2009 DNA integrity and semen quality in men with low seminal antioxidant levels. Mutation Research **665** 29-36.

Song G & Lewis V 2008 Mitochondrial DNA integrity and copy number in sperm from infertile men. Fertility and Sterility **90** 2238-2244.

St John JC, Cooke ID & Barratt CLR 1997 Mitochondrial mutations and male infertility. Nature Medicine **3** 124-125.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M et al. 1999 Molecular characterization of mitochondrial apoptosis–inducing factors. Nature **397** 441-446.

Tesarik J, Mendoza C & Greco E 2002 Paternal effects acting during the first cycle

731 of human preimplantation development after ICSI. Human Reproduction **1** 189-202**.**

732 **Troiano L, Granata AR, Cossarizza A, Kalashnikova G, Bianchi R, Pini G,**
733 **Tropea F, Carani C & Franceschi C** 1998 Mitochondrial membrane potential and DNA
734 stainability in human sperm cells: a flow cytometry analysis with implications for male
735 infertility. Experimental Cell Research **241** 384-393.

736 **Varum S, Bento C, Sousa AP, Gomes-Santos CS, Henriques P, Almeida-**
737 **Santos T, Teodósio C, Paiva A, & Ramalho-Santos J** 2007 Characterization of human
738 sperm population using conventional parameters, surface ubiquitination and apoptotic
739 markers. Fertility and Sterility **87** 572-583.

740 **Venkatesh S, Deecaraman M, Kumar R, Shamsi MB & Dada R** 2009 Role of
741 reactive oxygen species in the pathogenesis of mitochondrial DNA(mtDNA) mutations in
742 male infertility. Indian Journal of Medical Research **129** 127-137.

743 **Wang X, Sharma RK, Gupta A, George V, Thomas AJ, Falcone T & Agarwal A**
744 2003 Alterations in mitochondria membrane potential and oxidative stress in men: a
745 prospective observational study. Fertility and Sterility **80** 844-850.

746 **Whittington K & Ford VCL** 1999 Relative contribution of leukocytes and of
747 spermatozoa to reactive oxygen species production in human sperm suspensions.
748 International Journal of Andrology **22** 229-235.

749 **Whittington K, Harrison SC, Williams KM, Day JL, Maclaughlin EA, Hull MGR**
750 **& Ford VCL** 1999 Reactive oxygen species (ROS) production and the outcome of
751 diagnostic tests of sperm function. International Journal of Andrology **22** 236-242.

752 **World Health Organization** 2010 WHO Laboratory Manual for the Examination of
753 Human Semen, 5th edn. WHO Library Cataloguing-in-Publication Data, Geneva,
754 Switzerland.

755 **Yakes FM & Van Houten B** 1997 Mitochondrial DNA damage is more extensive

and persists longer than nuclear DNA damage in human cells following oxidative stress: Proceedings of the National Academy of Sciences of the United States of America **94** 514-519.

Zidi-Jrah I, Hajlaoui A, Mougou-Zerelli S, Kammoun M, Meniaaoui I, Dallem A, Brahem S,Fekih M, Bibi M, Saad A et al. 2016 Relationship between sperm aneuploidy, sperm DNA integrity, chromatin packaging, tradional semen parameters, and recurrent pregnancy loss. Fertility and Sterility **105** 58-65.

FIGURE LEGENDS

Figure 1. Basal, FMLP-stimulated, and PMA-stimulated ROS production in controls and patients (open bars) with asthenozoospermia. Bars represent median and the 25th and 75th percentiles. Vertical lines represent range. Vertical axis is in logarithmic scale. Bars in the right vertical axis indicate medians in patients.

Figure 2. Four representative products of long PCR analysis of human sperm for determining mtDNA integrity. Lanes 1 and 2: results of long PCR of mtDNA of sperm from 2 controls. Lanes 3 and 4: results of long PCR of mtDNA of sperm from 2 representative patients with asthenozoospermia. Lanes 1 and 2 show a high-intensity 8.7-kb band, which represents full-length wild-type mtDNA, indicating the presence of normal intact mtDNA. Lanes 3 and 4 show low-intensity 8.7-kb bands, indicating the presence of low amount of full-length mtDNA, and smaller bands, indicating deletions in mtDNA. HindIII/λ was used as a size marker.

Figure 3. Frequency (%) of abnormal biochemical, genomic, and cytofluorimetric parameters in sperm of patients with asthenozoospermia.

Table 1 Descriptive analysis

	CONTROLS	*PATIENTS*	*p-VALUE*
SEMEN VARIABLES	**(n = 22)**	**(n = 37)**	
CYTOLOGICAL			
Concentration ($\times 10^6$/ml)*	119.3 ± 49 (39–235)	69 ± 33.6 (20–150)	p < 0.0002
Total sperm number ($\times 10^6$)*	439.3 ± 196.5 (126–750)	214.2 ± 108.8 (60–440)	p < 0.0001
Progressive motility (%) (a + b)*	47.4 ± 9.1 (30–68)	11.8 ± 4.8 (2–20)	p < 0.0001
Nonprogressive motility (%) (c)*	22.8 ± 11.7 (5–46)	61.5 ± 6.5 (52–72)	p < 0.0001
Non-motile sperm (%) (d)*	30.2 ± 8.9 (14–48)	26.6 ± 6.5 (16–42)	p < 0.12
Normal morphology (%)*	21.2 ± 5.4 (10–32)	19.8 ± 7.8 (8–40)	p < 0.50
Leukocytes ($\times 10^6$/ml) **	0 (0–0.4) 0–0.9	1 (0.7–1.5) 0.3–7	p < 0.0001
Flow cytometry			
Viable spermatozoa*	73.5 ± 7.8 60.2–86.1	66.2 ± 19.9 20.7–93.1	p < 0.11
Spermatozoa with PS externalization (%)**	1.9 (1.2–2.6) 0.04–13.8	3.2 (1.3–4.9) 0.12–40.3	p < 0.13
Spermatozoa in late apoptosis (%)**	6.0 (2.0–7.8) 0.1–14.7	7.1 (4.4–12.6) 0.1–32.0	p < 0.23
Necrotic spermatozoa (%)**	16.0 (12.2–23.4) 2.2–30.4	13.7 (6.3–20.5) 2.2–54.7	p < 0.39
Spermatozoa with DNA fragmentation (%)**	2.4 (1.4–3.2) 0.5–4.0	2.0 (0.9–5.0) 0.6–34.6	p < 0.6
Spermatozoa with high (normal) MMP values (%)*	86.1± 7.9 72.2–97.8	55.3 ± 21.7 15.5–95.8	p < 0.0001
Spermatozoa with abnormal chromatin compactness (%)**	15.4 (11.4–17.8) 8.4–18.9	15.2 (11.4–21) 4.3–39.3	p < 0.57

* Values expressed as mean ± SD, with range in parentheses

** Values expressed median (25%–75%), with range in the second line

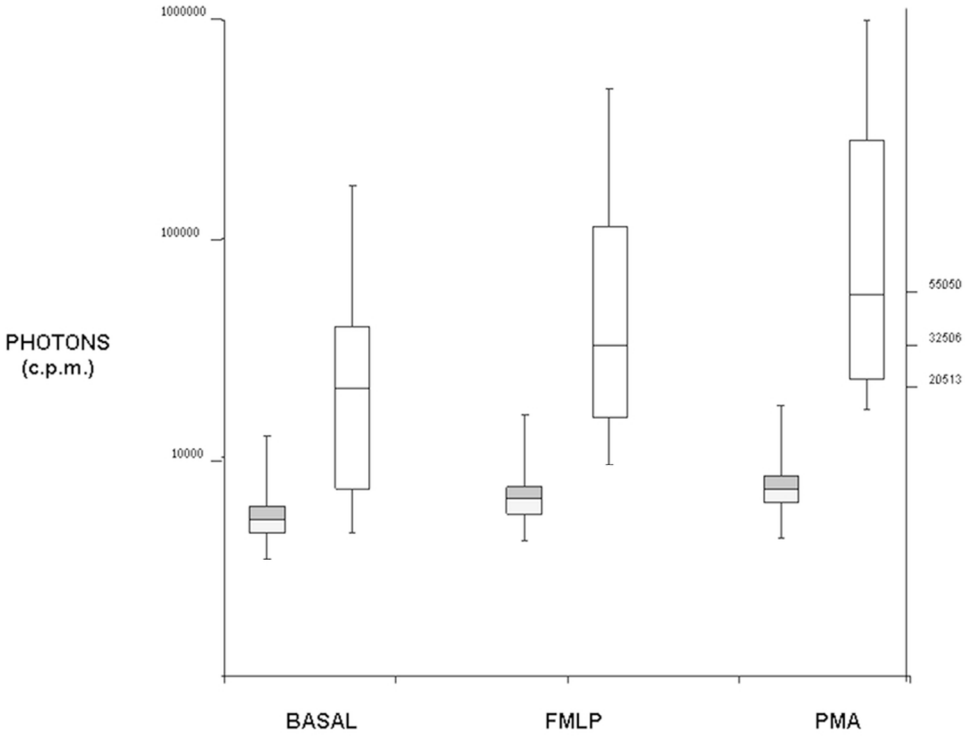
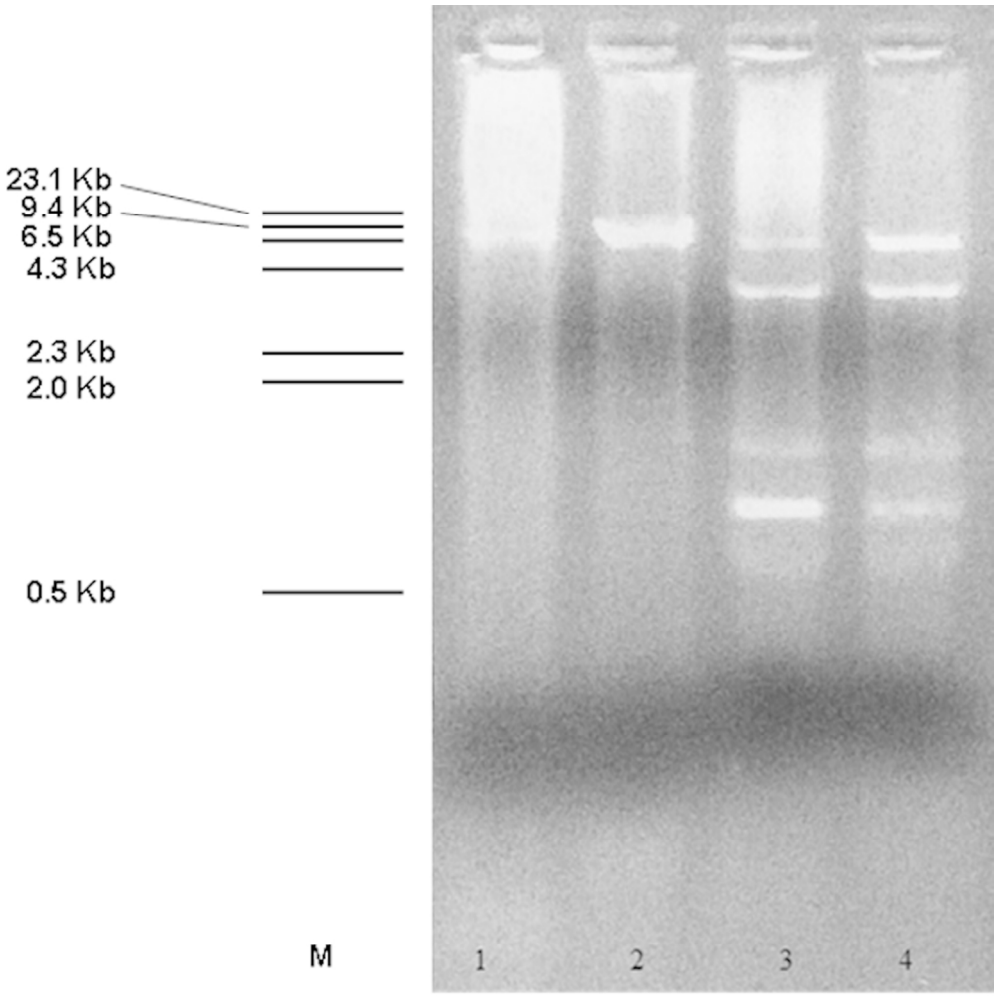


Figure 1

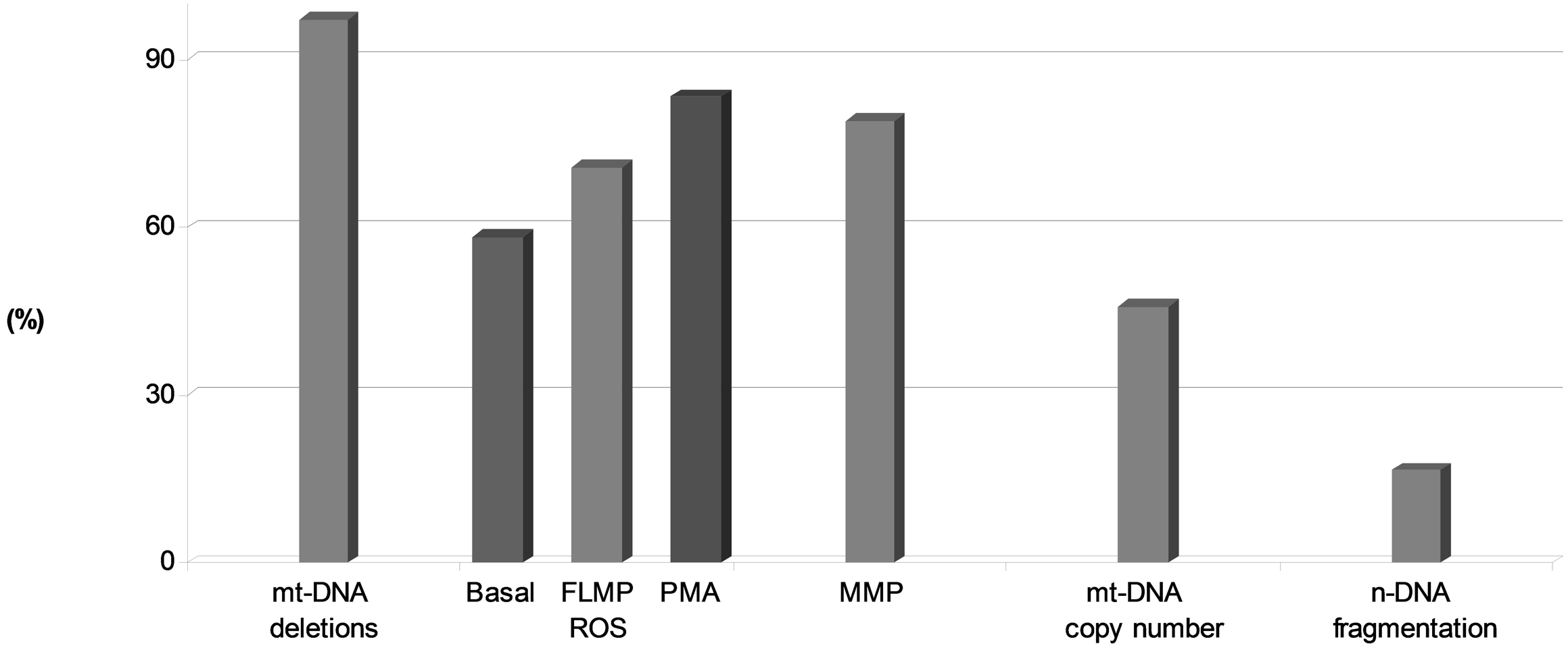
38x29mm (600 x 600 DPI)



1-2 NORMAL SAMPLES
3-4 ASTENOZOOSPERMIC SAMPLES
M λ DNA /Hind III

Figure 2

114x131mm (300 x 300 DPI)


(%)
90
60
30
0
mt-DNA deletions
Basal
FLMP ROS
PMA
MMP
mt-DNA copy number
n-DNA fragmentation