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

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Abstract

The multifactorial pathological condition, that is, severe low sperm motility is a frequent cause of infertility. However, mechanisms underlying the development of this condition are not completely understood. Single abnormalities have been reported in sperm of patients with asthenozoospermia. In this 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.
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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

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sperm with poor sperm quality (Kao *et al.* 1995, 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-Panloup *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 Iuliis 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).

Endogenous ROS production or ROS formed in leucocytes present in semen affect sperm (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, this 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.

Materials and methods

Chemicals and reagents

All chemicals used in this study were purchased from Sigma, 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). The 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). AnnexinV, 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, and TOPO TA Cloning (TOPOT-Cl) Kit and AccuPrime Pfx (AcP-Pfx) DNA polymerase were purchased from Invitrogen. iQ SYBR Green Kit was from Thermo Fisher.

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 ($a+b \leq 20\%$) 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 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, and 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 two-step discontinuous Percoll gradient (90%/45%) obtained by diluting isotonic Percoll (90 mL Percoll supplemented with 10 mL of 10× 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 500***g*** 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 600***g*** 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 were discarded.

Measurement of ROS production

Aliquots of 5–10 × 10^6 sperm were washed with two volumes BWW and were centrifuged at 300***g*** 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 leucocyte-specific chemiluminescent probe (Krausz *et al*. 1992, 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 leucocytes and sperm (Ford 1990), resulting in a sustained increase in the chemiluminescent signal (Krausz *et 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 this 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 darkness 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-labelled 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 labelled with 1 µL annexin V-FITC and 5 µL PI, was incubated in the darkness 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 darkness 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 darkness 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 reported previously (Perdichizzi *et al*. 2007). Briefly, the assay was conducted on aliquots of about 1 × 10^6 washed sperm, which were labelled 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 labelling.

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) in triplicate.

Long-range PCR for determining mtDNA integrity

Long-range PCR (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′-AAGGATCCTCTAGAGCCCACTG TAAAG-3′),
- reverse primer (5′-TTGGATCCAGTGCATACCGCC AAAAG-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 at −80°C in a freezer.

The amount of mtDNA and *GAPDH* was determined using two 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) 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.

Statistical analysis

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

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

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 leucocyte infiltration ($P < 0.0001$; Table 1). In contrast, ejaculates of only 6 controls (27.2%) showed leucocyte 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,

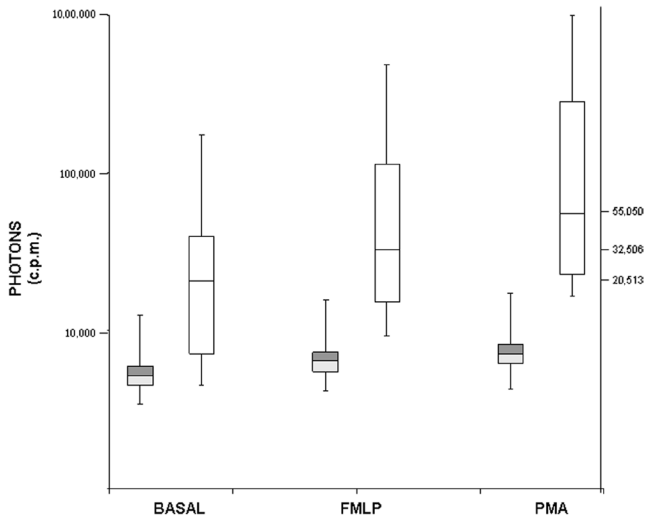

Figure 1 Basal, FMLP-stimulated and PMA-stimulated ROS production in controls and patients (open bars) with asthenozoospermia. Bars represent median and the 25th and 75th percentiles. Vertical lines represent range. Vertical axis is in logarithmic scale. Bars in the right vertical axis indicate medians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

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 leucocyte 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). Figure 2 shows the representative products of long PCR from 2 normozoospermic controls and 2 patients.

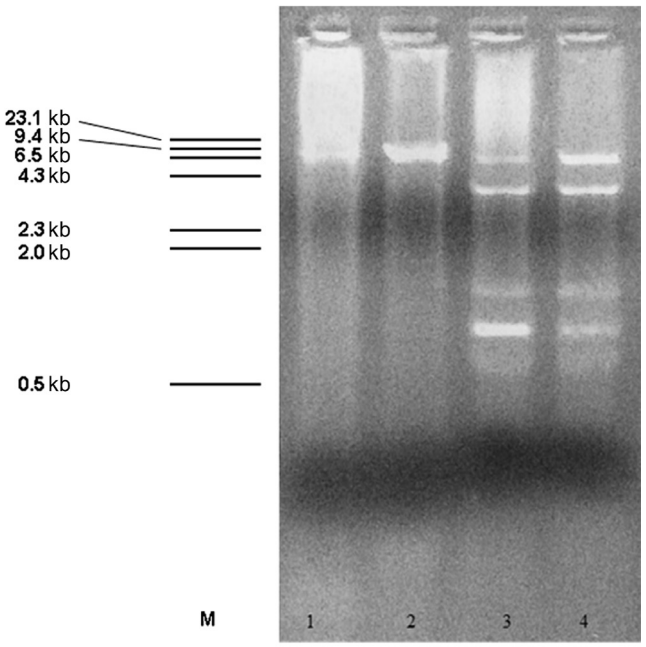

Figure 2 Four representative products of long PCR analysis of human sperm for determining mtDNA integrity. Lanes 1 and 2: results of long PCR of mtDNA of sperm from 2 controls. Lanes 3 and 4: results of long PCR of mtDNA of sperm from two representative patients with asthenozoospermia. Lanes 1 and 2 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.

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 leucocytes 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 two 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% and 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 among the sperm of the two 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 non-conventional biofunctional sperm parameters varied in patients with asthenozoospermia (Fig. 3).

Table 1 Descriptive analysis.

	Controls ($n=22$)		**Patients** ($n=37$)		
Semen variables	Values	Range	Values	Range	***P* value**
Cytological					
Concentration (×10⁶/mL)*	119.3±49	39–235	69±33.6	20–150	<0.0002
Total sperm number (×10⁶)*	439.3±196.5	126–750	214.2±108.8	60–440	<0.0001
Progressive motility (%) (a+b)*	47.4±9.1	30–68	11.8±4.8	2–20	<0.0001
Nonprogressive motility (%) (c)*	22.8±11.7	5–46	61.5±6.5	52–72	<0.0001
Non-motile sperm (%) (d)*	30.2±8.9	14–48	26.6±6.5	16–42	<0.12
Normal morphology (%)*	21.2±5.4	10–32	19.8±7.8	8–40	<0.50
Leukocytes (×10⁶/mL)**	0 (0–0.4)	0–0.9	1 (0.7–1.5)	0.3–7	<0.0001
Flow cytometry					
Viable sperm*	73.5±7.8	60.2–86.1	66.2±19.9	20.7–93.1	<0.11
Sperm with PS externalization**	1.9 (1.2–2.6)	0.04–13.8	3.2 (1.3–4.9)	0.12–40.3	<0.13
Sperm in late apoptosis (%)**	6.0 (2.0–7.8)	0.1–14.7	7.1 (4.4–12.6)	0.1–32.0	<0.23
Necrotic sperm (%)**	16.0 (12.2–23.4)	2.2–30.4	13.7 (6.3–20.5)	2.2–54.7	<0.39
Sperm with DNA fragmentation (%)**	2.4 (1.4–3.2)	0.5–4.0	2.0 (0.9–5.0)	0.6–34.6	<0.6
Sperm with high (normal) MMP values (%)*	86.1± 7.9	72.2–97.8	55.3±21.7	15.5–95.8	<0.0001
Sperm with abnormal chromatin compactness (%)**	15.4 (11.4–17.8)	8.4–18.9	15.2 (11.4–21)	4.3–39.3	<0.57

*Values expressed as mean±S.D.; **Values expressed median (25–75%).

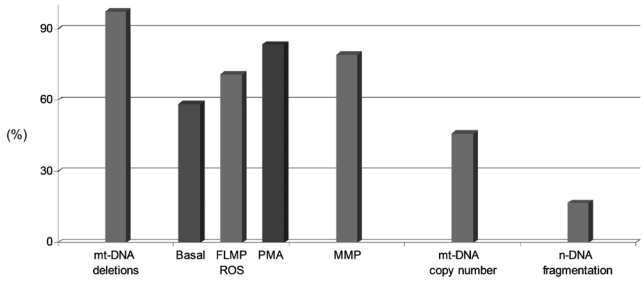

Figure 3 Frequency (%) of abnormal biochemical, genomic and cytofluorimetric parameters in sperm of patients with asthenozoospermia.

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 with controls. This result indicated that ROS was overproduced under basal condition irrespective of its source, that is, leucocytes or sperm, which was consistent with the results of Whittington and Ford (1999). As from leucocyte-specific FMLP agonist stimulation, leucocytes, 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 cannot be specifically compartmentalized to some extent to leucocytes or sperm, as the probe is a stimulus for both leucocytes and sperm. However, consistently PMA-stimulated ROS production were elevated significantly over those after FMLP, suggesting some amount of ROS production by sperm.

In this 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 reported previously (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 & Van Houten 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 (leucocytes) 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). Therefore, as the generation of these reactive free radicals overwhelms the defence 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-Jrah *et al*. 2016). This was also observed in patients with asthenozoospermia included in this 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 this 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 the 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 harbour cellular abnormalities, which may jeopardize oocyte fertilization and embryonic development depending on the severity of these abnormalities.

Declaration of interest

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

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Authors' contribution statement

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 PhD 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.

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