SPANDIDOS PUBLICATIONS
MOLECULAR MEDICINE REPORTS 10: 1875-1880, 2014

Oxygen free radicals and mitochondrial signaling in oligospermia and asthenospermia

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Received February 14, 2014; Accepted May 9, 2014

DOI: 10.3892/mmr.2014.2428

Abstract. The aim of this study was to investigate the roles of oxygen free radicals and mitochondrial signaling in semen disorders, in particular, how this induces low concentrations and reduced motility of sperm. Ejaculate samples were obtained from 120 young adult males (mean age, 28.7±5.3 years) with normal semen (n=30), oligospermia (n=30), asthenospermia (n=30) and oligoasthenozoospermia (n=30). The malondialdehyde (MDA) content, total superoxide dismutase (T-SOD) activity and glutathione peroxidase (GSH-Px) activity of the sperm samples were determined by enzymatic assays. Mitochondrial membrane potential (MMP) was determined by flow cytometric detection of accumulated membrane-permeable JC-1 fluorescent dye. The mRNA and protein expression levels of apoptosis-associated genes [Bcl-2, Bax, cytochrome *c* (Cyt C) and caspase-3] were measured by quantitative polymerase chain reaction and western blotting. Intergroup differences were evaluated by Student's t-test. The sperm samples from all semen disorder groups exhibited significantly lower T-SOD content and GSH-Px activity (all $P<0.05$ versus normal sperm), and the extent of reduction revealed a disorder-associated trend (asthenospermia < oligospermia ≈ oligoasthenozoospermia). By contrast, the semen disorder groups had significantly higher MDA content (all $P<0.05$ versus normal sperm); the extent of this increase also revealed a disorder-associated trend (asthenospermia > oligospermia ≈ oligoasthenozoospermia). The sperm from patients with semen disorders also exhibited significantly lower MMP than normal sperm, as evidenced by lower mean ratios of $JC\text{-}1^+$ sperm per group. The semen disorder groups had significantly higher Bax, Cyt C and caspase-3 mRNA and protein expression levels in the sperm samples, but significantly lower levels of Bcl-2 (all $P<0.05$ versus normal sperm). However, only the extent of increases in Cyt C and caspase-3 exhibited a disorder-associated trend (oligospermia > asthenospermia). In conclusion, oxygen free radicals may be involved in reduced sperm concentration and motility, possibly through effects on the mitochondrial apoptotic signaling pathway. Perturbed mitochondrial release of Cyt C may be the distinguishing molecular feature between oligospermia and asthenospermia.

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Key words: oxygen free radicals, mitochondrial signaling, oligospermia, asthenospermia, oligoasthenozoospermia, cytochrome *c*

Introduction

The most frequent causes of male infertility are a low sperm concentration (oligospermia) and reduced sperm motility (asthenospermia) (1). These two semen disorders frequently occur in combination, a condition known as oligoasthenozoospermia (2). While the effects of such disorders on the possibility of fertilization are well-known, for example, a reduced likelihood of sperm reaching the egg in the oviduct, the causative mechanisms remain largely unknown.

Extensive efforts to elucidate the pathogeneses of oligospermia and asthenospermia have suggested the involvement of chromosome abnormalities, perturbed gene regulation, environmental factors, infection- and immune-related factors, and endocrine dysfunction (3). A molecular study demonstrated that the mitochondrial apoptotic signaling pathways are also important in male infertility (4). Thus far, however, the majority of studies of apoptotic factors have assessed sperm in the testicular tissue (5); studies of ejaculated spermatozoa, particularly those from male patients diagnosed with oligospermia and/or asthenospermia, are rare.

An important component of mitochondria-dependent apoptosis is the development of associations among oxygen free radicals and various apoptosis-inducing factors. Notably, a substantial proportion (up to 40%) of males diagnosed with semen disorders exhibit high levels of reactive oxygen species (ROS) (6). A number of common environmental factors are known to stimulate ROS production, and are introduced through routine medical care (e.g. synthetic hormones and antibiotics), the workplace environment (e.g. plasticizers and ionizing radiation) and lifestyle practices (e.g. tobacco smoking and alcohol consumption). Furthermore, various studies of oligospermia and asthenospermia cases have

Table I. Characteristics of ejaculate specimens in semen disorder patients.

Group	n	Sperm count[b], x10^6 sperm/ml	Fast progressive motility[b], %	Mean age, years[c]
Normal semen[a]	30	≥15	≥32	28.6±5.6
Oligospermia	30	<15	≥32	28.4±4.3
Asthenospermia	30	≥15	<32	28.5±4.3
Oligoasthenozoospermia	30	<15	<32	29.3±7.1

[a]Infertility issue attributed to a factor associated with the female partner. [b]Sperm count and fast progressive motility were assessed by standard semen analysis. [c]No significant differences were identified in mean ages among the groups.

demonstrated close associations among the conditions and these environmental factors (7). The pathogenic potential of stimulated ROS has also been demonstrated in certain disease conditions (e.g. traumatic brain injury and myocardial ischemia) and involves induction of the mitochondrial apoptotic signaling pathway (8,9).

The present study was designed to investigate the potential pathogenic role of oxygen free radicals and the mitochondria-dependent apoptotic signaling pathway in the development of oligospermia, asthenospermia and oligoasthenozoospermia. Malondialdehyde (MDA) content, total superoxide dismutase (T-SOD) activity and glutathione peroxidase (GSH-Px) activity were determined for the investigation of oxygen free radicals. The mRNA and protein expression levels of apoptosis-related genes [Bcl-2, Bax, cytochrome *c* (Cyt C) and caspase-3] were measured to investigate the mitochondrial signaling pathway.

Materials and methods

Collection of ejaculate specimens. Young adult males between the ages of 21 and 37 were recruited from the Department of Andrology in the Third Affiliated Hospital of Zhengzhou University (Zhengzhou, China) for study participation. A total of 120 participants were selected according to a previous diagnosis of normal sperm or semen disorder using the sperm count (sperm/ml) and fast progressive motility (%) findings from standard semen analysis (10,11), presented in Table I. When all samples were processed, one measure from each specimen was used immediately for the MDA, T-SOD, GSH-Px and mitochondrial membrane potential (MMP) assays, and the other measure was stored at -80˚C for subsequent use in quantitative polymerase chain reaction (qPCR) and western blotting.

The study was approved by the local ethics committee of the Third Affiliated Hospital of Zhengzhou University and all donors provided written informed consent.

Isolation of seminal plasma and sperm. The liquefied semen sample from each participant was subjected to Percoll non-discontinuous density gradient (40 and 80%) centrifugation at 800 x g for 20 min. Subsequent to collection of the seminal plasma (upper layer) for MDA, T-SOD and GSH-Px assays, the accumulated sperm (bottom layer) were washed three times with phosphate-buffered saline (PBS; 0.01 mol/l; pH 7.4) and resuspended in fresh PBS to a final concentration of 30x10^6 sperm/ml. Apart from the aliquot removed for use in the MMP assay, all prepared sperm samples were stored at -80˚C until use.

Assessment of MDA and antioxidant enzyme activity. Commercially available kits (MDA test kit, T-SOD test kit, GSH-Px test kit; all Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) were used to assess MDA concentration (as determined by the thiobarbituric method), T-SOD activity (as determined by the xanthine oxidase colorimetric method) and GSH-Px activity (as determined by the enzymatic reaction method).

Assessment of MMP. An aliquot equal to 2% of the total sperm sample volume from each participant was washed and stained with the membrane-permeant fluorescent dye JC-1 (Beijing Fanbo Biochemicals Co., Ltd., Beijing, China). The JC-1-stained sperm (10,000 cells for each sample) were then subjected to flow cytometric analysis (FACSCalibur; Becton-Dickinson, Franklin Lakes, NJ, USA) using an excitation wavelength of 488 nm and gating by forward scattered light and side scatter light. The fluorescence intensities detected in fluorescence channels FL1 (green) and FL2 (red) were analyzed by the accompanying BD CellQuest software. The MMPs of sperm samples were classified as JC-1$^+$%, the percentage of sperm emitting orange-red fluorescence, which indicated the proportion of cells in R4 (12).

Quantitative measurement of apoptosis-associated gene expression levels. Total RNA was extracted from the sperm sample of each participant using the UltraPure RNA kit from CWBio Biotech Co. (Beijing, China) and was quantified by UV spectrophotometry (260 nm; ND-100 Nanodrop unit; Thermo, Wilmington, DE, USA). Reverse transcription was performed with the First Strand cDNA Synthesis kit from Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China) and applied as a template in qPCR using the Prism 9700 StepOne™ Real-Time PCR system (Applied Biosystems Inc., Carlsbad, CA, USA). The primers used for gene-specific amplification are listed in Table II; all primers were synthesized by the Sangon Biotech Co. (Shanghai, China). The thermal cycling conditions were as follows: One cycle of 95˚C for 10 min, 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The $2^{-\Delta\Delta CT}$ method was used to calculate the transcript expression levels relative to those of GAPDH, which served as an internal control.

Table II. Gene-specific primers used.

Gene	GenBank ID	Primer sequence, 5'-3'	
		Forward	Reverse
Bcl2	NM_000633.2	GTGGATGACTGAGTACCTGAACC	AGACAGCCAGGAGAAATCAAAC
Bax	NM_004324.3	TTTTGCTTCAGGGTTTCAT	ACACTCGCTCAGCTTCTTG
Cyt C	NM_006003.2	GCCTCAATGTCCCTGCTTCT	AGCACTCATGCTGGAAACGA
Caspase-3	NM_004346.3	ATCACAGCAAAAGGAGCAGTTT	ACACCACTGTCTGTCTCAATGC
GAPDH	NM_002046.3	TCGTGGAAGGACTCATGACC	AGGGATGATGTTCTGGAGAG

Cyt C, cytochrome *c*.

Table III. MDA content in seminal plasma from each group.

Group	n	MDA content (nmol/ml)
Normal semen	30	6.47±1.73
Oligospermia	30	7.28±1.06[a]
Asthenospermia	30	8.06±1.85[bc]
Oligoasthenozoospermia	30	7.54±1.56[a]

[a]P<0.05 and [b]P<0.01, vs. the normal semen group; [c]P<0.05, vs. oligospermia group. MDA, malondialdehyde.

Table IV. T-SOD and GSH-PX activity in seminal plasma from each group.

Group	n	T-SOD (U/ml)	GSH-Px (units)
Normal semen	30	81.70±10.93	105.20±16.07
Oligospermia	30	75.96±10.43[a]	96.24±14.78[a]
Asthenospermia	30	69.81±16.07[b]	88.67±21.21[b]
Oligoasthenozoospermia	30	74.31±11.39[a]	93.61±18.05[a]

[a]P<0.05, [b]P<0.01, vs. normal semen group. T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase.

Table V. Sperm MMP of each group (mean ± standard deviation).

Group	n	MMP (JC-1$^+$%)
Normal semen	30	71.08±19.43
Oligospermia	30	55.95±21.92[a]
Asthenospermia	30	57.26±18.03[a]
Oligoasthenozoospermia	30	53.28±20.80[a]

[a]P<0.01, vs. the normal semen group. MMP, mitochondrial membrane potential.

Quantitative measurement of apoptosis-associated protein expression levels. The remaining sperm sample from each participant was used to isolate total sperm protein (13) for analysis by western blotting. The total sperm protein samples were resolved by SDS-PAGE and electro-transferred to nitrocellulose membranes. Non-specific binding sites were blocked by incubating the transferred membrane in 1X Tris-buffered saline with 10% bovine serum albumin. The apoptosis-associated proteins were probed by incubation with primary antibodies (anti-Bcl-2 antibody, ab47489; anti-Bax antibody, ab54829; anti-Cyt C antibody, ab90529; anti-caspase-3 antibody, ab59388; and anti-β-actin antibody, ab15263; all from Abcam, Cambridge, UK) for 60 min at 37˚C, followed by incubation with the appropriate secondary antibodies (horseradish peroxidase-labeled goat anti-rabbit IgG; Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) for 60 min at 37˚C. Immunoreactivity was detected by enhanced chemiluminescence using an ECL kit (Beijing ComWin Biotech Co., Ltd., Beijing, China) and analyzed by densitometry (Naturegene Life Sciences Co., Ltd., Hong Kong, China). Data were obtained from at least three individual experiments performed in triplicate and the expression levels of the apoptosis-associated proteins were normalized to those of β-actin.

Statistical analysis. Statistical analyses were performed with the SPSS software suite, version 17.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation and inter-group differences were evaluated by Student's t-test. P<0.05 was considered to indicate a statistically significance difference.

Results

Sperm from males with semen disorders have increased MDA content and reduced antioxidant enzyme activity. The seminal plasma specimens of all three semen disorder groups exhibited significantly higher MDA content than the specimens from normal semen (oligospermia, P<0.05; asthenospermia, P<0.01 oligoasthenozoospermia, P<0.05). Comparison of the semen disorder-associated increases indicated that the patients with asthenospermia had significantly higher levels of MDA than counterparts with low sperm concentrations, particularly oligospermia (P<0.05; Table III). The extent of this increase

Table VI. Normalized mRNA expression levels of Bcl-2, Bax, Cyt C and caspase-3.

Group	Bcl-2	Bax	Cyt C	Caspase-3
Normal semen	1.00±0.12	1.00±0.13	1.00±0.19	1.00±0.16
Oligospermia	0.87±0.20[a]	1.14±0.22[a]	1.26±0.15[c]	1.24±0.14[c]
Asthenospermia	0.88±0.17[a]	1.11±0.16[a]	1.13±0.21[bd]	1.11±0.20[ad]
Oligoasthenozoospermia	0.86±0.20[a]	1.12±0.19[a]	1.15±0.21[b]	1.16±0.19[b]

[a]P<0.05, [b]P<0.01 and [c]P<0.001 vs. the normal semen group; [d]P<0.01 vs. the oligospermia group. Cyt C, cytochrome *c*.

Table VII. Protein expression levels of Bcl-2, Bax, Cyt C and caspase-3.

Group	Bcl-2	Bax	Cyt C	Caspase-3
Normal semen	0.89±0.09	0.78±0.11	0.76±0.11	0.83±0.09
Oligospermia	0.80±0.12[b]	0.87±0.11[b]	0.90±0.11[c]	1.01±0.15[c]
Asthenospermia	0.82±0.10[b]	0.84±0.06[b]	0.82±0.07[bd]	0.89±0.10[ad]
Oligoasthenozoospermia	0.79±0.14[b]	0.86±0.11[b]	0.85±0.13[b]	0.93±0.14[b]

[a]P<0.05, [b]P<0.01 and [c]P<0.001 vs. the normal semen group. [d]P<0.01 vs. the oligospermia group. Cyt C, cytochrome *c*.

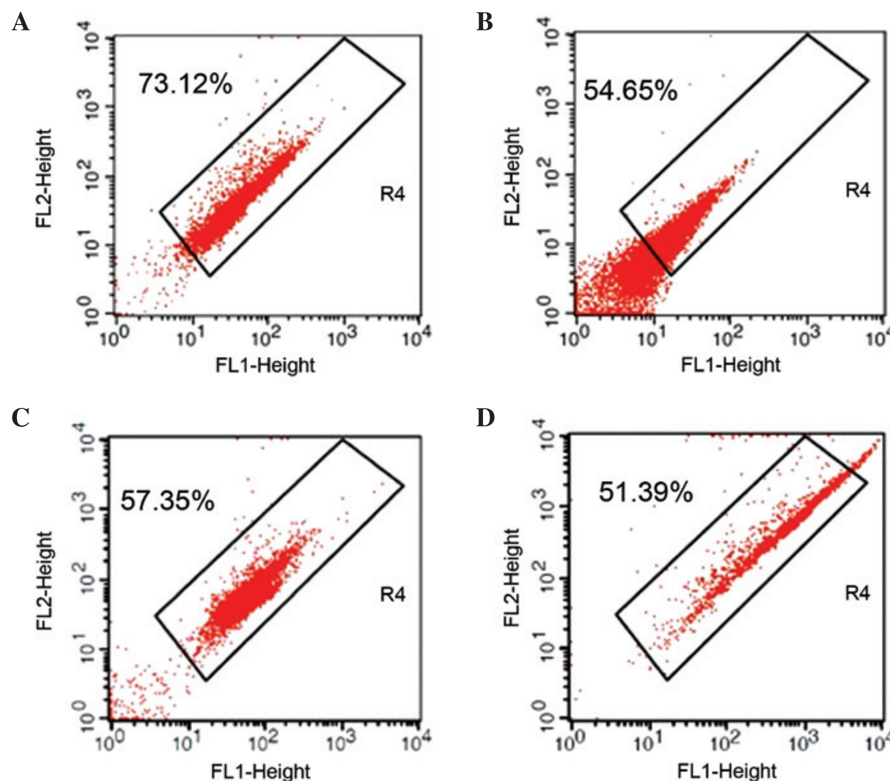

Figure 1. Sperm mitochondrial membrane potential (MMP) of the normal semen group and semen disorder groups. The ratio of R4 cells (JC-1+%) indicates the percentage of sperm with normal MMP. Flow cytometric data are shown for single representative patients from the (A) normal semen group, (B) oligospermia group, (C) asthenospermia group and (D) oligoasthenozoospermia group.

revealed a disorder-associated trend (asthenospermia > oligospermia ≈ oligoasthenozoospermia).

In addition, samples from all three semen disorder groups exhibited significantly lower T-SOD and GSH-Px antioxidant activity compared with the normal semen samples (oligospermia, P<0.05; asthenospermia, P<0.01; oligoasthenozoospermia, P<0.05; Table IV) and the extent of reduction revealed a disorder-associated trend

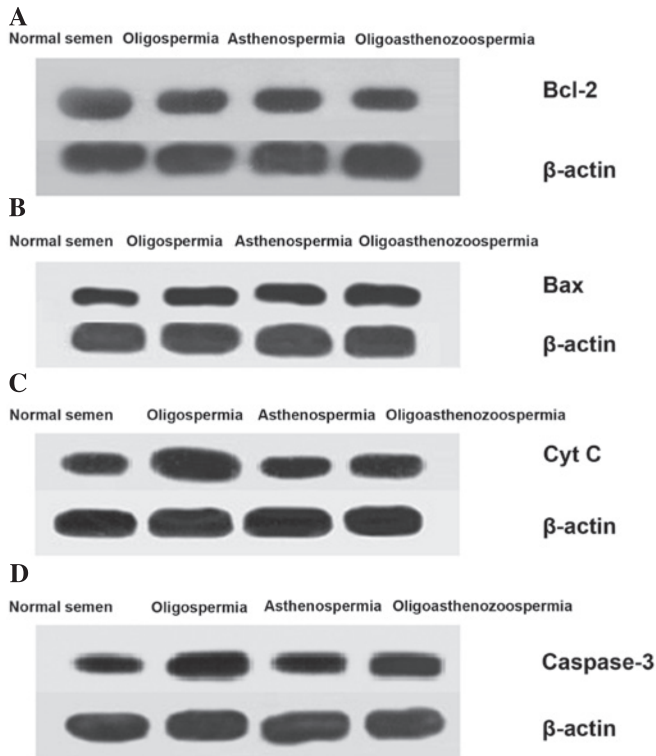

Figure 2. Bcl-2, Bax, Cyt C and caspase-3 protein expression levels in sperm from each group. Western blot analysis of (A) Bcl-2, (B) Bax, (C) Cyt C and (D) caspase-3 from single representative patients from the indicated groups. Cyt C, cytochrome *c*.

(asthenospermia < oligospermia ≈ oligoasthenozoospermia). Comparison of the semen disorder-associated decreases indicated that the patients with asthenospermia had the lowest levels of T-SOD and GSH-Px.

Sperm from males with semen disorders have reduced MMP expression levels. Sperm from normal semen exhibited a significantly higher JC-1$^+$% than sperm from patients with semen disorders, as evidenced the higher mean ratio of JC-1$^+$ compared with disorder groups (normal vs. oligospermia, 71.08±19.43 vs. 55.95±21.92%, P<0.01; normal vs. asthenospermia, 71.08±19.43 vs. 57.26±18.03%, P<0.01; and normal vs. oligoasthenozoospermia, 71.08±19.43 vs. 53.28±20.80%, P<0.01). Comparison of the semen disorder-associated reductions indicated that the patients with oligoasthenozoospermia had lower JC-1$^+$% than counterparts with the single-feature disorders, oligospermia and asthenospermia (Table V, Fig. 1); however, the differences among the groups did not reach the threshold for statistical significance (P>0.05).

Sperm from males with semen disorders exhibited perturbed expression levels of apoptosis-associated factors. Sperm from patients with semen disorders exhibited significantly downregulated expression levels of Bcl-2 mRNA and significantly upregulated expression levels of Bax mRNA compared with the sperm from normal semen (all, P<0.05). The protein expression levels of Bcl-2 (Fig. 2A) and Bax (Fig. 2B) followed the same trend, with statistically significant differences observed between the semen disorder groups and the normal semen group (all P<0.01; Tables VI and VII).

In addition, the mRNA expression levels of the apoptotic factors Cyt C and caspase-3, located downstream of Bcl-2 and Bax, were significantly upregulated in the patients with semen disorders compared with the normal semen group [oligospermia, P<0.001; asthenospermia, P<0.01 (Cyt C) and P<0.05 (caspase-3); oligoasthenozoospermia, P<0.01]. Furthermore, the extent of Cyt C and caspase-3 mRNA upregulation was significantly higher in the oligospermia group than in the asthenospermia group (P<0.01; Table VI). The same trends were observed in the upregulated expression levels of Cyt C (Fig. 2C) and caspase-3 (Fig. 2D) proteins, with significant differences between the semen disorder groups compared with the normal semen group [oligospermia, P<0.001; asthenospermia, P<0.01 (Cyt C) and P<0.05 (caspase-3); oligoasthenozoospermia, P<0.01]. Significant differences were also detected between the Cyt C and caspase-3 protein expression levels in the oligospermia group and the asthenospermia group (P<0.01; Table VII).

Discussion

The Bcl-2 protein family members function as pro-apoptotic and anti-apoptotic signaling factors. Among these factors, Bcl-2 and Bax are the key regulators of mitochondria-dependent apoptosis. When an apoptotic activation signal is received, Bax oligomerizes and inserts into the mitochondrial membrane; the consequent change in MMP facilitates the release of Cyt C into the cytosol, where it interacts with the apoptotic protease activating factor Apaf-1, which contains a caspase-recruiting domain. Recruitment of the caspase-9 precursor initiates the caspase cascade, which includes downstream caspase-3 activation, a critical mediator of the ultimate apoptotic outcome (14,15).

In the male reproductive tract, excessive ROS may result in a state of oxidative stress that is detrimental to the quality and integrity of seminal fluid and/or sperm (16). However, the question remains whether this acts as an apoptosis-activation signal, initiating the mitochondria-dependent apoptotic signaling pathway described above. The present study revealed that semen samples from patients with oligospermia, asthenospermia and oligoasthenozoospermia exhibited abnormalities in factors associated with this signaling pathway. In particular, all patients with these semen disorders exhibited a substantial reduction in Bcl-2 levels and a corresponding increase in Bax levels, in addition to increased levels of Cyt C and caspase-3; these results suggest that the mitochondria-dependent apoptotic signaling pathway may contribute to infertility in these patients. Furthermore, the abnormal MDA concentrations and antioxidant enzyme activity in the sperm from patients with semen-disorders suggest that excessive oxygen free radicals may be responsible for stimulating this signaling pathway. Further studies are required to obtain direct evidence for this hypothesis.

The commonalities in the perturbed expression levels of apoptosis-related factors in oligospermia and asthenospermia may aid in explaining why these two conditions frequently occur in combination, i.e. as oligoasthenozoospermia. The present study elucidated certain distinctive molecular features that may differentiate oligospermia from

asthenospermia. In particular, oligospermia pathogenesis was predominantly associated with apoptosis (i.e. significantly higher Caspase-3) while asthenospermia pathogenesis was mainly associated with oxidative damage (i.e. significantly higher MDA content). Notably, the results of the present study for the oligoasthenozoospermia semen samples indicated generally equal pathogenic roles of apoptosis and oxidative damage.

The significant increase in MDA content may be explained by an unknown environmental factor that was shared among the particular patient population of the present study. Undetected oxidative damage may have resulted in mitochondrial oxidation respiratory chain dysfunction, but detailed analysis of this hypothesis was beyond the scope of this study.

The present study demonstrated that the oligospermia semen samples had a significantly higher level of Cyt C than the asthenospermia semen samples. This result suggests that the mitochondrial release of Cyt C may indicate a demarcation point in the apoptotic signaling pathway that differentiates the pathogenesis of oligospermia from asthenospermia. Mitochondria-dependent apoptosis occurs in three stages: Early, intermediate and late. The rapid release of Cyt C into the cytosol occurs at the intermediate stage and is critical to the activation of the caspase cascade (17). This stage is also exponentiated by a positive feedback loop (18). In oligospermia, which was demonstrated to be dominated by apoptosis, the mitochondrial release of Cyt C may be the cause of the apoptotic fate of the sperm. In asthenospermia, which was demonstrated to be dominated by a mitochondrial oxidative respiratory chain overload, the release of Cyt C may be induced by oxidative stress-induced injury.

Cyt C is a cation capable of combining with a superoxide anion that is normally produced as a by-product of the respiratory chain, thereby consuming the cytoplasmic Cyt C (19). The present study found that asthenospermia semen samples exhibited significantly lower levels of Cyt C than oligospermia semen samples. It is possible that oligospermia pathogenesis seldom involves a perturbation in the respiratory chain, as compared with asthenospermia pathogenesis.

A change in MMP is considered an early indicator of apoptosis (20) and MMP has been shown in previous studies to be positively correlated with sperm motility (21). In the present study, abnormal MMP was readily and rapidly detected by JC-1 staining and flow cytometry, and this technique may be a useful supplementation to the standard semen analysis for diagnosing male infertility (22). However, the infertility-related reduction in JC-1^{+}% was not identified to be significantly different between the oligospermia and asthenospermia semen samples, indicating that this technique may not be a useful means to diagnose the particular semen disorder (between these two conditions).

In conclusion, the common semen disorders of low sperm concentration and reduced sperm motility may be associated with perturbations in oxygen free radical levels and mitochondria-dependent apoptotic signaling. The distinctive pathogenic mechanisms of oligospermia and asthenospermia appear to involve apoptosis and oxidative damage respectively, and mitochondrial release of Cyt C may be the demarcation point between the two conditions.

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