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Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility

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BACKGROUND: Reactive oxygen species (ROS)-induced damage of membrane phospholipids and DNA in human spermatozoa has been implicated in the pathogenesis of male infertility. In this study, variations in ROS production, DNA structure (as measured by the sperm chromatin structure assay) and lipid composition, were studied in human spermatozoa at different stages of maturation. METHODS: Sperm subsets were isolated by discontinuous density gradient centrifugation of semen samples obtained from healthy donors and from infertility patients. RESULTS: DNA damage and ROS production were highest in immature spermatozoa with cytoplasmic retention and abnormal head morphology, and lowest in mature spermatozoa. Docosahexaenoic acid and sterol content were highest in immature germ cells and immature spermatozoa, and lowest in mature spermatozoa. The relative proportion of ROS-producing immature spermatozoa in the sample was directly correlated with DNA damage in mature spermatozoa, and inversely correlated with the recovery of motile spermatozoa. There was no correlation between DNA damage and sperm morphology in mature spermatozoa. CONCLUSIONS: The high levels of ROS production and DNA damage observed in immature spermatozoa may be indicative of derangements in the regulation of spermiogenesis. DNA damage in mature spermatozoa may be the result of oxidative damage by ROS-producing immature spermatozoa during sperm migration from the seminiferous tubules to the epididymis.

Key words: chromatin/docosahexaenoic acid/ROS/spermatozoa/sperm preparation

Introduction

One in six couples of reproductive age present with infertility (Hull *et al*., 1995). A number of aetiologies have been identified as causes of male infertility including, gene mutations, aneuploidy, infectious diseases, ejaculatory duct occlusion, varicocele, radiation, chemotherapy and erectile dysfunction (Padron *et al*., 1997a,b; Wang *et al*., 1997; Carbone *et al*., 1998; Hendin *et al*., 1999; Mak *et al*., 1999). However, nearly 50% of infertile men are classified as idiopathic (Sherins, 1995). Damage induced by reactive oxygen species (ROS) generated by spermatozoa endogenously has been proposed as a major factor in male infertility (Aitken, 1994; Aitken and Fisher, 1994; de Lamirande and Gagnon, 1994; Sharma and Agarwal, 1996).

Docosahexaenoic acid (DHA), a major polyunsaturated fatty acid in human spermatozoa (Poulos *et al*., 1973; Alvarez and Storey, 1995; Zalata *et al*., 1998), accounts for up to 30% of phospholipid-bound fatty acid and 73% of polyunsaturated fatty acids (Alvarez and Storey, 1995). DHA is thought to play a major role in regulating membrane fluidity in spermatozoa, and is the main substrate of lipid peroxidation, accounting for 90% of the overall rate of lipid peroxidation in human spermatozoa (Alvarez and Storey, 1995). Oxidation of phospholipid-bound DHA has been shown to be the major factor that determines the motile lifespan of spermatozoa *in vitro* (Alvarez and Storey, 1995), and also results in acrosomal loss and DNA oxidation (Fraga *et al*., 1991, 1996).

The rate of lipid peroxidation of spermatozoa *in vitro* is determined by oxygen concentration and temperature in the extracellular medium, ROS production, the activity of antioxidant enzymes in spermatozoa, and the content of phospholipid-bound DHA (Alvarez *et al*., 1987; Alvarez and Storey, 1989, 1995). The equilibrium between these factors determines the overall rate of lipid peroxidation *in vitro*.

Sperm maturation involves the remodelling of sperm membrane components (Jones, 1989) leading to the acquisition of motility (Esponda, 1991; Hegde, 1996) and the ability of

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spermatozoa to undergo the zona-induced acrosome reaction (Alvarez and Storey, 1995) during sperm migration through the seminiferous tubules and epididymis (Jones, 1989; Amann *et al*., 1993). It has been reported previously that there is a decrease in DHA content in human spermatozoa during the process of sperm maturation (Ollero *et al*., 2000). This may be part of the genomically regulated cellular maturational steps which take place within the adluminal compartment. These events are completed by the time spermatozoa arrive at the epididymis (Huszar *et al*., 1998). However, if that fails to occur, then immature spermatozoa in the ejaculate would exhibit cytoplasmic retention and a high rate of lipid peroxidation and DNA fragmentation (Aitken *et al*., 1994; Huszar and Vigue, 1994; Twigg *et al*., 1998a).

Sperm maturation also involves significant nuclear remodelling during the process of spermiogenesis, resulting in extensive protamination and chromatin condensation. Chromatin packaging might require endogenous nuclease activity to create and ligate nicks that facilitate protamination and chromatin condensation. These nicks are thought to provide relief of torsional stress and to aid chromatin arrangement during the displacement of histones by the protamines (McPherson and Longo, 1992, 1993a,b).

It has been reported extensively that fractionation of spermatozoa by density gradient centrifugation results in a significant improvement in the quality of spermatozoa recovered in the pellet. The concentration of motile spermatozoa recovered in the pellet is significantly higher than in the lower density layers. In contrast, the percentage abnormal forms is significantly higher in spermatozoa from the lower density layers compared with spermatozoa from the pellet (Menkveld *et al*., 1990; Mortimer and Mortimer, 1992; Chen and Bongso, 1999; Ollero *et al*., 2000). More recently, the presence of a significant number of spermatozoa with DNA damage in ejaculated spermatozoa has been reported (Sakkas *et al*., 1999); fractionation of ejaculated spermatozoa by density gradient centrifugation resulted in a significant reduction in the number of spermatozoa with DNA damage recovered in the pellet (Larson *et al*., 1999; Sakkas *et al*., 1999).

The objectives of this study were to: (i) determine the quality of sperm subsets isolated from the different fractions of a three-layer ISolate discontinuous density gradient, as assessed by ROS production, chromatin structure and lipid composition; and (ii) compare these sperm parameters in the different fractions in samples obtained from normal healthy donors and from patients being evaluated for infertility.

Materials and methods

Semen samples

Following Institutional Review Board approval, semen samples were collected from males undergoing infertility screening ($n = 32$) and from normozoospermic healthy donors ($n = 16$). This same patient population was used in a previous study (Gil-Guzman *et al*., 2001). Samples with a sperm concentration $<1\times10^6$/ml or any detectable leukocytes in semen were excluded from the study. All specimens were collected by masturbation at the clinical andrology laboratory after an abstinence period of 48–72 h. After liquefaction, semen analysis was performed using computer-assisted semen analysis (CASA) (Cell-Trak, version 4.24; Motion Analysis Corporation, Palo Alto, CA, USA) (Esteves *et al*., 1998) to measure sperm concentration, percentage motility and motion characteristics. Smears were prepared for the assessment of sperm morphology. Myeloperoxidase staining (Shekarriz *et al*., 1995) was performed to evaluate the leukocyte concentration in the specimen.

Classification of semen samples

Semen samples from patients were classified as samples with normal or abnormal semen parameters, based on WHO criteria (World Health Organization, 1999). Specimens with a sperm count $<20\times10^6$/ml, sperm motility <50%, or percentage normal forms <14% (Kruger *et al*., 1986) were considered abnormal.

Density gradient centrifugation

Aliquots (0.5–1 ml) of the liquefied semen were loaded onto a 47, 70 and 90% discontinuous ISolate gradient (Irvine Scientific, Santa Ana, CA, USA) and centrifuged at 500 *g* for 20 min at room temperature. The resulting interfaces between seminal plasma and 47% (Fraction 1), 47% and 70% (Fraction 2), 70 and 90% (Fraction 3), and the 90% pellet (Fraction 4) were aspirated, and transferred to separate test tubes. An aliquot of each fraction was used to assess sperm concentration and motility by phase-contrast microscopy, and morphology by brightfield microscopy of Diff-Quik-stained slides. Sperm suspensions from the different ISolate fractions were diluted in one volume of Biggers–Whitten–Whittingham medium (BWW) and centrifuged at 500 *g* for 7 min. The pellet was then resuspended in 1 ml of BWW and an aliquot used to determine the total number of spermatozoa and round cells. Aliquots from each fraction were examined for sperm concentration, percentage motility (by CASA), sperm morphology, leukocyte concentration and ROS production.

Measurement of ROS

Basal or unstimulated ROS levels were measured by the conventional chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St Louis, MO, USA) as the probe (Hendin *et al*., 1999). Measurements were made using a Berthold luminometer (Autolumat LB 953, Wallac Inc., Gaithersburg, MD, USA). Luminol (5 mmol/l) prepared in dimethylsulphoxide (DMSO; Sigma Chemical Co.) was added to 400 µl of the washed sperm suspension. The chemiluminescent signal was monitored for 15 min, and results were expressed as $\times10^6$ counted photons per minute (c.p.m.). Despite the fact that all ISolate fractions were negative for the presence of leukocytes, based on the myeloperoxidase test, it is still possible that small numbers of leukocytes ($<5\times10^4$/ml) could be contaminating these fractions. In order to rule out the presence of leukocytes, the different fractions were incubated with 25 mmol/l luminol supplemented with 12.4 U horseradish peroxidase (Type VI, 310 U/mg; Sigma Chemical Co.) for 5 min to sensitize the assay for the generation of extracellular hydrogen peroxide. After allowing 5 min to capture the basal luminol-dependent signal, cells were stimulated with formyl-methionyl-leucyl-phenylalanine (FMLP) prepared as a 10 mmol/l stock solution in DMSO. The signal was monitored for 5 min to determine the magnitude of the peak chemiluminescence response and to allow the system to return to baseline (Aitken *et al*., 1996). The sperm suspensions were stimulated with 4 µl of 100 nmol/l 12-myristate, 13-acetate phorbol ester (PMA) and monitored for 15 min to assess the residual capacity of the cell population for ROS generation.

Sperm morphology

Smears of raw semen and spermatozoa from the different ISolate fractions were prepared for sperm morphology assessment. The

smears were fixed and stained using the Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw, IL, USA). Immediately after staining, the smears were rinsed in distilled water and air-dried. Smears were scored for sperm morphology by brightfield microscopy using strict criteria (Kruger *et al.*, 1986).

Sperm chromatin structure assay

The sperm chromatin structure assay (SCSA) was carried out as described previously (Evenson and Jost, 1994). Frozen semen samples were thawed in a 37°C water bath, and immediately diluted with TNE buffer (0.15 mol/l NaCl, 0.01 mol/l Tris, 0.001 mol/l EDTA, pH 7.4) to $1–2\times10^6$ spermatozoa/ml. As reported previously, DNA structure is not affected by the freezing–thawing process (Evenson *et al.*, 1989, 1994). A 400 µl aliquot of the acid-detergent solution (0.08 mol/l HCl, 0.15 mol/l NaCl, 0.1% Triton X-100, pH 1.2) was admixed with 200 µl of the diluted sample. After 30 s, spermatozoa were stained by adding 1.2 ml of acridine orange (AO) stain solution containing 6 µg/ml of AO (chromatographically purified; Cat. # 04539, Polysciences Inc., Warrington, PA, USA) per ml buffer [0.037 mol/l citric acid; 0.126 mol/l Na_2HPO_4; 0.0011 mol/l EDTA (di-sodium), 0.15 mol/l NaCl, pH 6.0] (Darzynkiewicz *et al.*, 1975; Evenson *et al.*, 1985). Immediately afterwards, the sample was placed on the flow cytometer for 2.5 min to allow for hydrodynamic and stain equilibrium before data were collected on 7000 cells per sample (Ortho Diagnostic Inc., Westwood, MA, USA) with a Lexel 100 mW argon ion laser operated at 35 mW interfaced to a Cicero data handling unit with PC-based Cyclops Software (Cytomation, Fort Collins, CO, USA). The extent of DNA denaturation was quantified by the calculated parameter $\alpha_t = [\alpha_t$ red/(red + green) fluorescence] (Darzynkiewiez *et al.*, 1975). Normal, native chromatin remains structurally intact and produces a narrow α_t distribution. DNA in spermatozoa with abnormal structure has increased red fluorescence (Evenson *et al.*, 1980, 1985) with an α_t distribution which is broader, having a higher mean channel ($X\alpha_t$) and a larger percentage of cells outside the main population of cells ($COMP\alpha_t$). Standard deviation of α_t ($SD\alpha_t$) describes the extent of chromatin structure abnormality within a given population (Evenson *et al.*, 1980, 1985, 1991). The elevated green fluorescence (HGRN) is considered to reflect the lack of complete chromatin condensation, and is not necessarily correlated with cytoplasmic retention or sperm architectural remodelling.

It is noteworthy, that the SCSA described herein measured the susceptibility of sperm nuclear DNA to denaturation *in situ*. This susceptibility has been correlated with the presence of DNA strand breaks that may be derived in part by ROS-induced damage and possibly by a unique, abortive apoptotic mechanism (Gorczyca *et al.*, 1993; Sailer *et al.*, 1995; Aravindan *et al.*, 1997). Therefore, the designation 'DNA damage' or 'damaged DNA' used throughout the text refers to this susceptibility to DNA strand breaks (Evenson, 1999a). The SCSA is primarily a measure of DNA integrity and, secondarily, provides information on the extent of chromatin condensation.

Extraction, purification and analysis of sperm lipids

Sperm suspensions from the different ISolate fractions were diluted with one volume of Dulbecco's phosphate-buffered saline (PBS) and centrifuged at 800 *g* for 8 min. The pellet was then resuspended in 1 ml of PBS, and an aliquot was used to determine the total number of spermatozoa and immature germ cells. This centrifugation process was repeated and the pellet resuspended in 500 µl of PBS. Lipids were extracted by liquid–liquid extraction with 6 volumes of chloroform:methanol (2:1, v/v), centrifuged at 800 *g* for 3 min, and the resulting lower phase aspirated. An aliquot of 20 µl of a 0.25 mg/ml solution of heptadecanoic acid (17:0) in chloroform:methanol (2:1, v/v) was added as an internal standard to those samples used for fatty acid analysis by gas chromatography. Samples were transmethylated as described elsewhere (Alvarez and Storey, 1995). Fatty acid methyl esters were analysed by gas chromatography using a Hewlett Packard 5890A II gas chromatograph (Alvarez and Storey, 1995). Fatty acid methyl ester peaks were identified by comparison of retention times of standard mixtures and mass spectrometry, and quantified using methylheptadecanoate as the internal standard. In a separate experiment, lipid extracts from the different sample preparations were fractionated into neutral lipids, free fatty acids and phospholipids by aminopropyl column chromatography (Alvarez and Touchstone, 1992).

In order to confirm the stereospecific location of polyunsaturated fatty acids at the sn-2 position of phospholipids, the phospholipid fraction obtained by aminopropyl column chromatography was hydrolysed with phospholipase A_2, lipids extracted by liquid–liquid extraction with chloroform:methanol (2:1, v/v), and the resulting free fatty acid fraction isolated by aminopropyl column chromatography, methylated, and analysed by gas chromatography as indicated above.

Cholesterol and desmosterol content in the different sperm fractions were analysed by micro high-performance thin-layer chromatography (HP-TLC), as described previously (Alvarez and Storey, 1995; Ollero *et al.*, 2000). Following extraction, the lower phases were evaporated to dryness and resuspended in 50 µl of chloroform:methanol (1:1, v/v). Aliquots (4 µl) of the different samples and of the cholesterol and desmosterol standards at a concentration of 0.1 mg/ml were applied to $AgNO_3$-impregnated HP-K plates, developed in chloroform:acetone (95:5, v/v), and stained with the $CuSO_4$ reagent, as described previously (Alvarez and Storey, 1995). The resulting bands were scanned at 400 nm in the reflectance mode using a Shimadzu CS-9000U spectrodensitometer (Shimadzu Scientific, Inc., Columbia, MD, USA).

Statistical analysis

A power analysis was performed to determine the minimum sample size. Based on preliminary data, using an α value of 0.05 and a β value of 0.1 (90% power), the minimum sample size required was 16 samples per group (total 48 samples). Student's *t*-test (for normally distributed variables) and Wilcoxon sum-rank tests (for non-normal variables) were used to compare groups and fractions. Coefficients of correlation were calculated using Spearman correlation analysis. These correlations were considered clinically meaningful at $r > 0.1$. To determine the effect of ROS on various semen parameters, base-10 logarithms of ROS levels were used to normalize the distribution, and then multiplied by the percentage recovery of the corresponding ISolate fraction. This is referred to throughout the text as 'relative ROS levels'. The correlations between the various measures with non-standardized ROS, the logarithm of ROS, and the log relative ROS levels in each fraction, were tested independently. All hypothesis tests were two-tailed with statistical significance assessed at the $P < 0.05$ level. Statistical computations were calculated using SPSS 10 for Windows software (SPSS Inc, Chicago, IL, USA).

Results

ROS production

ROS production in samples from donors and patients was highest in spermatozoa from fraction 2 (47/70% interface), and lowest in spermatozoa from fraction 4 (90% pellet). Differences in ROS production between spermatozoa from fractions 1 (seminal plasma/47% interface) and 2 were not statistically significant. In contrast, significant differences in

Table I. Fatty acid content in human spermatozoa from the different ISolate fractions[a]

Fatty acid	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Palmitic acid	2.588 ± 2.027	1.241 ± 0.442	0.847 ± 0.259	1.321 ± 0.837
Stearic acid	1.376 ± 1.325	0.711 ± 0.414	0.553 ± 0.323	1.208 ± 0.988
Oleic acid	0.350 ± 0.362	0.130 ± 0.068	0.087 ± 0.063	0.162 ± 0.129
Linoleic acid	0.699 ± 0.633	0.246 ± 0.113	0.153 ± 0.052	0.219 ± 0.158
γ-Linolenic acid	0.466 ± 0.370	0.175 ± 0.062	0.106 ± 0.028	0.132 ± 0.054
Arachidonic acid	0.411 ± 0.321	0.171 ± 0.068	0.111 ± 0.026	0.131 ± 0.043
Eicosapentaenoic acid	0.008 ± 0.019	0.001 ± 0.001	0.001 ± 0.004	0.001 ± 0.002
Docosahexaenoic acid	2.370 ± 1.231	1.158 ± 0.344	0.816 ± 0.125	0.806 ± 0.269
Total fatty acid	7.967± 6.139	3.833 ± 1.341	2.675 ± 0.767	3.209 ± 2.370

[a]Values are nmol/10^6 spermatozoa (mean ± SD; n =12).

ROS production were seen between spermatozoa from fractions 2 and 3 (70/90% interface) ($P = 0.004$) and 2 and 4 ($P < 0.04$).

There were no statistically significant differences in ROS production between spermatozoa from the different fractions in samples obtained from donors and from patients with normal semen parameters. In contrast, differences in ROS production in spermatozoa from the different fractions in samples obtained from donors and patients with abnormal semen parameters were statistically significant ($P < 0.02$). Differences in ROS production by the different sperm fractions from patients with normal and abnormal semen parameters were only statistically significant for fraction 2 ($P < 0.02$).

ROS production by spermatozoa from fraction 2 was correlated with the concentration of spermatozoa with cytoplasmic retention and abnormal head morphology in this fraction, as previously reported (Gil-Guzman *et al*., 2001). This correlation was statistically significant in both patients and donors ($P < 0.0001$).

Fatty acid content

The fatty acid profile of sperm subsets from the different ISolate fractions is shown in Table I. DHA content in spermatozoa from fraction 1, expressed in nmol/10^6 spermatozoa, was more than two-fold higher than that found in spermatozoa from fraction 4 ($P < 0.001$). As expected, >85% of the DHA in spermatozoa from all fractions was esterified to the sn-2 position of phospholipids, as determined by aminopropyl column chromatography, enzymatic hydrolysis with phospholipase A_2 and gas chromatography.

The total fatty acid content in spermatozoa from fraction 1 was more than two-fold higher than that found in spermatozoa from fraction 4 ($P < 0.001$) (Table I). No significant differences in total fatty acid or DHA content were found in spermatozoa from fractions 3 and 4. Neither were any statistically significant differences found in phospholipid-bound DHA content in spermatozoa from ISolate fraction 4 between samples with high and low sperm recoveries in this fraction. No significant differences in DHA content was observed in spermatozoa from fraction 4 in samples obtained from donors or patients with either normal or abnormal semen parameters.

Sterol content

The cholesterol and desmosterol content in spermatozoa from the different ISolate fractions is shown in Table II. Cholesterol content was five-fold higher in fraction 1 compared with fraction 4 ($P = 0.002$). The cholesterol content in spermatozoa from fraction 1 was 2.5- and 4.5-fold higher than that found in spermatozoa from fractions 2 ($P = 0.016$) and 3 ($P < 0.0001$) respectively. No significant differences in cholesterol content were found between fractions 3 and 4. Desmosterol content in spermatozoa from fraction 1 was about seven-fold higher than that found in spermatozoa from fraction 4 ($P = 0.002$). No significant differences in desmosterol content were found between fractions 1, 2 and 3.

Chromatin structure

The level of green fluorescence (HGRN) (which is indicative of the accessibility of double-stranded DNA to acridine orange stain) in spermatozoa from the different fractions is shown in Table III. The highest values were observed in spermatozoa from fraction 1, and the lowest in spermatozoa from fraction 4 in samples obtained from both donors and patients. This is consistent with the fact that the highest percentage of immature germ cells is found in fraction 1, whilst fraction 4 consists (for the most part) of mature, morphologically normal spermatozoa with highly condensed chromatin that is resistant to AO DNA staining by five-fold relative to round spermatids (Evenson and Melamed, 1983). Differences in HGRN values in spermatozoa from fraction 1 in samples obtained from donors and from patients with either normal or abnormal semen parameters were statistically significant ($P < 0.05$). No significant differences were found in HGRN values obtained in spermatozoa from fractions 2, 3 or 4 in samples from donors and patients with normal semen parameters.

There was a significant inverse correlation between sperm concentration in semen and HGRN values in spermatozoa from fraction 1 ($P < 0.03$). In contrast, no significant correlation was found between HGRN values and relative ROS levels in any of the ISolate fractions. HGRN values in fraction 1 were not correlated with sperm motility or morphology (data not shown).

The mean COMPα_t values in spermatozoa from the different fractions are shown in Figure 1. The highest values were observed in spermatozoa from fraction 1 and the lowest in spermatozoa from fraction 4 in sperm samples from both donors and patients with normal semen parameters. Differences in COMPα_t values in spermatozoa from fractions 1 and 4 in samples obtained from either donors or patients with normal semen parameters were statistically significant ($P < 0.05$). In

Table II. Cholesterol and desmosterol content in human spermatozoa from the different ISolate fractions

	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Cholesterol[a]	2.078 ± 1.668	0.812 ± 0.247	0.466 ± 0.126	0.390 ± 0.167
Desmosterol[b]	0.710 ± 0.625	0.921 ± 1.525	0.542 ± 0.670	0.095 ± 0.118

[a]Values are nmol/10^6 spermatozoa (mean ± SD; n =12).
[b]Values are pmol/10^6 spermatozoa (mean ± SD; n =12).

Table III. Percentage of spermatozoa with high green fluorescence (HGRN) in the different ISolate fractions

	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Donors	14.2 ± 5.3	10.8 ± 6.8	7.21 ± 5.0	5.8 ± 2.8
Pt Abnormal SP	29.14 ± 16.5	15.36 ± 11.4	10.1 ± 8.8	7.0 ± 6.5
Pt Normal SP	21.5 ± 12.2	12.5 ± 8.7	7.0 ± 3.4	5.1 ± 2.2

[1]Values represent the mean ± SD of 16 different experiments.
Pt = patients; SP = semen parameters.

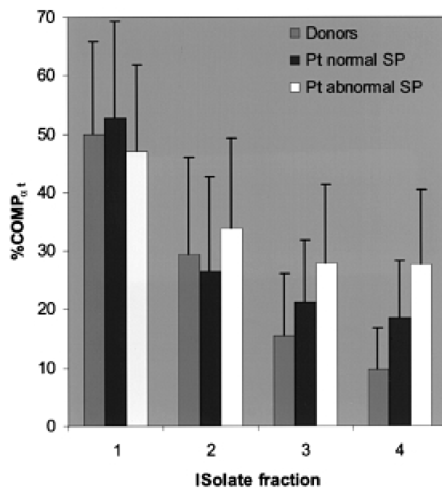

Figure 1. COMPα_t values in the different ISolate fractions in samples obtained from normal healthy donors and patients with normal and abnormal semen parameters. Values represent the mean of 16 different samples per group. Error bars indicate standard deviations.

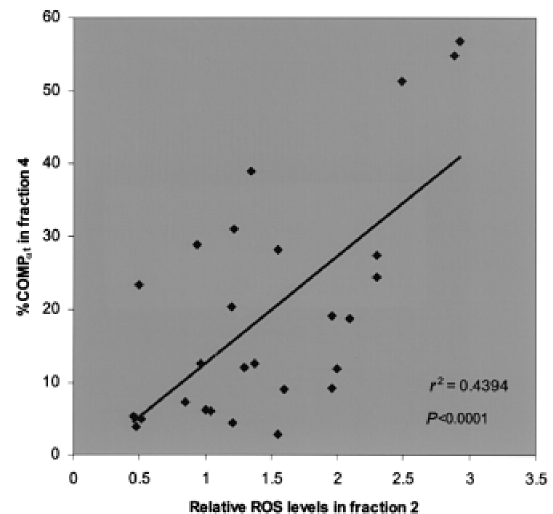

Figure 2. Correlation between reactive oxygen species (ROS) production in fraction 2 and COMPα_t values in spermatozoa from fraction 4.

contrast, there were no statistically significant differences in COMPα_t values in spermatozoa from fractions 1 and 4 compared with samples obtained from patients with abnormal semen parameters. The mean COMPα_t values in spermatozoa from fraction 4 from samples obtained from donors and from patients with normal semen parameters were 9.0 ± 7.1% and 18.5 ± 9.9% respectively. These differences were statistically significant (P = 0.03). Furthermore, there was a bimodal distribution of COMPα_t values in samples obtained from patients with normal semen parameters with peaks at 8.0 ± 5.0% and 23.7 ± 6.9%. This is consistent with the fact that some of the males presenting for infertility screening have normal chromatin parameters comparable with those observed in healthy sperm donors. The mean COMPα_t values in spermatozoa from fraction 4 from samples obtained from patients with abnormal semen parameters was 27.6 ± 8.2%. Differences in COMPα_t values in spermatozoa from fraction 4 from samples obtained from donors or patients with normal semen parameters versus patients with abnormal semen parameters were statistically significant ($P < 0.03$) (data not shown).

COMPα_t values in spermatozoa from fraction 4 were highly correlated with relative ROS levels in fraction 2 ($r^2 = 0.44$; $P < 0.0001$) (Figure 2), and this was applicable to samples obtained from both donors and patients.

Receiver-operating characteristics analysis indicated that a cut-off value of relative ROS levels in fraction 2 of 1.2 would result in maximal sensitivity and specificity for chromatin damage in spermatozoa from fraction 4, as measured by COMPα_t (Figure 3). A positive result was defined as a value $\geqslant$9% COMPα_t in spermatozoa from fraction 4 (which is the mean value observed in spermatozoa from fraction 4 from samples obtained from normal healthy donors), and a negative result as <9%. The sensitivity of a relative ROS value in

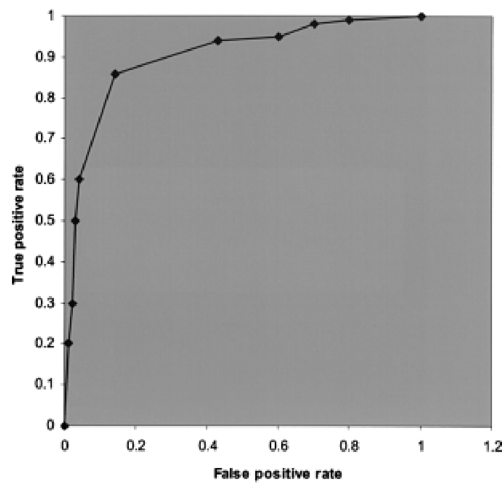

Figure 3. Receiver-operating characteristics analysis of relative ROS levels in fraction 2 and COMPα_t. The calculated area under the curve was 0.92 (SD = 0.02, 95% CI = 0.86 to 0.98).

Table IV. Performance characteristics of relative ROS levels in fraction 2 in the prediction of COMPα_t in spermatozoa from fraction 4

	≥9% COMPα_t in fraction 4	<9% COMPα_t in fraction 4	Total	
<1.2	3	11	14	PPV = 78%
≥1.2	16	1	17	NPV = 94%
Total	19	12	31	
	SENS = 84%	SPEC = 92%		

NPV = negative predictive value; PPV = positive predictive value; SENS = sensitivity; SPEC = specificity.

fraction 2 of 1.2 was 84% and the specificity 92%. The negative predictive value was 94% and the positive predictive value 78% (Table IV). The overall accuracy was 88%.

No significant correlation was observed between sperm morphology and COMPα_t values in spermatozoa from fraction 4.

Discussion

The results of the current study revealed significant differences in ROS production, lipid content and chromatin structure in ejaculated human spermatozoa, and marked changes in these parameters during the process of sperm maturation. DNA damage was greatest in samples containing high levels of ROS-producing spermatozoa, and this was in good agreement with previous reports (Aitken *et al*., 1994, 1997; Twigg *et al*., 1998a).

An unexpected finding of this study was the presence of a significant number of mature spermatozoa with damaged DNA in fraction 4 in samples obtained from patients presenting for infertility screening. These patients also presented with high relative ROS levels in fraction 2. Three main hypotheses can be postulated to explain this observation: (i) that a defect in spermiogenesis leads to both an increase in ROS-producing immature spermatozoa and sperm DNA damage; hence DNA damage occurs before spermiation; (ii) that DNA damage occurs after spermiation; and (iii) a combination of both.

With regard to the first hypothesis, in a number of studies (McPherson and Longo, 1992, 1993a,b) it has been proposed that the presence of endogenous nicks in ejaculated spermatozoa may be indicative of incomplete maturation during spermiogenesis. These authors postulated that chromatin packaging might necessitate endogenous nuclease activity to create and ligate nicks that facilitate protamination. These nicks are thought to provide relief of torsional stress and to aid chromatin arrangement during the displacement of histones by the protamines. A second explanation that is now gaining wider acceptance is that the presence of spermatozoa with damaged DNA in the ejaculate may be indicative of the occurrence of apoptosis during spermatogenesis (Billig *et al*., 1996; Pentikainen *et al*., 1999; Sakkas *et al*., 1999). This hypothesis is consistent with apoptosis-related DNA strand breaks in the early immature spermatozoon, but does not really explain how breaks would be created in cytoplasmic-free spermatid stages unless the possibility is considered of activation of a stored endonuclease. However, although this hypothesis would explain the presence of DNA damage in immature spermatozoa with abnormal head morphology or cytoplasmic retention, it does not explain how mature, motile spermatozoa devoid of cytoplasmic retention and with normal head morphometry (Sailer *et al*., 1996) isolated in fraction 4, have damaged DNA. Nevertheless, it is still possible that DNA damage may be the result of incomplete or defective packaging of sperm chromatin during the process of spermiogenesis independently of nuclear remodelling.

The second hypothesis postulates that chromatin damage in mature spermatozoa occurs after spermiation. Since mature and ROS-producing immature spermatozoa, with or without cytoplasmic retention, are highly packed in both the seminiferous tubules and in the epididymis, it is conceivable that co-existence of these spermatozoa during migration from the seminiferous tubules to the epididymis could result in oxidative DNA damage of mature spermatozoa. Therefore, DNA damage of morphologically normal spermatozoa may occur prior to, or during, transit of spermatozoa through the epididymis where further chromatin condensation takes place (Bedford *et al*., 1973). This is consistent with the observation that centrifugation of semen samples containing high levels of ROS-producing spermatozoa results in significant DNA damage of mature spermatozoa (Twigg *et al*., 1998b). This is also consistent with the fact that in-vitro exposure of mature spermatozoa to high levels of ROS resulted in significant DNA damage (Aitken *et al*., 1998; Lopes *et al*., 1998). In addition, this hypothesis is also supported by the negative predictive value of relative ROS values in fraction 2 in terms of DNA damage of mature, morphologically normal spermatozoa in fraction 4 (Table IV).

The third hypothesis postulates that the DNA damage observed in spermatozoa from fraction 4 is the result of a combination of both, alterations in the regulation of spermiogenesis (leading to defective chromatin packaging) and ROS-induced damage of mature spermatozoa after spermiation.

Another important finding of this study was the relatively high COMPα_t values observed in immature spermatozoa with cytoplasmic retention and abnormal head morphology isolated

in fractions 1 and 2. Elongating spermatids have been reported to have the highest levels of DNA nicks. Nick/ligation of DNA plays an important role in the reorganization of sperm nuclear chromatin that occurs during the process of spermiogenesis. Alterations in the regulation of spermiogenesis could result in unrepaired DNA strand breaks. Therefore, the high levels of DNA damage observed in spermatozoa from fractions 1 and 2 might be indicative of derangements in the normal regulation of spermiogenesis and also of the inefficiency of this process under normal conditions. This is consistent with the high COMPα_t values observed in spermatozoa from fractions 1 and 2 in samples obtained from normal healthy donors. However, the recovery of spermatozoa with high COMPα_t values in these fractions was significantly higher in patients than in donors ($P < 0.001$). This suggests that in these males the regulation of spermiogenesis may be even less efficient than under normal conditions, resulting in an increased release of immature spermatozoa with DNA damage into the seminiferous tubules.

It is also noteworthy that HGRN values in spermatozoa from fraction 1—which contains, for the most part, double-stranded DNA-containing immature germ cells—were inversely correlated with sperm concentration in semen. In addition, HGRN values in spermatozoa from fraction 1 were significantly higher in patients with abnormal semen parameters compared with donors or patients with normal semen parameters (Table III). Therefore, this increase in HGRN values in fraction 1 might be indicative of an up-regulation of spermatogenesis and immature germ cell production in these patients in response to a reduction in the overall sperm production.

Another important finding emerging from this study was the significant changes in DHA and cholesterol content observed during the process of sperm maturation. DHA and cholesterol contents were significantly lower in mature spermatozoa from fraction 4 compared with immature spermatozoa and immature germ cells isolated from ISolate fractions 1 and 2, and this was in good agreement with a previous report using Percoll gradient fractionation (Ollero *et al*., 2000). Phospholipid-bound DHA is known to play a key role in regulating membrane fluidity in biological membranes. Therefore, DHA removal from the sperm membranes during the process of sperm maturation would be expected to lead to a decrease in membrane fluidity. However, since there is a concomitant decrease in saturated fatty acid (Table I), cholesterol and desmosterol content (Table II)—which would tend to make the sperm membranes less rigid—the overall sperm membrane fluidity may not significantly change or even increase, despite a two-fold decrease in DHA content. The other important physiological consequence of the decrease in DHA content during sperm maturation is that removal of DHA from human sperm membranes during the process of sperm maturation will decrease the susceptibility of spermatozoa towards oxidative damage (Alvarez and Storey, 1995). This suggests that during the process of sperm maturation, spermatozoa retain a critical level of DHA resulting in: (i) optimal membrane fluidity required to support sperm motility and the early steps of fertilization; and (ii) minimal risk of oxidative damage to the sperm membranes and DNA.

Table V. Percentage recovery of motile spermatozoa in fraction 4

	Mean ± SD	Range	*n*
Donors	27.9 ± 9.7	22–42	16
Pt normal SP	23.2 ± 8.6	11–39	16
Pt abnormal SP	12.1 ± 8.9	5–23	16

n = number of samples; Pt = patients; SP = semen parameters.

If the characteristics of spermatozoa obtained in fraction 4 were to represent those of sperm cells that have matured adequately in terms of cytoplasmic and nuclear remodelling, motility, chromatin structure and morphology, one would expect a higher fraction of these cells in semen samples obtained from sperm donors compared with males presenting for infertility screening. As shown in Table V, the mean percentage recovery of motile spermatozoa in fraction 4 from samples obtained from sperm donors was 27.9 ± 9.7% compared with 23.2 ± 8.6% in males with normal semen parameters, and 12.1 ± 8.9% in males with abnormal semen parameters—in good agreement with our previous report using Percoll gradient fractionation (Ollero *et al*., 2000). Differences between donors (or patients with normal semen parameters) and patients with abnormal semen parameters were statistically significant ($P < 0.001$). In contrast, differences in percentage recovery between normal healthy donors and patients with normal semen parameters were not statistically significant. This is consistent with the notion that not all males presenting for infertility screening have a fertility problem. In contrast, differences in the recovery rates between donors (or patients with normal semen parameters) and patients with abnormal semen parameters were statistically significant ($P < 0.0001$).

Despite the significant differences in the overall recovery of spermatozoa in fraction 4 in samples obtained from sperm donors compared with males with normal or abnormal semen parameters, no statistically significant differences in DHA content, morphology or motility were found in spermatozoa from fraction 4 from samples obtained from either donors or patients with normal semen parameters; this lends additional support to the notion that the sperm cells isolated in fraction 4 correspond to the more mature spermatozoa. In addition, the higher the percentage of immature spermatozoa in fractions 1 and 2, the lower the percentage of cells in fraction 4. Therefore, since for the most part, spermatozoa recovered in fraction 4 represent those spermatozoa that have matured adequately in terms of membrane remodelling, morphology and motility, the recovery of spermatozoa in fraction 4 could be used as an indicator of the efficiency of spermatogenesis in males presenting for infertility screening. However, COMPα_t values (which reflect abnormal chromatin structure) in mature spermatozoa from fraction 4 were not correlated with either DHA content or sperm morphology in this fraction. Furthermore, COMPα_t values were correlated with relative ROS levels in fraction 2, suggesting that the DNA damage observed in mature spermatozoa from fraction 4 may be the result, at least

in part, of oxidative damage of these spermatozoa by high ROS-producing spermatozoa after spermiation. Therefore, the presence of a critical concentration of ROS-producing spermatozoa in the sample (relative ROS levels) may lead to DNA damage of mature spermatozoa. This is in agreement with our second hypothesis.

Based on the fact that: (i) the recovery of mature, morphologically normal spermatozoa in fraction 4 is indicative of the efficiency of spermiogenesis; and (ii) the relative ROS levels in ISolate fraction 2 were directly correlated with COMPα_t values (and therefore with DNA damage) and inversely correlated with the recovery of motile spermatozoa in fraction 4, it is possible that the ratio of percentage recovery of motile spermatozoa in fraction 4 (%REC-F4) to the relative ROS levels in fraction 2 (REL-ROS-F2) could be used as a sperm quality index (SQI), and as a surrogate of semen analysis (% REC-F4/REL ROS-F2). Using this ratio, the mean SQI value in samples obtained from healthy donors was 27.5, 21.9 in patients with normal semen parameters, and 6.7 in patients with abnormal semen parameters. Differences in SQI values between donors or patients with normal semen parameters versus patients with abnormal semen parameters were statistically significant ($P = 0.0001$). However, ~25% of the males with low relative ROS levels in fraction 2 had $\geqslant$9% COMPα_t in fraction 4, indicating that the SQI may not always be a reliable estimate of chromatin integrity in spermatozoa from the ISolate pellet.

The implications of these findings in the diagnosis and treatment of male infertility deserve consideration. Since not all the spermatozoa in fraction 4 had DNA damage, as measured by the SCSA, it might be speculated that insemination of oocytes *in vitro* or *in vivo* with spermatozoa from fraction 4 should result in successful pregnancies, since some of these spermatozoa do not have 'apparent' DNA damage. However, as has been reported previously, COMPα_t values in the semen sample are indicative of more extensive DNA damage within the sperm population (the 'tip of the iceberg' effect) (Evenson, 1999). That is, although only a fraction of the spermatozoa had strand breaks that resulted in single-stranded DNA, following exposure to the SCSA acid treatment, the remainder of the spermatozoa may still have some uncompensated DNA damage, despite the lack of expression of single-stranded DNA under these assay conditions. This is supported by one report in which no pregnancies were produced in natural cycles that used spermatozoa with $\geqslant$30% COMPα_t values in neat semen (Evenson *et al*., 1999), and by another report (Larson *et al*., 2000) in which no pregnancies were produced in IVF/intracytoplasmic sperm injection (ICSI) cycles that used spermatozoa with $\geqslant$27% COMPα_t values in neat semen.

In-vitro fertilization of metaphase II oocytes with spermatozoa that have damaged DNA could potentially lead to failed fertilization, defective embryo development, implantation failure or early abortion (Genesca *et al*., 1992; Parinaud *et al*., 1993; Twigg *et al*., 1998b; Evenson *et al*., 1999). Fully competent, mature oocytes have functional DNA repair mechanisms that allow repair of DNA damage in defective spermatozoa during fertilization (Brandiff and Pedersen, 1981; Matsuda and Tobari, 1988). However, immature oocytes (or oocytes from older women) may not be capable of repairing DNA damage in spermatozoa. This is consistent with the observation that insemination with donor spermatozoa of oocytes from older women, who have previously had embryos of poor quality after insemination with defective spermatozoa from their male partners, often results in a significant improvement in embryo quality and pregnancy rate (Genesca *et al*., 1992; Obasaju *et al*., 1999). Therefore, one of the implications of this study is that the relative ROS levels in spermatozoa from fraction 2, following ISolate gradient fractionation of ejaculated spermatozoa, could be used as a biomarker to identify those males who are at higher risk for the production of spermatozoa with damaged DNA. The other important implication is that selection of spermatozoa with intact DNA using in-vitro isolation techniques would be of great benefit to these couples. Currently available gradient fractionation techniques do not allow the isolation of mature spermatozoa with intact DNA from ejaculated spermatozoa as mature spermatozoa with intact or damaged DNA achieve their isopycnic separation in the gradient pellet.

In summary, the results of the current study indicate that there is significant variation in lipid content, ROS production, chromatin structure, morphology and motility in subsets of human spermatozoa at different stages of maturation. ROS production was highest in immature spermatozoa with cytoplasmic retention, and lowest in mature spermatozoa. The relative proportion of ROS-producing immature spermatozoa in fraction 2 was directly correlated with COMPα_t values in spermatozoa from fraction 4, and inversely correlated with the recovery of motile, mature spermatozoa in fraction 4. Therefore, oxidative damage of mature spermatozoa by ROS-producing immature spermatozoa during sperm migration from the seminiferous tubules to the epididymis may be an important cause of male infertility. This suggests that perhaps interventions directed to: (i) increase antioxidant levels in immature germ cell membranes during spermatogenesis; and (ii) isolate spermatozoa with intact DNA by in-vitro separation techniques should be of particular benefit to those patients in which a defect in the normal regulation of spermiogenesis and spermiation leads to an abnormal increase in the production of ROS-producing immature spermatozoa. Studies are currently under way to determine the in-vivo incorporation of α-tocopherol in immature germ cell membranes from mouse testis during the spermatogenic cycle following administration of oral α-tocopherol, and the development of in-vitro techniques to isolate mature spermatozoa with intact DNA.

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