

Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation

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BACKGROUND: Reactive oxygen species (ROS)-mediated damage to human spermatozoa has been implicated in the pathogenesis of male infertility. Although ROS production by human spermatozoa has been extensively studied, the cell-to-cell variation in ROS production by spermatozoa at different stages of maturation has never been investigated. **METHODS:** In this study, we determined ROS production by subsets of human spermatozoa at different stages of maturation isolated by density gradient centrifugation of ejaculated spermatozoa obtained from healthy donors and from patients attending a clinic for infertility screening. **RESULTS:** Four different fractions were obtained. ROS production was highest in immature spermatozoa with abnormal head morphology and cytoplasmic retention and lowest in mature spermatozoa and immature germ cells ($P < 0.01$). ROS production was highest in immature spermatozoa from males with abnormal semen parameters compared with donors ($P < 0.0001$) or patients with normal semen parameters ($P = 0.015$). ROS production by immature spermatozoa was inversely correlated with the recovery of motile, mature spermatozoa in the high density fraction 4 ($P = 0.01$). **CONCLUSIONS:** The results of this study indicate that there is significant cell-to-cell variation in ROS production in subsets of spermatozoa at different stages of maturation and that 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.

Key words: ISolate gradient/lipid peroxidation/ROS/sperm maturation/spermatozoa

Introduction

Male infertility is a multi-factorial condition encompassing a wide variety of aetiologies including gene mutations, chromosomal abnormalities, infectious diseases, excurrent duct occlusion, varicocele, radiation, chemotherapy, and impotence (Padron *et al.*, 1997; Wang *et al.*, 1997; Carbone *et al.*, 1998; Hendin *et al.*, 1999; Mak *et al.*, 1999). However, nearly 50% of infertile men do not have an identifiable cause of infertility and are classified as idiopathic (Sherins *et al.*, 1995). Decreases in sperm concentration and motility as well as morphological alterations are frequent findings in the semen analyses of these patients (Sigman *et al.*, 1992). Many of these alterations have recently been linked to high levels of reactive oxygen species (ROS) (Aitken, 1994; Aitken and Fisher, 1994; de Lamirande and Gagnon, 1994; Sharma and Agarwal, 1996). In addition, a decrease in the total antioxidant capacity (TAC) of seminal plasma has also been linked to the reduction of sperm quality (Lewis *et al.*, 1994; Palan and Naz, 1996; Smith *et al.*, 1996; Sharma *et al.*, 1999).

The overall rate of lipid peroxidation of spermatozoa *in vitro* is mainly determined by oxygen concentration and temperature in the extracellular medium, ROS production, the presence of antioxidant enzymes in spermatozoa, and the concentration of membrane-bound docosahexaenoic acid (DHA) (Alvarez and Storey, 1995). The higher the temperature and oxygen concentration in the medium, the higher the rate of lipid peroxidation as measured by malondialdehyde production (Alvarez *et al.*, 1987). Conversely, the higher the activity of the antioxidant enzymes, the lower the rate of lipid peroxidation (Alvarez *et al.*, 1987). The balance between these factors determines the overall rate of lipid peroxidation *in vitro*. Although ROS are important mediators of normal sperm function and are involved in the induction and development of sperm hyperactivation, capacitation and acrosome reaction (Jones and Mann, 1976; Aitken, 1995; de Lamirande and Gagnon, 1995a,b; Griveau *et al.*, 1995), an increase in ROS production above normal levels results in lipid peroxidation and ROS-induced membrane damage leading to loss of sperm

motility (Alvarez and Storey, 1982), inactivation of glycolytic enzymes (Alvarez and Storey, 1984), damage to the acrosomal membranes (Alvarez and Storey, 1984), and DNA oxidation (Fraga *et al.*, 1991), which render the sperm cell unable to fertilize the oocyte or produce a viable pregnancy.

Sperm maturation involves a broad range of events, which occur in the seminiferous epithelium and during sperm migration through the seminiferous tubules and epididymis (Amann *et al.*, 1993; Jones, 1989). These events include membrane and nuclear remodelling (Clermont, 1963), leading to sperm differentiation, the acquisition of motility (Esponda, 1991; Hegde, 1996) and the ability of spermatozoa to undergo the zona-induced acrosome reaction (Alvarez and Storey, 1995; Yeung *et al.*, 1996; Toshimori, 1998), which require an increase in membrane fluidity (Flechon, 1985; Myles *et al.*, 1987). We have recently reported that there is a significant loss of DHA in human spermatozoa during the process of sperm maturation (Ollero *et al.*, 2000). This may be part of the genomically regulated cellular maturational steps that take place within the adluminal compartment of the seminiferous epithelium. By the time spermatozoa arrive at the epididymis, these events are completed (Huszar *et al.*, 1997, 1998). If these events do not occur, immature spermatozoa in the ejaculate would exhibit cytoplasmic retention and a high rate of lipid peroxidation (Aitken *et al.*, 1994; Huszar and Vigue, 1994). Huszar and Vigue reported high levels of malondialdehyde and high creatine kinase activity in oligozoospermic patients (Huszar and Vigue, 1994). Increased production of ROS has been reported in patients whose spermatozoa have excess residual cytoplasm (Aitken *et al.*, 1994). There have been several additional reports suggesting a possible metabolic alteration related to irregular spermatogenesis (Aitken and Clarkson, 1987; Sukcharoen *et al.*, 1995; Gomez *et al.*, 1996; Yeung *et al.*, 1996). In addition, a close correlation has been reported between ROS production and the stage of development, being highest in immature spermatozoa (Fisher and Aitken, 1997).

The objectives of this study were (i) to determine ROS production by subsets of human spermatozoa at different stages of maturation isolated by discontinuous density gradient centrifugation of ejaculated spermatozoa and (ii) to investigate whether ROS production by these sperm subsets differs in males with normal and abnormal semen parameters.

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 ($n = 16$). Samples with a sperm concentration $<1 \times 10^6/\text{ml}$ or any detectable leukocytes in semen were excluded from this 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 a computer-assisted semen analyser (Cell-Trak, version 4.24 Motion Analysis Corporation, Palo Alto, CA, USA) to measure sperm concentration, percentage motility, and motion characteristics (Esteves *et al.*, 1999). Smears were prepared for the assessment of sperm morphology. Myeloperoxidase staining

(Shekarriz *et al.*, 1995) was performed to evaluate leukocyte concentration in the specimen.

Classification of semen samples

Semen samples from patients were classified as samples with normal and abnormal semen parameters based on World Health Organization criteria. Specimens with a sperm count $<20 \times 10^6/\text{ml}$, sperm motility $<50\%$ (WHO, 1999), and percentage normal forms $<14\%$ (Kruger *et al.*, 1986) were considered abnormal.

Density gradient centrifugation

Aliquots of 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. 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 computer-assisted semen analysis, sperm morphology, leukocyte concentration, and ROS production.

Measurement of reactive oxygen species

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). Five millimolar luminol prepared in dimethylsulphoxide (DMSO; Sigma) was added to 400 μl of the washed sperm suspension. The chemiluminescent signal was monitored for 15 min and results were expressed as 10^6 counted photon per minute (cpm) per 20×10^6 spermatozoa. 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 ($<50\,000/\text{ml}$) 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) 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.

Measurement of total antioxidant capacity

Total antioxidant capacity in seminal plasma was measured using a modified enhanced chemiluminescence assay (E.Gil-Guzman *et al.*, unpublished data). Aliquots of seminal plasma were stored at -76°C until analysis. Following thawing at room temperature, seminal plasma samples were diluted 1:20, v/v, with distilled water and filtered through a 0.2 μm Millipore filter (Allegiance HealthCare Corporation, McGaw Park, IL, USA). Signal reagent was prepared by adding 110 μl of luminol prepared in 282.1 mmol/l DMSO, 10 μl of

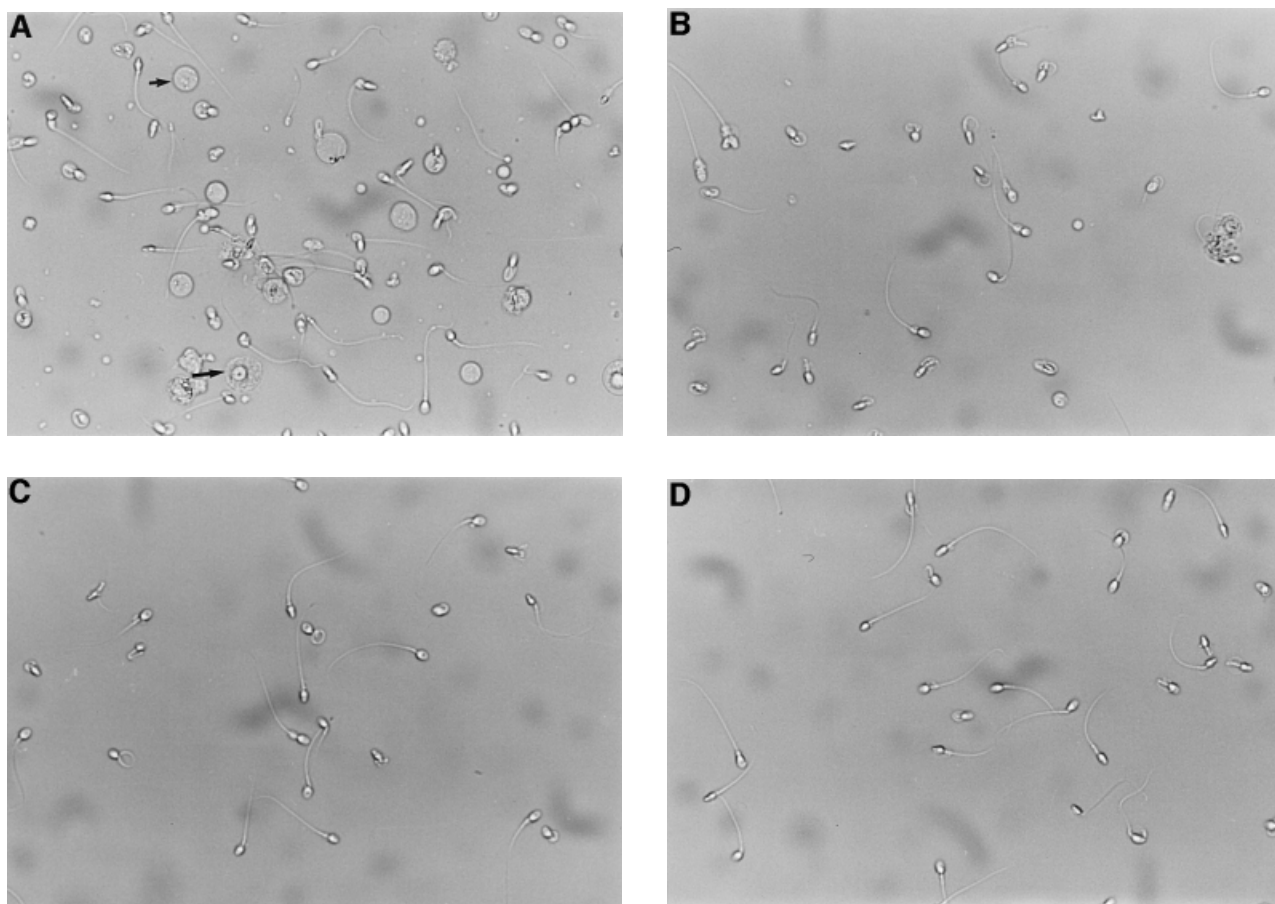


Figure 1. Phase-contrast micrography of human spermatozoa from the different ISolate fractions. (A) Fraction 1; primary spermatocytes and round spermatids are indicated by a large and small arrow respectively. (B) Fraction 2. (C) Fraction 3. (D) Fraction 4. Magnification $\times 200$.

p-Iodophenol (Fluka-Aldrich/Sigma) prepared in 41.72 mmol/l DMSO, and 30 μ l of 8.8 mol/l H_2O_2 (Sigma), to 10 ml of 100 mmol/l Tris buffer (pH 8.0). Horseradish peroxidase (HRP, mouse IgG HRP-linked whole antibody from sheep; Amersham LifeScience Inc., Arlington Ht, IL, USA) was diluted 1:500 to prepare HRP working solution. Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble α -tocopherol analogue, was added as the standard at concentrations between 12.5 and 75 μ mol/l. With the luminometer in the kinetic mode, 100 μ l of signal reagent and 100 μ l of HRP were added to 700 μ l of distilled water and mixed. The solution was equilibrated to the desired level of chemiluminescence output (between 2 and 3×10^7 cpm) for 108 s. One hundred microlitres of the prepared seminal plasma was added immediately to the signal reagent and HRP, and the chemiluminescence signal measured. Suppression of luminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence was recorded. The antioxidant capacity of the seminal plasma was expressed in molar Trolox equivalents. One trolox equivalent is equal to 1 mmol/l.

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 Park, IL, USA). Immediately after staining, the smears were rinsed in distilled water and air-dried. Smears were scored for sperm morphology using strict criteria (Kruger *et al.*, 1986).

Statistical analysis

A power analysis was performed to determine the minimum sample size. Based on preliminary data, using an alpha value of 0.05 and a beta value of 0.1 (90% power), the minimum sample size required was of 16 samples per group (total of 48 samples) to detect a 1 unit difference in the log-scale ROS values. The Student *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 Pearson and Spearman correlations. 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 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. Statistics were analysed using SPSS 10 for Windows software (SPSS Inc., Chicago, IL, USA).

Results

Histochemical analysis

The histochemical analysis of the different sperm fractions obtained following ISolate density gradient centrifugation is shown in Figure 1. Fraction 1 was enriched in immature

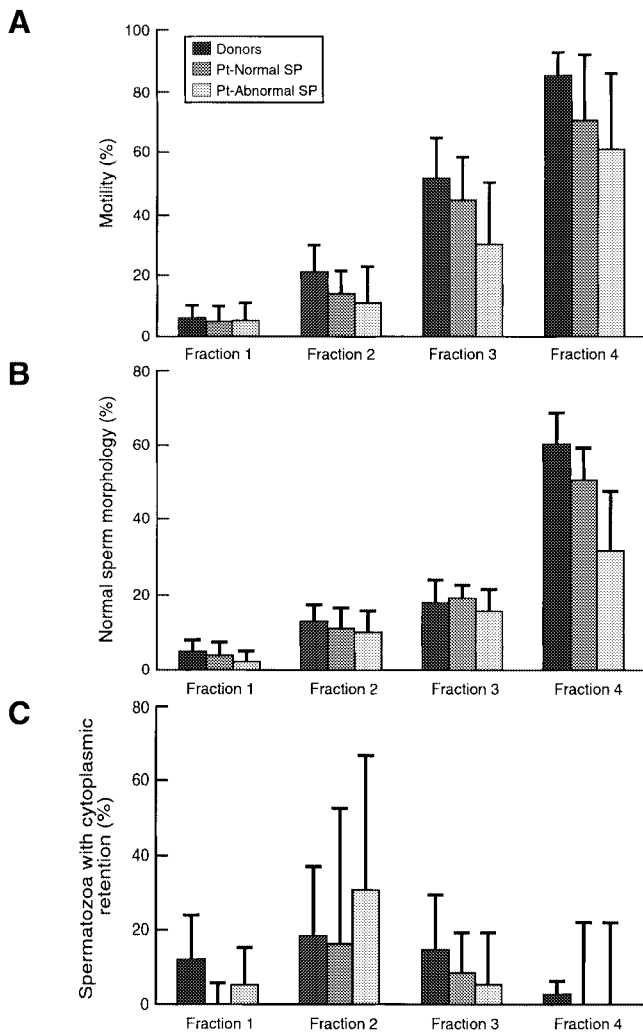


Figure 2. Percentage motility and morphology in the different ISolate fractions from donors and from patients with normal and abnormal semen parameters. Values represent the mean of 16 different samples per group. Error bars represent SD. (A) Percentage motility. (B) Normal sperm morphology. (C) Percentage spermatozoa with cytoplasmic retention.

germ cells and immature spermatozoa with abnormal head morphology and cytoplasmic retention (Figure 1A); fraction 2 contained, mostly, immature spermatozoa with cytoplasmic retention (Figure 1B); fraction 3 contained a mixture of morphologically normal and abnormal spermatozoa (Figure 1C); and fraction 4 contained, for the most part, morphologically normal spermatozoa (Figure 1D), consistent with previous reports (Ollero *et al.*, 2000). The cytoplasmic retention mentioned herein refers to the retention of cytoplasmic and membranous material in the head and proximal region of the midpiece of spermatozoa and is, therefore, different from the distal cytoplasmic droplet observed in spermatozoa during epididymal maturation.

Motility and morphology

Percentage sperm motility and morphology in the different ISolate fractions is shown in Figure 2A and B respectively. Motility was significantly lower in spermatozoa from fractions 1 and 2 compared with spermatozoa from fractions 3 and 4

($P < 0.01$). In contrast, the percentage normal forms was significantly lower in spermatozoa from fractions 1, 2 and 3 compared with spermatozoa from fraction 4 ($P < 0.01$), also consistent with previous reports (Chen and Bongso, 1999).

Percentage spermatozoa with cytoplasmic retention in the different ISolate fractions is shown in Figure 2C. The percentage of these spermatozoa in fraction 2 was significantly higher in samples obtained from patients with abnormal semen parameters compared with either donors or patients with normal semen parameters ($P = 0.023$).

ROS production

ROS production by subsets of human spermatozoa isolated by density gradient centrifugation of semen samples obtained from healthy donors and from patients with normal and abnormal semen parameters is shown in Table I. ROS production in samples from donors and patients was highest in spermatozoa from fraction 2 and lowest in spermatozoa from fraction 4. Differences in ROS production were only statistically significant between spermatozoa from fractions 2 and 3, and 2 and 4 (donors: $P < 0.001$ and $P < 0.0001$; patients with abnormal semen parameters: $P = 0.002$ and $P < 0.001$; and patients with normal semen parameters: $P < 0.04$ and $P < 0.004$).

Differences in ROS production in spermatozoa from the different fractions from samples obtained from donors and from patients with normal semen parameters were not significantly different. In contrast, differences in ROS production in spermatozoa from the different fractions from samples obtained from donors and patients with abnormal semen parameters were significantly different ($P < 0.002$). Differences in ROS production by the different spermatozoa fractions from patients with normal and abnormal semen parameters were statistically significant for all fractions ($P < 0.016$) (Table I).

ROS production in fraction 2 was highly correlated with the concentration of spermatozoa with cytoplasmic retention in this fraction ($P < 0.0001$) (Figure 3A). This correlation between ROS production in fraction 2 and concentration of sperm cytoplasmic retention was statistically significant in both patients and in donors. In contrast, ROS production in fractions 3 and 4 was not correlated with the concentration of spermatozoa with cytoplasmic retention in these fractions (Figure 3B).

ROS production in fraction 1 showed no significant correlation with the concentration of immature germ cells in this fraction (Figure 4). In fact, ROS production in cell suspensions highly enriched in round spermatids and free from immature spermatozoa was very low, comparable with that found in fraction 4 from either donors or patients (data not shown).

ROS production in spermatozoa from the different ISolate fractions in samples obtained from patients in response to FMLP and PMA is shown in Table II. Stimulation of spermatozoa from fraction 2 with 100 nmol/l PMA resulted in a significant increase in ROS production compared with luminol control ($P = 0.03$). In contrast, stimulation of mature spermatozoa from fraction 4 with PMA did not result in a significant increase in ROS production. Stimulation of spermatozoa from either fraction with 0.2 mmol/l FMLP did not result in any

Table I. Reactive oxygen species (ROS) levels in subsets of human spermatozoa isolated by density gradient centrifugation

Subjects	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Donors	5.51 ± 0.74 ^a	5.96 ± 0.78	4.63 ± 1.26	3.81 ± 1.26
Patients with abnormal SP	6.28 ± 0.58	6.93 ± 0.85	5.86 ± 0.89	5.25 ± 0.93
<i>P</i> values (donors and patients with abnormal SP)	0.003	0.002	0.003	<0.0001
Patients with normal SP	5.07 ± 1.80	5.69 ± 1.37	4.63 ± 1.46	4.22 ± 1.29
<i>P</i> values (patients with normal versus abnormal SP)	0.016	0.007	0.007	0.015

^aValues represent mean ± SD of 16 samples and are expressed as log₁₀ of ROS concentrations. Significant differences were seen in different fractions in donors: 1 versus 3 (*P* = 0.02); 1 versus 4 (*P* < 0.0001); 2 versus 3 (*P* = 0.001); 2 versus 4 (*P* < 0.0001). Significant differences were seen in different fractions in patients with abnormal SP: 1 versus 2 (*P* = 0.017); 1 versus 4 (*P* < 0.0001); 2 versus 3 (*P* = 0.002); 2 versus 4 (*P* < 0.0001). Significant differences were seen in different fractions in patients with normal SP: 2 versus 3 (*P* = 0.04); 2 versus 4 (*P* < 0.004). No significant differences were seen in any of the fractions between the donors and patients with normal SP. SP = semen parameters.

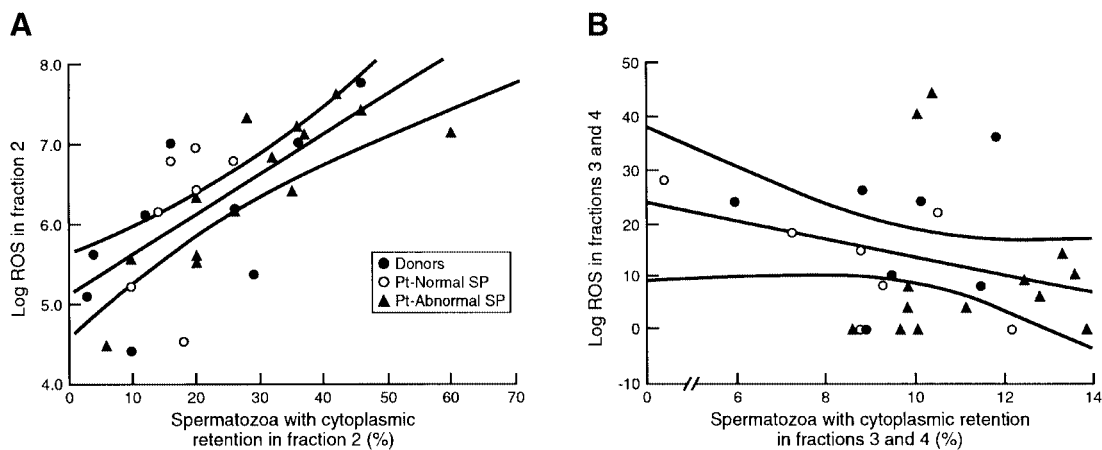


Figure 3. Correlation between reactive oxygen species (ROS) production and the concentration of immature spermatozoa with cytoplasmic retention. (A) Isolate fraction 2, *R*² = 0.53; (B) Isolate fractions 3 and 4, *R*² = 0.019.

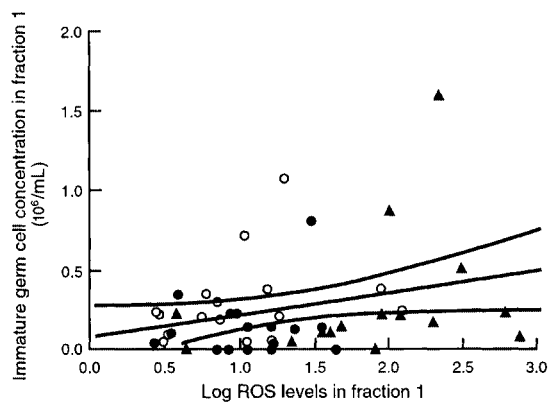


Figure 4. Correlation between log reactive oxygen species (ROS) concentrations in fraction 1 and immature germ cell concentration in fraction 1 (×10⁶/ml), *R*² = 0.002. ●, patients with normal semen parameters; ▲, patients with abnormal semen parameters; ○, donors.

significant increase in ROS production, confirming the absence of leukocytes in these fractions. FMLP specifically stimulates receptors present in leukocytes which are absent in spermatozoa. PMA, on the other hand, is known to induce ROS production by a protein kinase C-mediated mechanism.

There was a direct correlation between relative ROS levels in fraction 2 and spermatozoa with abnormal morphology (Figure 5) (*P* = 0.002).

Relative ROS levels in fraction 2 were inversely correlated with the percentage recovery of motile spermatozoa in fraction 4 (Figure 6) (*P* = 0.001).

Total antioxidant capacity

The mean TAC value in samples from donors was 742 ± 328 trolox equivalents. In patients with normal semen parameters the mean TAC value it was 528 ± 393, whereas in patients with abnormal semen parameters was 513 ± 323 trolox equivalents. Both patients with normal and abnormal semen parameters showed significantly lower TAC values compared with donors (*P* = 0.05). No differences were seen between patients with normal and abnormal semen parameters. An

Table II. Differential response to FMLP and PMA stimulation in different subsets of human spermatozoa

	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Luminol (5 mmol/l)	5.5 ± 0.4	6.4 ± 0.50	4.9 ± 0.4	2.4 ± 0.8
Luminol + HRP	5.6 ± 0.6	6.6 ± 0.3	5.2 ± 0.3	3.0 ± 1.1
FMLP (0.2 mmol/l)	5.7 ± 0.2	6.5 ± 0.5	5.1 ± 0.3	2.9 ± 1.1
PMA (100 nmol/l)	6.3 ± 0.8	7.6 ± 0.5 ^a	4.7 ± 1.7	3.6 ± 1.3

Values represent mean ± SD and are expressed as log₁₀ of reactive oxygen species concentrations.

^aSignificantly different from the luminol control ($P = 0.03$).

HRP = horseradish peroxidase; FMLP = formyl-methionyl-leucyl-phenylalanine; PMA = 12-myristate, 13-acetate phorbol ester.

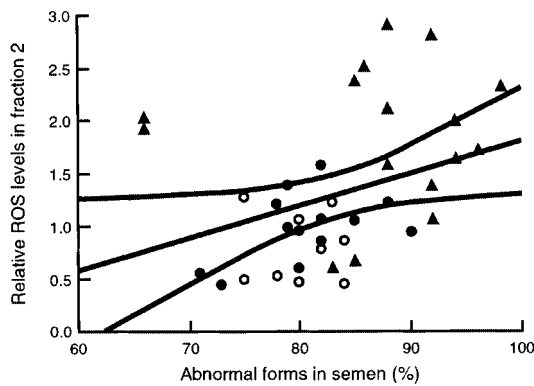


Figure 5. Correlation between the percentage abnormal forms in semen and the relative reactive oxygen species (ROS) concentrations in fraction 2, $R^2 = 0.64$. ●, patients with normal semen parameters; ▲, patients with abnormal semen parameters; ○, donors.

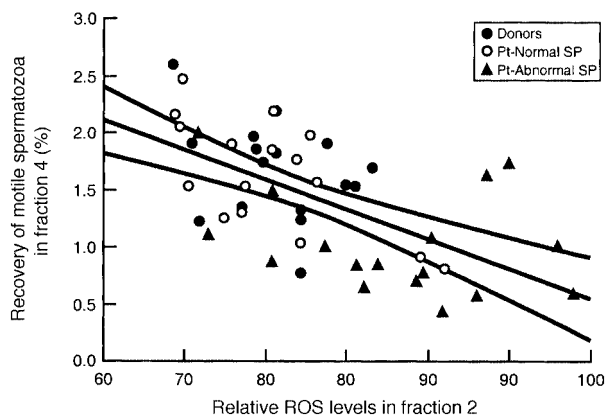


Figure 6. Correlation between the relative reactive oxygen species (ROS) concentrations in fraction 2 and the recovery of motile spermatozoa in fraction 4, $R^2 = 0.57$. ●, patients with normal semen parameters; ▲, patients with abnormal semen parameters; ○, donors.

inverse correlation was found between the relative ROS levels in fraction 2 and the TAC values of seminal plasma ($P = 0.02$) (Figure 7). The higher the relative ROS levels in fraction 2, the lower the TAC values in semen.

Discussion

The most relevant findings emerging from this study are the significant differences in the cell-to-cell production of ROS

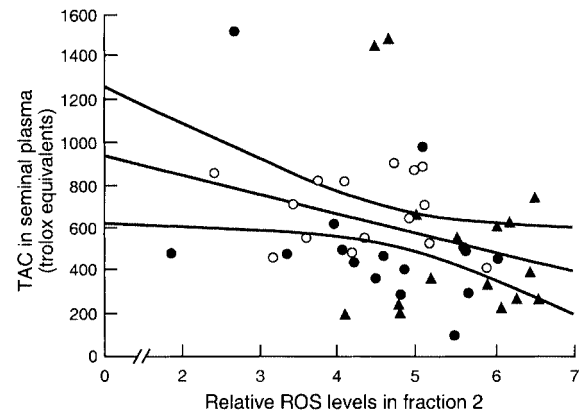


Figure 7. Correlation between total antioxidant capacity (TAC) values in the seminal plasma and the relative reactive oxygen species (ROS) concentrations in fraction 2, $R^2 = 0.55$. ●, patients with normal semen parameters; ▲, patients with abnormal semen parameters; ○, donors.

by ejaculated human spermatozoa and the marked changes in ROS production during the process of sperm maturation. ROS production was highest in immature spermatozoa with abnormal head morphology and cytoplasmic retention and lowest in immature germ cells and in mature spermatozoa, in good agreement with previous reports (Aitken *et al.*, 1994, 1997).

Previous studies have suggested that a defect in the normal regulation of spermiogenesis may lead to the abnormal production of immature spermatozoa with cytoplasmic retention that produce high levels of ROS (Aitken and Clarkson, 1987; Sukcharoen *et al.*, 1995; Yeung *et al.*, 1996; Gomez *et al.*, 1996). During the last steps of spermiogenesis (steps 17-19 in the rat and 7-8 in the human), there is marked membrane remodelling and cytoplasm dehydration of the elongating spermatid. In step 19 spermatid in the rat, spermatids release the residual body into the Sertoli cell during the process of spermiation (Clermont, 1963). Failure of testicular spermatozoa to shed the residual body into the Sertoli cell leads to the production of testicular spermatozoa with proximal cytoplasmic retention. The presence of high levels of NADPH oxidase (Aitken *et al.*, 1997) and glucose-6-phosphate dehydrogenase activity (Aitken *et al.*, 1994), which leads to NADPH biosynthesis in spermatozoa (Storey *et al.*, 1998), and DHA (Ollero *et al.*, 2000) in the residual body and in close proximity to sperm mitochondria, which produce superoxide anion and hydrogen peroxide (Holland and Storey, 1981; Holland *et al.*,

1982), could result in the production of increased levels of highly toxic oxygen radicals (Griveau and Le Lannou, 1997). However, other factor(s) may also be required for this increase in ROS production since spermatozoa with cytoplasmic retention from fractions 3 and 4 did not produce increased levels of ROS. This suggests that spermatozoa from fraction 2 may have other specific defect(s) that lead to the observed increase in ROS production. This defect(s) would confer these spermatozoa an overall lower cell density, which would explain, at least in part, why they reach their isopycnic separation in the lower density fractions 1 and 2.

In addition, it is also possible that immature spermatozoa with abnormal head morphology recovered in fraction 2 may produce excessive levels of ROS independently of proximal cytoplasmic retention. This is consistent with the reports by Weese *et al.* (1993) and Ford *et al.* (1997) and with our results that show that stimulation of sperm suspensions enriched in immature spermatozoa with abnormal head morphology from fraction 2 with 100 nmol/l PMA resulted in a 10–100-fold increase in ROS production compared to unstimulated spermatozoa (Table II). In contrast, no significant increase in ROS production was observed after stimulation of mature spermatozoa from fraction 4 with PMA. This is also consistent with our results that show a direct correlation between the percentage abnormal forms in semen and the relative ROS levels in fraction 2 (Figure 5) implying that dysregulation of normal spermiogenesis may lead to the release of high ROS-producing immature spermatozoa into the seminiferous tubules. Therefore, seminiferous tubules containing steps 17–19 spermatids in the rat (7 and 8 in the human) would be at greater risk for oxidative damage. This is supported by the fact that expression of Cu/Zn-superoxide dismutase mRNA in rat seminiferous tubules occurs in a stage-specific manner with the highest expression occurring at stages VI through VIII (steps 17–19) which are precisely those stages in which spermatids with residual bodies are found in the seminiferous epithelium (Jow *et al.*, 1993).

Another fundamental question that remains to be answered is why there are such marked differences in the concentration of ROS-producing spermatozoa with cytoplasmic retention in fraction 2 from males with normal and abnormal semen parameters. Is this related to a defect in the regulation of normal spermiogenesis in these males? Perhaps an important clue to the answer to this question might be provided by comparing the prevalence of spermatozoa with abnormal head morphology and cytoplasmic retention in ejaculated spermatozoa from man and other mammalian species in relation to the duration of spermiogenesis. In the rat, a total of 19 steps are required for round spermatids to become differentiated spermatozoa. Of these 19 steps, eight (12–19) involve significant remodelling of the nucleus and cytoplasm. In the mouse and Rhesus monkey, spermiogenesis spans a total of 16 steps, whereas in dog, stallion and bovine spermatozoa it spans 12 steps (Clermont, 1963). The prevalence of spermatozoa with abnormal head morphology and cytoplasmic retention in ejaculated spermatozoa from these species is relatively low. In contrast, in man, a species in which there is a relatively high prevalence of immature spermatozoa with head abnormalities

and cytoplasmic retention in the ejaculate, spermiogenesis spans 8 steps of which only two involve remodelling of the nucleus and cytoplasm. Therefore, it is conceivable that human spermatozoa, because of a less rigorous quality control, are more prone to derangements in normal spermiogenesis. However, despite the release of immature spermatozoa with cytoplasmic retention into the seminiferous epithelium that occurs under normal conditions, the percentage of these spermatozoa in semen samples from donors was significantly lower than that observed in samples from patients with abnormal semen parameters (Figure 2C). This suggests that in these males the control of spermiogenesis is even less efficient than that observed under normal conditions, resulting in the release of significantly higher numbers of immature spermatozoa with cytoplasmic retention into the seminiferous tubules.

What is the significance of the high prevalence of ROS-producing spermatozoa in the pathogenesis of male infertility? The relative ROS level in fraction 2 was negatively correlated in our study with the recovery of motile spermatozoa in fraction 4 (Figure 6). As indicated previously, spermatozoa in fraction 4 represent, for the most part, mature motile spermatozoa. Therefore, the higher the concentration of immature spermatozoa in fraction 2, the lower the concentration of mature spermatozoa in fraction 4. Given the fact that spermatozoa are highly packed in both the seminiferous tubules and in the epididymis, it is conceivable that co-existence of ROS-producing immature spermatozoa with mature spermatozoa during migration from the seminiferous tubules to the epididymis could result in oxidative damage of mature spermatozoa. If that were the case, this could result in loss of motility and DNA damage of mature spermatozoa. This is consistent with the results of our study where a decrease in motility recovery in fraction 4 was found in samples containing a high concentration of ROS-producing spermatozoa (Figure 6). This is also consistent with the observation that centrifugation of semen samples containing high levels of ROS-producing spermatozoa results in significant cross-damage of mature spermatozoa (Guérin *et al.*, 1989). In addition, this is in agreement with the report by Sakkas *et al.*, where they found a significant number of mature spermatozoa with DNA damage in the 90% PureSperm gradient pellet (Sakkas *et al.*, 1999). However, it is important to emphasize that cross-damage of mature spermatozoa by immature spermatozoa *in vivo* or *in vitro* may only occur under those conditions in which the concentration of high ROS-producing immature spermatozoa is above a critical threshold. This is consistent with the fact that the recovery of mature motile spermatozoa was inversely correlated with relative ROS levels in fraction 2 (which is a composite of both ROS production and the concentration of ROS-producing spermatozoa) and not with ROS levels alone. This may explain why, in the report by Plante *et al.*, co-incubation of immature and mature did not result in significant loss of motility during the 5 h incubation time (Plante *et al.*, 1994). Perhaps in their study, the concentration of high ROS-producing immature spermatozoa was below this critical threshold. This is consistent with the fact that these authors used immature spermatozoa isolated from samples obtained from healthy

donors which, as shown in our study, produce lower ROS levels and are present in lower concentrations.

It is noteworthy that semen samples obtained from males with abnormal semen parameters—which had the lowest sperm concentration—had the highest relative ROS levels in fraction 2. The ratio of immature germ cells to differentiated spermatozoa in these samples was not significantly different to that observed in donors or in patients with normal semen parameters, thus suggesting that there is not a block in spermatogenesis in these patients. Therefore, there exists the distinct possibility that perhaps prolonged exposure of the seminiferous epithelium to high levels of ROS-producing immature spermatozoa with cytoplasmic retention could lead to damage of the seminiferous tubules, testicular atrophy, and a reduction in total sperm production. This is consistent with the observation that varicocele has been reported to be associated with both higher levels of ROS production (Weese *et al.*, 1993) and progressive testicular atrophy (Lipshultz and Corriere, 1977; Okuyama *et al.*, 1988; Pinto *et al.*, 1994). This suggests that perhaps young males with high relative ROS levels in fraction 2 may be at higher risk for testicular atrophy. This hypothesis is amenable to experimental testing by monitoring the presence of potential damage of the seminiferous tubules by testicular ultrasound or testicular biopsy in these males.

An unexpected finding in this study was the strong inverse correlation between the relative ROS levels in fraction 2 and TAC values in seminal plasma (Figure 7). These results indicate that the higher the relative ROS levels in fraction 2, the lower the TAC values in seminal plasma, thus suggesting that this may be associated with an increase in the consumption of soluble, non-enzymatic antioxidants in semen. This also suggests that prolonged in-vitro incubation of semen samples containing a high proportion of high ROS-producing immature spermatozoa prior to sperm processing, e.g. gradient centrifugation, may lead to the depletion of antioxidants in seminal plasma and to an increased risk of oxidation of mature spermatozoa.

In summary, the results of this study indicate that there is significant cell-to-cell variation in ROS production in subsets of human spermatozoa at different stages of maturation. ROS production was highest in immature spermatozoa with abnormal head morphology and cytoplasmic retention and lowest in mature spermatozoa and immature germ cells. The relative proportion of high ROS-producing immature spermatozoa in fraction 2 was 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 and post-ejaculation may be an important cause of male infertility. This suggests that perhaps interventions directed to increase antioxidant levels in the spermatozoa and Sertoli cell membranes during spermatogenesis may be of particular benefit to these patients in which a defect in the normal regulation of spermiogenesis and spermiation leads to an abnormal increase in ROS-producing immature spermatozoa, as previously suggested (Suleiman *et al.*, 1996). Studies are currently underway to determine the in-vivo incorporation of α -tocopherol in mouse

immature germ cells and epididymal spermatozoa following oral administration of this antioxidant for the duration of the spermatogenic cycle.

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