

Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility

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Objective: To evaluate ejaculated spermatozoa from patients with male factor infertility for the role of cytochrome c and caspases 9 and 3 (the proteins known to mediate apoptosis) and to examine association between semen quality and apoptosis in the presence of oxidative stress.

Design: Prospective study.

Setting: Male infertility clinic at a tertiary health care center.

Patient(s): Semen specimens from 35 patients with idiopathic infertility and 8 normal healthy donors.

Main Outcome Measure(s): Levels of ROS, cytochrome c, and caspases 9 and 3 and semen variables.

Result(s): Compared with normal donors, infertile patients had significantly higher levels (expressed as median [25th and 75th percentiles]) of ROS (4.15×10^6 counted photons per minute [cpm] [0.26, 40.16×10^6 cpm] vs. 0.06×10^6 cpm [0.02, 0.29×10^6 cpm]; $P < .01$), cytochrome c (2.78 [2.21, 43.65 vs. 1.5 [1.25, 2.2]; $P < .01$), caspase 9 (2.52 [0.9, 4.28] vs. 6 [4.85, 7.63]; $P < .006$), and caspase 3 (0.56 [0.32, 1.02] vs. 1.69 [1.66, 2.67]; $P < .01$). Semen variables (motility, concentration, and morphology) were negatively correlated with caspase 9 and 3 ($P < .05$). Reactive oxygen species was positively correlated with cytochrome c ($r = .43$; $P < .03$), caspase 9 ($r = .56$; $P < .001$), and caspase 3 ($r = .65$; $P < .01$).

Conclusion(s): Infertile men have decreased sperm variables induced by higher ROS levels in semen. A positive relationship exists between increased sperm damage by ROS and higher levels of cytochrome c and caspases 9 and 3, which indicate positive apoptosis in patients with male factor infertility. (Fertil Steril® 2003; 80:531–5. ©2003 by American Society for Reproductive Medicine.)

Key Words: Human spermatozoa, reactive oxygen species, cytochrome c, caspases, apoptosis

Oxidative stress due to excessive production of reactive oxygen species (ROS) has been associated with defective sperm function and infertility (1–4). Reactive oxygen species are known to affect cellular lipids, proteins and DNA. Oxidative stress to the sperm DNA can have profound implications for normal embryonic development and long-term health of progeny (1, 5–7).

Apoptosis, or programmed cell death due to DNA fragmentation, is a distinctive form of eukaryotic cell death characterized by a series of morphologic and biochemical changes that result in elimination of cells from the tissues without eliciting an inflammatory response (8).

The role of apoptosis in gonadal tissue during spermatogenesis has recently attracted great research interest (9–10).

Studies in animal models have demonstrated that apoptosis is the underlying mechanism of germ cell death during normal spermatogenesis (11). Relatively high rates of apoptosis have been reported in testicular biopsies from infertile men with various degree of testicular insufficiency (12). An altered apoptotic process has been observed to be closely associated with male infertility (11, 13). In contrast, very few studies have reported apoptosis in the ejaculated spermatozoa. Gorczyca et al. (14) suggested that DNA strand breaks in

abnormal sperm were analogous to apoptosis in somatic cells. Other studies have linked apoptotic cell death with conventional semen parameters (6, 15).

The presence of phosphatidylserine, an apoptotic marker, has been confirmed during sperm apoptosis (16). About 20% of ejaculated spermatozoa are apoptotic, as indicated by externalization of the phosphatidylserine residues on annexin V staining (16).

Reports on apoptosis in ejaculated spermatozoa by other mediators, such as caspases, are lacking. Caspases are cysteine proteases that promote apoptosis in mammals (17). However, it is unclear whether the caspase-mediated pathway is involved in inducing apoptosis in ejaculated spermatozoa, and, if so, how it is influenced by oxidative stress.

We examined cytochrome c and caspases 9 and 3 in ejaculated spermatozoa obtained from patients with male factor infertility and evaluated the possible association between semen quality and apoptosis in the presence of oxidative stress.

MATERIALS AND METHODS

Collection and Preparation of Semen Samples

We studied 35 infertile patients attending our male infertility clinic and 8 normal healthy donors. The Institutional Review Board of the Cleveland Clinic Foundation approved the study, and written consent was obtained from all participants. Normal donors had an ejaculate volume of at least 2 mL and a sperm concentration of at least 20×10^6 /mL, of which at least 50% were motile and 30% had normal sperm morphology according to the World Health Organization (WHO) classification (18). Men with leukocytospermia ($>1 \times 10^6$ leukocytes/mL) were excluded.

Semen samples were obtained by masturbation after at least 48 hours of sexual abstinence. Samples were collected into sterile containers and allowed to liquefy at room temperature before analysis.

Semen Analysis

After liquefaction, a 5- μ L aliquot of the ejaculate was removed from each specimen and loaded on a counting chamber (MicroCell; Conception Technologies, La Jolla, CA). Specimens were analyzed for sperm concentration and motility according to WHO guidelines (18). Normal values for sperm variables were defined as a sperm concentration $\geq 20 \times 10^6$ /mL and motility $\geq 50\%$. Smears of raw semen were stained by using a Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGraw Park, IL) for assessment of sperm morphology. After staining, the smears were rinsed in distilled water and air-dried, and morphology was assessed by WHO classification and Tygerberg's strict criteria (18, 19).

Measurement of Reactive Oxygen Species

Aliquots of liquefied semen were centrifuged at $300 \times g$ for 7 minutes. Seminal plasma was discarded. The sperm

pellet was washed twice with phosphate-buffered saline (PBS) (pH, 7.4), and resuspended at a concentration of 20×10^6 sperm/mL. Levels of ROS were determined by a chemiluminescence assay using luminol (5-amino-2,3 dihydro-1,4 pthalazinedione; Sigma Chemical Co., St. Louis, MO) as the probe (20). Measurements were made using a Berthold luminometer (Autoluminat LB 953; Wallace, Inc., Gaithersburg, MD). Five mM of luminol prepared in dimethyl sulfoxide (DMSO) (Sigma Chemical Co.) was added to each of 400 μ L of sperm suspension, blank (PBS) and control (PBS and luminol).

Levels of ROS were determined by measuring chemiluminescence for 15 minutes. Results are expressed as counted photons per minute (cpm) per 20×10^6 sperm. Levels greater than 1×10^6 cpm in semen specimens were considered positive for ROS.

Western Blot Analysis for Cytochrome c and Caspase 9 and 3 Activities

Sperm pellet was washed twice in PBS, and cell lysate was prepared (1:1 [vol/vol]) in Quan's lysis buffer. This comprised of protease inhibitor cocktail (10 μ g/mL of aprotinin, 1 mM of phenylmethylsulfonyl fluoride, 50 μ g/mL of trypsin inhibitor, 5 mM of benzamidine) and lysis buffer (4 mM ethylenediamine tetraacetic acid, 1% NP-40, 20 mM HEPES, 10 μ g/mL).

After centrifugation, the supernatant was collected and protein content measured by Pierce BCA protein assay kit (Fisher Scientific, Pittsburgh, PA). Thirty micrograms of total lysate protein and 12 μ g of positive control (Jurkat whole-cell lysate for cytochrome c and caspase 3 [Santa Cruz Biotechnology, Inc., Santa Cruz, CA]), and untreated Hela cells for caspase 9 (Cell Signaling, Beverly, MA) were loaded and separated by one-dimensional SDS-PAGE before electrophoretic transfer to nitrocellulose.

Blots were incubated with anti-cytochrome C (1 μ g/mL), anti-caspase 9 (1:2,000), and anti-caspase 3 antibodies (1:200). Specific antibodies used for these apoptotic markers (caspases 3 and 9) by Western blot analysis detect only active forms. The cell signaling cleavage caspase 9 (Asp330) antibody (human specific) detects endogenous levels of large fragment (37 kDa with predomain 17kDa) of caspase 9 after cleavage at Asp330. This antibody does not recognize the uncleaved procaspase 9. We measured full length of active caspase 3 by using human-specific H277 antibody (Santa Cruz Biotechnology, Inc.) that reacts with p11, p17, and p20 subunits.

After incubation with peroxidase-conjugated anti-rabbit IgG (1:5,000 dilution) (Amersham Bioscience, Piscataway, NJ), the blot was washed with ECL Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ) and transferred on X-ray film. For cytochrome c data, the amount of expressed protein was quantified by scanning the blots by image densitometer IQmac (NIH Image 1.63, Scion Image

TABLE 1

Semen variables in donors and infertile patients.

Variable	Donors (n = 8)	Infertile patients (n = 35)	P value
Volume (mL)	2.10 (1.60, 3.00)	3.80 (3.00, 5.50)	.02
Round cells ($\times 10^6$ /ml)	1.26 (1.03, 1.65)	1.30 (0.60, 3.00)	.96
Concentration ($\times 10^6$ /mL)	56.90 (36.90, 116.20)	17.35 (9.20, 26.00)	.0001
Motility (%)	68.00 (52.00, 75.00)	37.00 (22.00, 57.00)	.004
Morphology by WHO criteria (%)	34.00 (33.00, 36.50)	25.00 (16.00, 31.00)	.0006
Morphology by Tygerberg's strict criteria (%)	9.00 (7.50, 13.00)	6.00 (3.00, 8.00)	.01

Note: Values are medians (25th, 75th percentile). WHO = World Health Organization.

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Corp., National Institutes of Health, Bethesda, MD), considering positive control as 1. For the caspase levels, we used 10 as the positive control value to compare the samples, since the positive control levels were greater than the semen samples. The Western blot pictures are representative blots from random patients and donors in the study.

Statistical Analysis

The Wilcoxon rank-sum test was used to compare semen analysis results in healthy donors and patients. $P < .05$ was considered significant. All summary statistics are presented as median (25th and 75th percentiles). All statistical analyses were performed by using SAS statistical software (SAS Institute, Cary, NC).

RESULTS

Semen Variables and Reactive Oxygen Species Levels

Table 1 shows results of classic semen analysis. Compared with donors, infertile patients had significantly poorer sperm concentration (56.9 [36.9, 116.2] vs. 17.35 [9.2, 26]; $P < .0001$) and reduced motility (37 [22, 57] vs. 68 [52, 75]; $P < .004$). Patients also had poorer sperm morphology than did donors.

Significantly higher levels of ROS were observed in patients (4.15×10^6 cpm [0.26, 40.16×10^6 cpm] compared with normal donors (0.06×10^6 cpm [0.02, 0.29]; $P < .01$).

Expression of Cytochrome c, Caspase 9, and Caspase 3

Protein was isolated from sperm, and Western blot analysis was performed. Compared with donors, patients had increased levels of cytochrome c (2.78 [2.21, 43.65] vs. 1.5 [1.25, 2.2]; $P < .01$) (Fig. 1A). Similarly, the representative Western blots from four patients show dense bands compared to the four bands from donors. The lowest cytochrome c activity was seen for the positive assay control composed of Jurkat whole-cell lysate.

Similarly, significantly higher levels of caspase 9 were seen in patients compared with donors (2.52 [0.9, 4.28] vs.

0.56 [0.32, 1.02]; $P < .006$) (Fig. 1B). The Western blots showed dense bands in the representative blots from three patients compared with the three bands representing the donors. The positive assay control consisted of HeLa cell whole-cell lysate.

Levels of caspase 3 were significantly higher in patients than donors (6 [4.85, 7.63] vs. 1.69 [1.66, 2.67]; $P < .01$) (Fig. 1C). The representative Western blots from four patients showed dense bands compared with the four bands representing the donors. The positive assay control consisted of Jurkat cell whole-cell lysate.

Apoptotic Factors, Semen Variables, and Reactive Oxygen Species

A negative correlation was observed between sperm motility, concentration, and sperm morphology (both by WHO and strict criteria) and levels of caspase 9 and 3 ($P < .05$). Levels of ROS were positively correlated with levels of cytochrome c ($r = .43$; $P < .03$), caspase 9 ($r = .56$; $P < .001$), and caspase 3 ($r = .65$; $P < .01$).

DISCUSSION

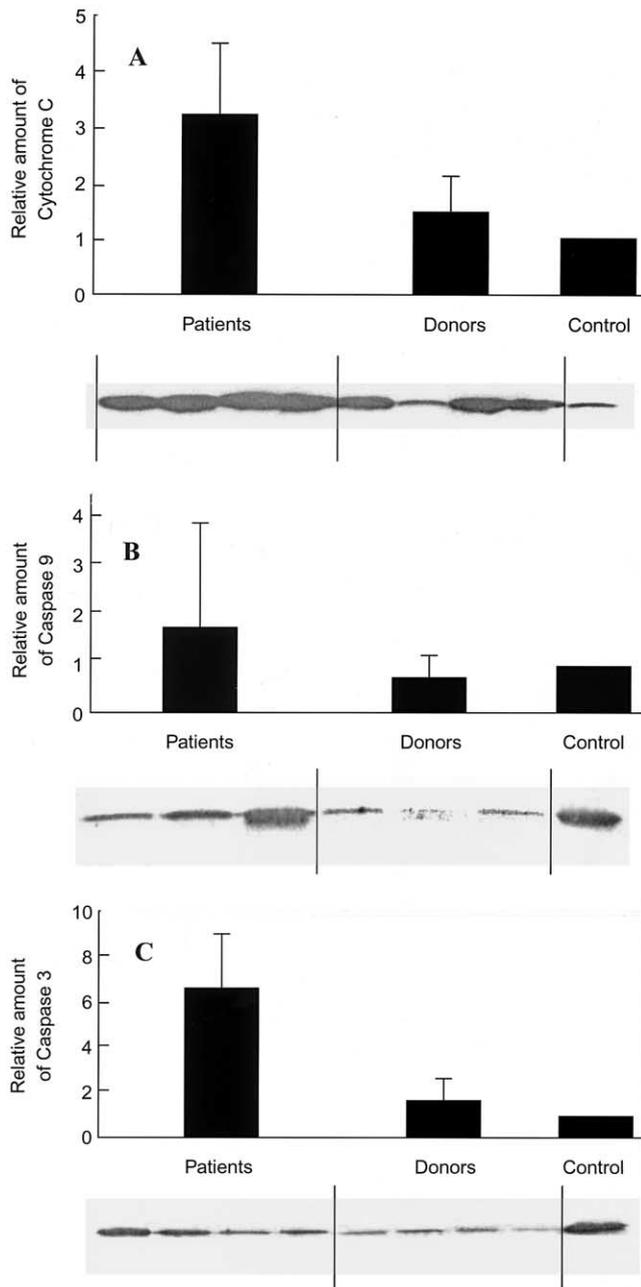
We observed elevated ROS levels in semen samples of infertile patients compared with normal donors. It is not clear how these high ROS levels, which reflect impaired mitochondrial function, ultimately lead to DNA damage.

Spermatozoa are rich in mitochondria. The integrity of mitochondria is established by the presence of cytochrome c in the inner membrane space. High cytochrome c levels in seminal plasma suggest significant mitochondrial damage by high ROS in the infertile men in our study. Release of series of such proteins from mitochondrial inner space is likely to accelerate the process of apoptosis, possibly leading to DNA damage. Early apoptosis cannot be detected by conventional semen parameters, but cells undergoing apoptosis experience externalization of phosphatidylserine (21).

In this preliminary study, we observed significantly elevated levels of cytochrome c in infertile patients. Considerable evidence exists that disruption of mitochondrial func-

FIGURE 1

Relative amounts of (A) cytochrome c, (B) caspase 9, and (C) caspase 3 in semen samples from infertile men and healthy donors, based on densitometry. Bars represent means (\pm SD). Below each histogram is a representative blot showing patients, donors, and assay controls. Assay controls represent Jurkat whole-cell lysate for cytochrome c and caspase 3 and untreated Hela cells for caspase 9.



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tions (e.g., loss of transmembrane potential, permeability transition, and release of cytochrome c leading to impaired electron transport) are important events in many apoptotic

cell deaths (22, 23). We also observed a concomitant decrease in mitochondrial membrane potential in infertile patients with elevated levels of reactive oxygen species compared with controls (24). Our results are also supported by a recent report describing activation of caspases in ejaculated spermatozoa (25). Our findings establish a role of apoptosis in ejaculated spermatozoa. It would be of interest to study caspase activity in immature and mature spermatozoa from these patients. Apoptotically labeled spermatozoa (annexin V) have been found in sperm fractions with both high and low motility (26), and can be found in spermatozoa with normal morphology (27, 28).

Conventional semen variables are not always sufficient in the assessment of sperm function and male infertility. In our attempt to link apoptosis (as an index of DNA damage) in spermatozoa with conventional semen variables, we observed a negative correlation between sperm concentration and motility and caspases 9 and 3. This finding confirmed those of other studies in which sperm motility was inversely correlated with apoptosis (16, 26, 29). Similarly, morphologic features of the sperm have been linked with apoptotic changes (30, 31).

Increased levels of caspase 9 and 3 were reported in spermatozoa from infertile patients examined after gradient separation into leukocyte-free fractions of high and low motility (32). Similar to our findings, caspases were demonstrated in ejaculated spermatozoa by using immunofluorescent microscopy, immunoblot analysis, and the fluorogenic substrate DEVD-afc. These findings contrast with those of Weil et al. (33), who reported that caspases may be lacking in ejaculated spermatozoa. When annexin V staining and DNA fragmentation were used, a significant positive correlation was seen between in situ active caspase-3 in the sperm midpiece and DNA fragmentation in the low motility fractions of patients, suggesting that caspase-dependent apoptotic mechanisms could originate in the cytoplasmic droplet or within mitochondria and function in the nucleus. These data suggest that in some ejaculated sperm, caspases are present and may function to increase PS translocation and DNA fragmentation.

Sakkas et al. (34) reported that DNA strand breaks and apoptotic markers did not exist together in the same mature spermatozoa. Ejaculated spermatozoa with apoptotic markers appear to have escaped programmed cell death in a process called *abortive apoptosis*. The presence of apoptotic proteins in the ejaculated spermatozoa may be linked to defects in cytoplasmic remodeling during later stages of spermatogenesis (34, 35). Therefore, it is important to identify spermatozoa that are positive for apoptotic markers. It is inappropriate to assume that DNA strand breaks are synonymous with apoptotic degradation (7).

In conclusion, we found a positive relationship between increased sperm damage, as indicated by increased oxidative stress, and caspase-mediated apoptosis in patients with male

factor infertility. The significant positive correlation of ROS with cytochrome c and caspases 9 and 3 suggests possible DNA damage through increased ROS production; however, we did not measure DNA damage. Likewise, we examined caspase-mediated apoptosis in washed, non-leukocytospermic ejaculated spermatozoa; identification of the extent and location of the site of apoptosis in immature and mature ejaculated spermatozoa is also important. Antioxidants aimed at reducing ROS production (during spermatogenesis, sperm storage or transit in the genital tract, or infection) may play a role in decreasing apoptosis and thus improving sperm quality and reducing DNA damage.

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