

# Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay

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**Objective:** To determine DNA damage as measured by the sperm chromatin structure assay (SCSA) in subsets of human spermatozoa at different stages of maturation in patients who are undergoing infertility evaluation.

**Design:** Prospective study.

**Setting:** Andrology laboratory at a tertiary care hospital.

**Patient(s):** Fifty-six patients undergoing infertility evaluation. Patients with normal semen parameters (n = 17), abnormal semen parameters (n = 29), leukocytospermia (n = 10), and a group of healthy fertile men (n = 18) were included in the study.

**Intervention(s):** None.

**Main Outcome Measure(s):** The shift of green (native DNA) to red (denatured, single-stranded DNA) fluorescence was measured and quantified using the expression  $\alpha_1$  (red fluorescence/[red + green fluorescence] per cell). Sperm DNA damage was examined in subsets of spermatozoa isolated by a three-step density gradient. The DNA damage was correlated with classic semen characteristics.

**Result(s):** Leukocyte concentration in semen was directly correlated with chromatin alterations in immature and mature sperm. Leukocyte concentration in semen was also directly correlated with immature germ cell concentration and the percentage of abnormal forms in semen.

**Conclusion(s):** The increase in chromatin alterations and DNA damage in sperm, as defined by the sperm chromatin structure assay from leukocytospermic samples may be related to alterations in the regulation of spermatogenesis. (Fertil Steril® 2002;78:319–29. ©2002 by American Society for Reproductive Medicine.)

**Key Words:** Leukocytes, spermatozoa, ROS, chromatin, SCSA, sperm maturation, ISolate gradient

A relatively large number of men attending fertility clinics exhibit leukocytospermia without any symptoms from their genital organs (1). Leukocytes are present in most ejaculates and are thought to play an important role in immunosurveillance (2, 3) and phagocytic clearance of abnormal sperm (4). Increased concentrations of leukocytes in semen provide an important clinical indicator of genital tract infection or inflammation (5). Males with symptomatic genital tract infections usually have leukocytospermia (6). However, leukocytospermia is not a reliable index of asymptomatic urogenital infection (7), and the relation-

ship between leukocytospermia and infection remains poorly defined. Prostatic secretions can contain high levels of leukocytes in cases of prostatitis (6). High levels of leukocytes have also been observed in the semen of vasectomized men with presumed subclinical prostatitis (8).

Leukocytospermia has been reported to be associated with an increase in immature germ cell concentration (4, 9) and abnormal sperm morphology (10–12). Although several reports have also found an association among leukocytospermia, a decrease in sperm concentration (10, 13), and sperm motility (14, 15) in semen,

other reports have not found any significant differences in these semen parameters (4, 16–17).

Leukocyte activation may be key to a relationship between inflammation and infertility (18). Cytokines affect Sertoli cell function, and alterations in cytokine levels in the testis could affect spermatogenesis (19). It has been proposed that one of the mechanisms by which leukocytospermia could lead to sperm dysfunction is related to sperm damage induced by reactive oxygen species (ROS) via activated leukocytes during or after ejaculation (14, 17, 20, 21). Another potential mechanism by which leukocytes could affect sperm function could be related to ROS-induced cross-damage of sperm by leukocytes during comigration from the seminiferous tubules to the epididymis. Nevertheless, definite evidence for the presence of significant numbers of leukocytes in the seminiferous tubules or epididymis from males with leukocytospermia is still lacking. Despite numerous reports showing an association between the presence of leukocytes in semen and sperm dysfunction, it still remains controversial whether leukocytospermia plays a role in the pathogenesis of male infertility. And perhaps one of the main hurdles in finding a definite answer to this question is the large heterogeneity in terms used to describe the condition, leukocyte subtypes in semen, and the magnitude of leukocytospermia.

In sperm, DNA damage has been reported as an indicator of alterations in the regulation of spermatogenesis and of poor pregnancy outcome (22–28). The increased sensitivity to DNA damage in abnormal spermatozoa is probably due to failed chromatin condensation, which makes the DNA more accessible to damage (29–31). An episode of influenza in a healthy male can result in an increase in DNA damage in sperm produced during that spermatogenic cycle; this DNA damage subsides after resolution of the influenza episode (32). Presence of DNA fragmentation in ejaculated spermatozoa has also been suggested to correlate with defects in spermatogenesis (33).

The objectives of this study were to determine DNA damage in subsets of human spermatozoa at different stages of maturation, as isolated by density gradient centrifugation of semen samples from males with leukocytospermia; to evaluate the standard semen parameters in patients with normal and abnormal semen parameters and patients with leukocytospermia as compared with healthy donors; and finally to determine ROS values in subsets of human spermatozoa and their role in DNA damage among individuals with leukocytospermia compared with healthy donors.

## MATERIALS AND METHODS

### Semen Samples

Following institutional review board approval, we collected semen samples from 56 male patients who were undergoing infertility screening and 18 normozoospermic

healthy donors (not of proven fertility in all cases). Leukocytospermia was defined as semen samples with a leukocyte concentration of greater than 1 million/mL (34).

All specimens were collected by masturbation at the clinical andrology laboratory after an abstinence period of 48 to 72 hours. After liquefaction, semen analysis was performed using a computer-assisted semen analyzer (Cell-Trak, version 4.24, Motion Analysis Corporation, Palo Alto, CA) (35) to measure sperm concentration, percent motility, and motion characteristics. Smears were prepared for the assessment of sperm morphology. Myeloperoxidase staining (36) 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 (WHO) criteria. Specimens with a sperm count of <20 million/mL, sperm motility of <50% (34), or percent normal forms of <14% (37) were considered abnormal.

### Density Gradient Centrifugation

Aliquots of 0.5 to 1.0 mL of the liquefied semen were loaded onto a 47%, 70%, and 90% discontinuous ISolate gradient (Irvine Scientific, Santa Ana, CA) prepared in Biggers-Whitten-Whittingham medium (BWW) and centrifuged at  $500 \times g$  for 20 minutes 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 bright-field microscopy of the stained slides.

Sperm suspensions from the different ISolate fractions were diluted in one volume of BWW and centrifuged at  $500 \times g$  for 7 minutes. The pellet was then resuspended in 1 mL of BWW and an aliquot used to determine the total number of spermatozoa and round cells. It is noteworthy that the term “leukocyte” used throughout this text only refers to neutrophils, macrophages, eosinophils, and basophils, as the myeloperoxidase assay does not identify lymphocytes or monocytes. In general, all other cell types that were not accounted for by the myeloperoxidase test were included as immature germ cells, as determined by standard histochemical analysis. Aliquots from each fraction were examined for sperm concentration, percentage of motility by CASA, sperm morphology, leukocyte concentration, ROS production, and DNA integrity by SCSA.

### 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) as the probe (38). Measurements were

made using a Berthold luminometer (Autolumat LB 953, Wallac Inc., Gaithersburg, MD). Ten microliters of 5-mM luminol prepared in dimethylsulfoxide (Sigma Chemical) was added to 400  $\mu$ L of the washed sperm suspension. The chemiluminescent signal was monitored for 15 minutes and results were expressed as  $\times 10^6$  counted photons per minute (cpm)/20  $\times 10^6$  spermatozoa.

Despite different ISolate fractions potentially being negative for the presence of leukocytes based on the myeloperoxidase test, it is still possible that small numbers of leukocytes (<50,000/mL) could be contaminating these fractions. To rule out the presence of leukocytes, the different fractions were incubated with 25-mM luminol supplemented with 12.4 U of horseradish peroxidase (Type VI, 310 U/mg; Sigma Chemical) for 5 minutes to sensitize the assay for the generation of extracellular hydrogen peroxide. The signal was monitored for 5 minutes to determine the magnitude of the peak chemiluminescence response and to allow the system to return to baseline (39). The sperm suspensions were then stimulated with 0.2-mM FMLP and the signal monitored for 15 minutes to assess the residual capacity of the cell population for ROS generation (i.e., FMLP specifically stimulates receptors present in leukocytes only, but absent in spermatozoa).

### Sperm Morphology

Smears of whole semen (raw) and from the different ISolate fractions (fractions 1 to 4) were prepared for sperm morphology assessment. The smears were fixed and stained using the Diff-Quik kit (Allegiance Healthcare Corporation, Inc., McGaw Park, IL). Immediately after staining, the smears were rinsed in distilled water and air dried. Smears were scored for sperm morphology using strict criteria (37).

### Sperm Chromatin Structure Assay

The sperm chromatin structure assay (SCSA) was carried out as previously described (40). Frozen semen aliquots obtained from raw and different fractions (fraction 1 to 4) from controls, from infertile patients, and from patients with leukocytospermia were thawed in a 37°C water bath, and immediately diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.4) to 1 to 2  $\times 10^6$  sperm cells/mL. Four hundred  $\mu$ L of the acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2) was admixed with 200  $\mu$ L of the diluted sample. After 30 seconds, spermatozoa were stained by adding 1.20 mL of acridine orange (AO) staining solution containing 6  $\mu$ g/mL of AO (chromatographically purified; Cat. no. 04539, Polysciences Inc., Warrington, PA) per mL buffer [0.037 M citric acid; 0.126 M Na<sub>2</sub>HPO<sub>4</sub>; 0.0011 M EDTA (disodium), 0.15 M NaCl, pH 6.0] (40, 41). Immediately afterward, the sample was placed on the flow cytometer for 2.5 minutes to allow for hydrodynamic and stain equilibrium before data were collected on 7,000 cells per sample (Ortho Diagnostic Inc., Westwood, MA) 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).

The extent of DNA denaturation was quantified by the calculated parameter  $\alpha_t$  [ $\alpha_t = \text{red}/(\text{red} + \text{green})$  fluorescence] (41). Sperm populations with normal chromatin structure produce a narrow  $\alpha_t$  distribution and have a small mean  $\alpha_t$  and ( $X\alpha_t$ ); the standard deviation of  $\alpha_t$  ( $SD\alpha_t$ ) describes the extent of chromatin structure abnormality within a population; and  $COMP\alpha_t$  is the percentage of cells outside the main population for  $\alpha_t$  (i.e., percentage of cells with denatured DNA) (26, 42). The DNA in spermatozoa with abnormal chromatin structure has increased red fluorescence, which yields an  $\alpha_t$  distribution that is usually broader, having a higher mean channel ( $X\alpha_t$ ), and a larger percentage of cells outside the main population of cells ( $COMP\alpha_t$ ).

It is noteworthy that the SCSA described herein measures 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 (43–45). Therefore, the designation “DNA damage” or “damaged DNA” used throughout the text refers to this susceptibility to DNA strand breaks (26). The SCSA is primarily a measure of DNA integrity and, secondarily, provides information on the extent of chromatin condensation and/or proper association of DNA with mature sperm nuclear proteins.

### Statistical Analysis

The Student *t*-test (for normally distributed variables) and Wilcoxon's rank sum tests (for non-normal variables) were used to compare groups and fractions. Coefficients of correlation were calculated using Spearman's rank correlation analysis. These correlations were considered clinically meaningful at the  $r > 0.1$ . The sample size in this study is sufficient to determine whether the correlation is significantly greater than the null hypothesis of  $r = 0.1$  (alternative hypothesis,  $r > 0.05$ ) with 90% power. All hypothesis tests were two-tailed with statistical significance assessed at the  $P < .05$  level. Statistical computations were calculated using SPSS 10 for Windows software (SPSS Inc, Chicago, IL).

## RESULTS

Based on the results of semen analysis, patients were assigned to three groups: [1] normal semen parameters ( $n = 17$ ), [2] abnormal semen parameters ( $n = 29$ ), and [3] leukocytospermia ( $n = 10$ ).

### Leukocyte and Immature Germ Cell Concentration

Patients with leukocytospermia presented a wide range of values for the semen parameters analyzed, which included both normal and abnormal scores. Sperm concentration in leukocytospermic samples ranged from 0.6 to 104.7  $\times$

**TABLE 1**

Semen parameters in leukocytospermic samples.

Sample no.	Sperm concentration (×10 <sup>6</sup> /mL)	Motility (%)	Morphology Kruger's strict criteria (%)	Leukocyte concentration (×10 <sup>6</sup> /mL)
1	35.60	53.0	6	2.60
2	0.60	42.3	0	18.80
3	48.80	67.7	9	2.80
4	32.80	62.5	7	1.20
5	66.20	71.3	6	11.90
6	32.20	44.0	3	9.40
7	5.43	24.5	0	18.90
8	13.60	83.5	0	4.00
9	46.70	62.0	3	6.20
10	104.70	72.0	5	5.00

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10<sup>6</sup>/mL and motility from 24.5% to 83.5%. Leukocyte values in these samples ranged between 1.2 to 18.9 × 10<sup>6</sup>/mL. Sperm morphology by Kruger's strict criteria was zero in three patients and ranged from 3% to 9% in the remaining patients (Table 1).

Leukocytes achieved their isopycnic separation (based on differences in their density) in fraction 2 following ISolate density gradient centrifugation of leukocytospermic samples. The mean percent recovery of leukocytes in fraction 2, as measured by the myeloperoxidase assay, was 96% ± 4.5%, with values ranging between 92% and 99%. The mean percent recovery of leukocytes in fraction 1 was 4% (range zero to 8%). No leukocytes were observed in fractions 3 or 4 by the myeloperoxidase assay. Leukocyte concentration in semen was directly correlated with immature germ cell concentration ( $r^2 = 0.69$ ) ( $P=.003$ ) and inversely correlated with the recovery of mature sperm in fraction 4 ( $r^2 = 0.81$ ;  $P <.0001$ ).

The concentration of immature germ cells (myeloperoxidase negative) was significantly higher in leukocytospermic samples ( $5.5 \pm 2.51 \times 10^6$ ) compared with samples obtained from either healthy donors ( $0.5 \pm 0.02 \times 10^6$ ) or patients with normal ( $0.61 \pm 0.08 \times 10^6$ ) or abnormal semen parameters ( $1.8 \pm 0.2 \times 10^6$ ) ( $P <.0001$ ).

Mean sperm density in raw samples and in different fractions for each study group is shown in Table 2. Significant differences were seen in sperm density in raw specimens from donors versus patients with abnormal semen parameters ( $P=.009$ ) and donors versus leukocytospermic group ( $P=.03$ ). Following separation on density gradient, significant differences were seen in fraction 1 between donors and patients with normal semen parameters ( $P=.0005$ ), donors and patients with abnormal semen parameters ( $P=.002$ ) and between patients with normal semen parameters and leukocytospermic group ( $P=.017$ ). Similarly significant differences were seen in sperm density in fraction 3 and fraction 4 between donors and patients with abnormal semen parameters ( $P=.01$  and  $.0007$ ) and between donors and leukocytospermic group ( $P=.018$  and  $.0001$ ).

**Motility and Morphology**

Percent sperm motility and morphology in raw semen and in the different ISolate fractions from the different groups of samples are shown in Figures 1 and 2, respectively. Motility was significantly lower in sperm from fractions 1 and 2 compared with sperm from fractions 3 and 4 ( $P <.01$ ). The mean percent recovery of sperm in the different ISolate fraction is shown in Table 3.

**ROS Production**

Significant differences were seen in the ROS production in different fractions between donors and patients with abnormal semen parameters and between patients with normal versus abnormal semen parameters. The ROS production in the different study groups is shown in Table 4. In patients

**TABLE 2**

Mean sperm density in raw sample and in different ISolate fractions.

Patients	Raw	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Donors (n = 18)	82.8 (31.3, 127.2)	15.0 (9.9, 27.7)	15.3 (10.5, 23.2)	16.5 (12.4, 28.5)	25.8 (15.6, 33.9)
Patients with normal SP (n = 17)	49.2 (32.4, 84.0)	3.9 (2.8, 8.1)	8.5 (3.6, 18.3)	12.6 (9.7, 15.8)	11.6 (7.7, 25.1)
Patients with abnormal SP (n = 29)	17.1 (14.4, 21.8)	4.5 (3.1, 8.5)	8.3 (6.7, 9.6)	7.5 (4.2, 11.6)	4.3 (3.4, 6.8)
Leukocytospermia (n = 10)	39.6 (32.4, 48.3)	12.1 (5.2, 25.3)	11.7 (8.3, 23.7)	8.1 (3.9, 12.1)	5.9 (4.8, 10.4)

Note: Values represent the median and interquartile (25% and 75%) values; SP = semen parameters.

In the raw sample, statistically significant differences were found between donors and patients with abnormal SP ( $P=.009$ ) and donors versus leukocytospermic patients ( $P <.03$ ): In patients with normal SP, 1 vs. 2 ( $P=.02$ ); 1 vs. 3 ( $P=.002$ ); 1 vs. 4 ( $P=.0006$ ).

● Statistically significant differences in fraction 1 between donors and patients with normal SP ( $P=.0005$ ); donors versus patients with abnormal SP ( $P=.002$ ); patients with normal SP versus leukocytospermia ( $P=.017$ ).

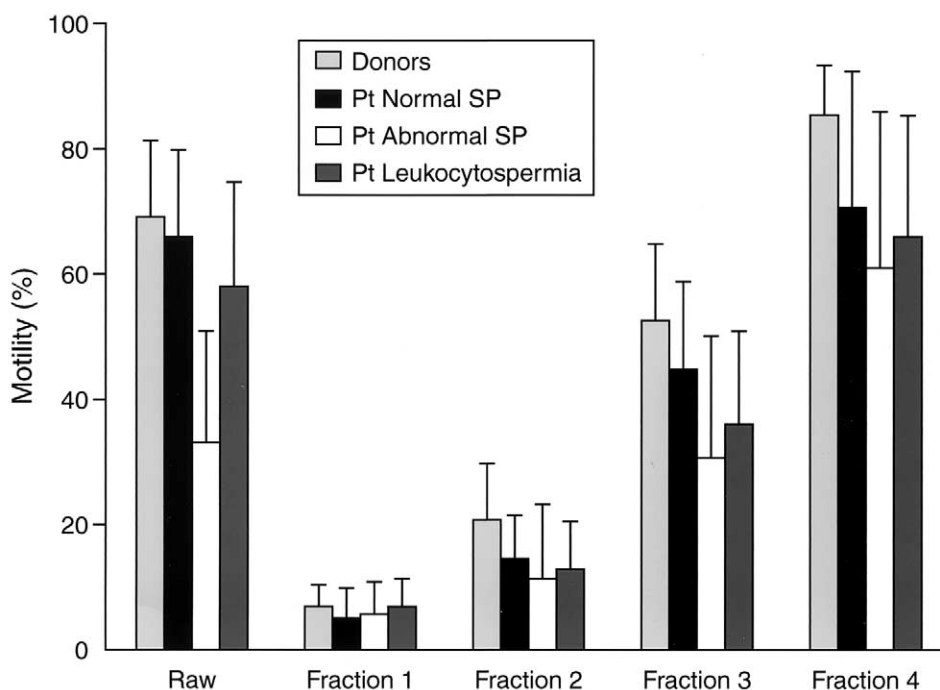
● Statistically significant differences in fraction 3 between donors and patients with abnormal SP ( $P=.01$ ); donors versus leukocytospermia ( $P=.018$ ).

● Statistically significant differences in fraction 4 between donors and patients with abnormal SP ( $P=.0007$ ); donors versus leukocytospermia ( $P=.0001$ ); patients with normal SP versus leukocytospermia ( $P=.04$ ).

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**FIGURE 1**

Percentage of motility in semen and the different ISolate fractions. Values represent the mean and the standard deviations.



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with abnormal semen parameters, ROS level ( $\log_{10}$  counted photons per minute) was highest in fraction 2 ( $6.93 \pm 0.85$ ) and lowest in fraction 4 ( $5.25 \pm 0.93$ ). Similarly, in the leukocytospermic group, ROS production was highest in fraction 2 ( $7.64 \pm 0.66$ ) and lowest in fraction 4 ( $5.92 \pm 1.27$ ). Differences in ROS production between sperm from fractions 1 and 2 ( $7.64 \pm 0.66$  and  $7.99 \pm 0.85$ ) were statistically significant ( $P < .001$ ). Differences in ROS values between sperm from fractions 2 and 3 ( $7.98 \pm 0.85$  vs.  $6.99 \pm 0.99$ ) ( $P = .03$ ) and 2 and 4 ( $7.99 \pm 0.85$  vs.  $5.92 \pm 1.38$ ) were statistically significant ( $P < .002$ ).

The ROS levels in fraction 2 were highly correlated with leukocyte concentration in this fraction ( $r^2 = 0.64$ ;  $P = .004$ ). No significant correlation was found between ROS levels in fraction 1 and leukocyte concentration in this fraction.

ROS production in spermatozoa from the different ISolate fractions in samples obtained from patients in response to FMLP did not result in any significant increase in ROS production, confirming the absence of leukocytes in these fractions. In patients with leukocytospermia, ROS levels in fractions 3 and 4 were not significantly different before or after FMLP stimulation ( $P = .45$ ) indicating that this fraction was not contaminated with leukocytes. The FMLP stimulation of fraction 2 from leukocytospermic samples resulted in a robust increase in ROS production ( $9.5 \pm 1.4$ ) compared

with the unstimulated control ( $7.98 \pm 0.77$ ) ( $P = .002$ ). Differences in mean ROS values in fraction 1 before and after FMLP stimulation were not statistically significant ( $P = .38$ ), although samples with a leukocyte concentration in semen  $> 6 \times 10^6/\text{mL}$  showed an increase in FMLP-stimulated ROS production in this fraction.

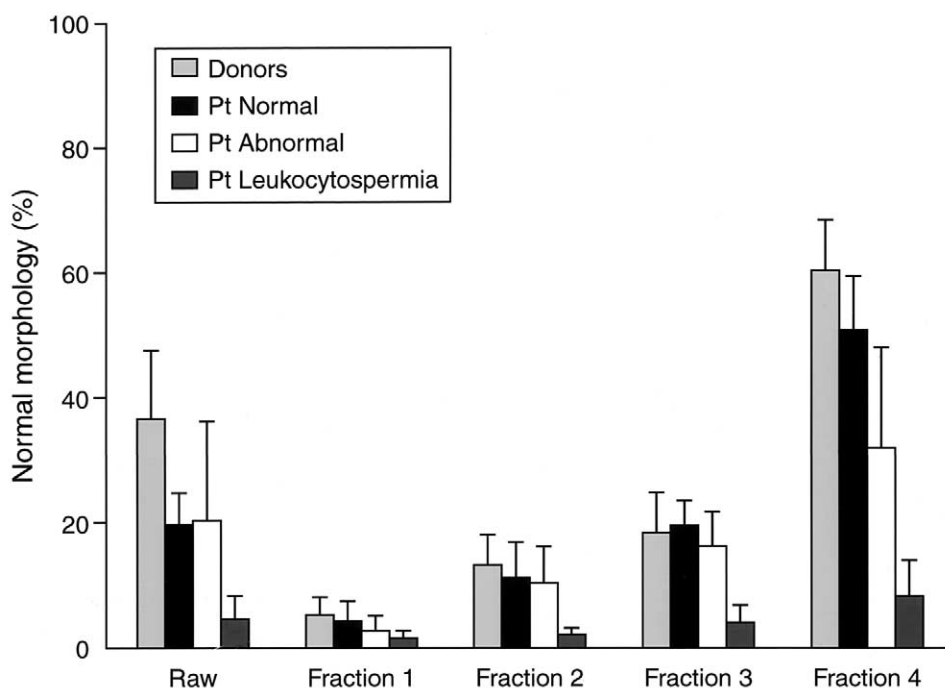
### SCSA-Defined DNA Damage

In the raw semen, the mean %COMP $\alpha_t$  values, which indicate the extent of chromatin structure abnormality, were comparable in donors ( $24.9\% \pm 10.2\%$ ) and patients with normal semen parameters ( $24.9\% \pm 9.6\%$ ) but were significantly different from patients with abnormal semen samples ( $34.5\% \pm 9.5\%$ ;  $P < .01$ ) and patients with leukocytospermia ( $39\% \pm 10.9\%$ ;  $P < .01$ ). No statistically significant differences were found in %COMP $\alpha_t$  values in raw semen from patients with abnormal semen parameters versus leukocytospermic samples (Fig. 3).

The mean %COMP $\alpha_t$  values in sperm from different ISolate fractions are shown in Figure 4. The highest values were observed in sperm from fraction 1 and the lowest in sperm from fraction 4 in sperm samples from all groups. Differences in %COMP $\alpha_t$  values in spermatozoa from fraction 4 in sperm samples obtained from either donors ( $9.0\% \pm 7.1\%$ ) or patients with normal semen parameters ( $18.5\% \pm 9.9\%$ ) were statistically significant ( $P < .03$ ). The mean

**FIGURE 2**

Percentage of normal morphology in semen and the different I isolate fractions. Values represent the mean and the standard deviations.



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%COMP $\alpha_t$  value in spermatozoa from fraction 4 from samples obtained from patients with abnormal semen parameters was 27.6%  $\pm$  8.2%. COMP $\alpha_t$  values in sperm from fraction 4 from leukocytospermic samples (31%  $\pm$  12.2%) were not significantly different from those with abnormal semen parameters ( $P=.02$ ).

The %COMP $\alpha_t$  values in sperm from fraction 4 were highly correlated with relative ROS levels in fraction 2 in samples from both donors ( $r^2 = 0.75$ ;  $P=.002$ ) or patients with or without leukocytospermia ( $r^2 = 0.79$ ;  $P < .001$ ). No significant correlation was found between relative ROS levels in fraction 2 and COMP $\alpha_t$  values in fractions 1 and 2 in

**TABLE 3**

Percent sperm recovery in the different isolate fractions.

Patients	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Donors (n = 18)	16.7 $\pm$ 8.1	17.2 $\pm$ 8.2	24.7 $\pm$ 8.7	32.9 $\pm$ 9
Patients with normal SP (n = 17)	11.6 $\pm$ 10	20.1 $\pm$ 7.8	30.0 $\pm$ 9.4	33.0 $\pm$ 8
Patients with abnormal SP (n = 29)	19.4 $\pm$ 10	27.7 $\pm$ 11.0	24.2 $\pm$ 9.8	20.2 $\pm$ 8
Leukocytospermia (n = 10)	36.3 $\pm$ 12	30.1 $\pm$ 8.0	16.7 $\pm$ 6.2	13.6 $\pm$ 5

Note: Values represent the percent recovery of sperm in each fraction compared to the total number of sperm loaded onto the gradient and are expressed as the mean  $\pm$  SD; SP = semen parameters.

- Donors: 1 vs. 3 ( $P=.012$ ); 1 vs. 4 ( $P < .0001$ ); 2 vs. 3 ( $P=.018$ ) and 2 vs. 4 ( $P < .0001$ ).
- Patients with normal SP: 1 vs. 2 ( $P=.03$ ); 1 vs. 3 ( $P < .0001$ ); 2 vs. 3 ( $P=.04$ ); 2 vs. 4 ( $P < .004$ ).
- Patients with abnormal SP: 1 vs. 2 ( $P=.03$ ); 2 vs. 4 ( $P < .04$ ).
- Leukocytospermic samples: 1 vs. 3, 1 vs. 4, 2 vs. 3, 2 vs. 4 ( $P < .0001$ ).

No statistically significant differences were seen in any of the fractions between donors and patients with normal SP. Statistically significant differences were seen in fraction 4 between patients with abnormal SP or leukocytospermia vs. donors or patients with normal SP ( $P < .01$ ). Statistically significant differences were seen in fraction 1 in patients with leukocytospermia vs. donors or patients with either normal or abnormal semen parameters.

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**TABLE 4**

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

Subjects	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Donors (n = 18)	5.51 ± 0.74	5.96 ± 0.78	4.63 ± 1.26	3.81 ± 1.26
Patients with abnormal SP (n = 29)	6.28 ± 0.59	6.53 ± 0.85	5.86 ± 0.89	5.25 ± 0.93
<i>P</i> values (donors and patients abnormal SP)	.009	.0502	.01	.003
Patients with normal SP (n = 17)	5.07 ± 1.80	5.69 ± 1.37	4.63 ± 1.46	4.22 ± 1.29
<i>P</i> values (patients with normal vs. abnormal SP)	.016	.007	.007	.015
Leukocytospermia (n = 10)	7.64 ± 0.66	7.99 ± 0.85	6.99 ± 0.99	5.92 ± 1.38
<i>P</i> values (donors vs. patients with leukocytospermia)	.0003	.0007	.001	.006
<i>P</i> values (patients with abnormal SP and leukocytospermia)	.001	.001	.01	.21

Note: Values are expressed as log<sub>10</sub> of ROS concentrations; SP = semen parameters.

- Statistically significant differences were seen in different fractions in donors: 1 vs. 3 (*P* = .02); 1 vs. 4 (*P* < .0001); 2 vs. 3 (*P* < .001); 2 vs. 4 (*P* < .0001).
- Statistically significant differences were seen in different fractions in patients with abnormal SP: 1 vs. 2 (*P* = .017); 1 vs. 4 (*P* < .0001); 2 vs. 3 (*P* = .002); 2 vs. 4 (*P* < .0001).
- Statistically significant differences were seen in different fractions in patients with normal SP: 2 vs. 3 (*P* = .04); 2 vs. 4 (*P* < .004).
- Statistically significant differences were seen in different fractions in leukocytospermia: 1 vs. 3 (*P* < .01); 1 vs. 4 (*P* < .002); 2 vs. 3 (*P* = .004); 2 vs. 4 (*P* < .002); 3 vs. 4 (*P* < .002).

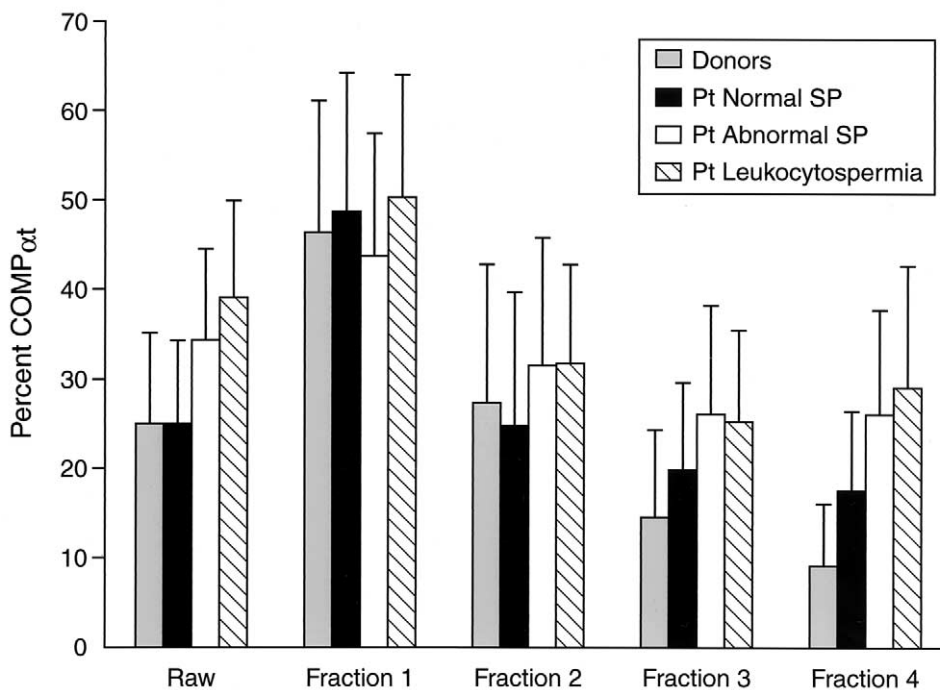
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samples obtained from donors and patients with normal or abnormal semen parameters. In contrast, leukocyte concentration in semen from leukocytospermic samples was significantly correlated with COMP<sub>αt</sub> values in all fractions (Figs. 4A–D).

No significant correlation was found between the age of the semen sample and sperm COMP<sub>αt</sub> values following incubation at room temperature for up to 4 hours in samples obtained from donors, patients, or leukocytospermic males.

**FIGURE 3**

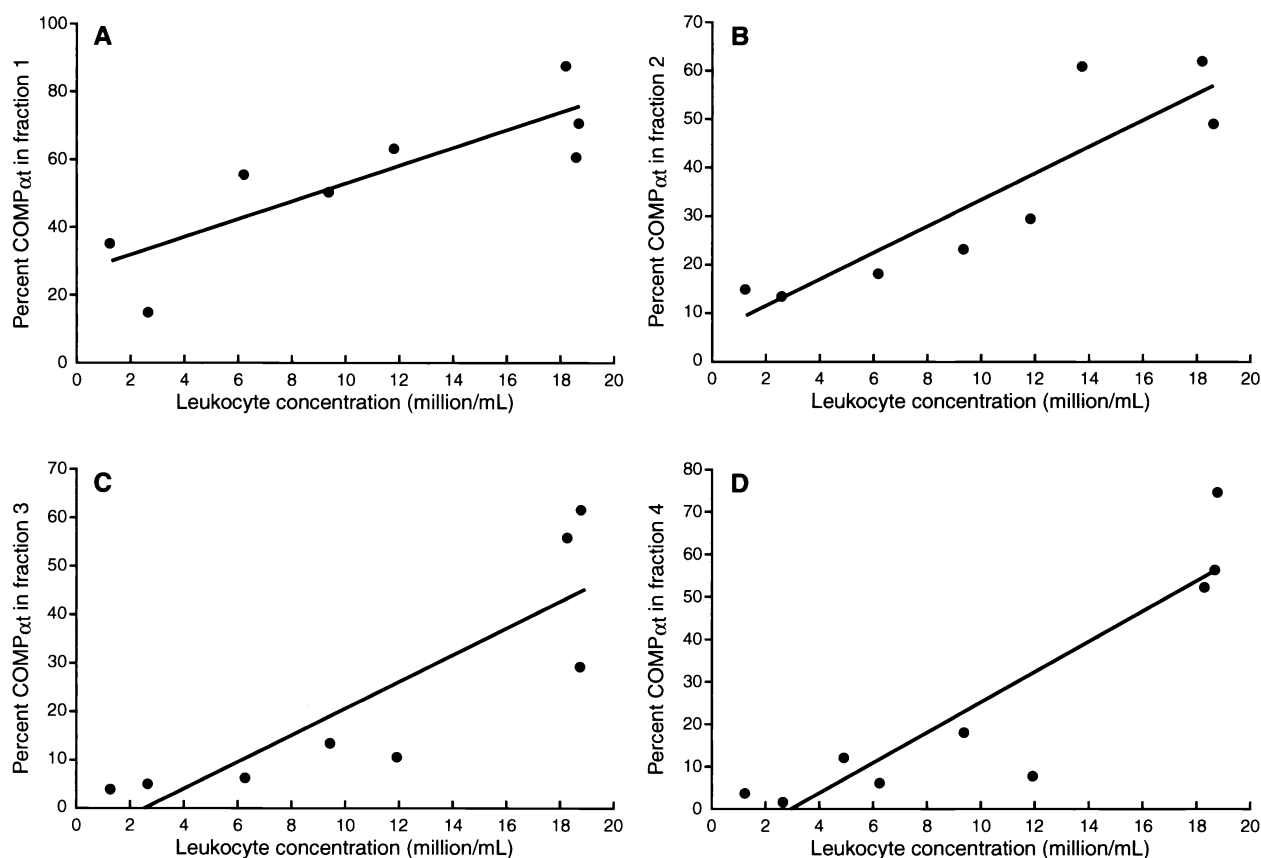
COMP<sub>αt</sub> values in the different ISolate fractions in samples obtained from leukocytospermic samples. Values represent the mean of at least 16 different samples per group. Error bars are the standard deviations.



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**FIGURE 4**

Correlation between leukocyte concentration in semen and COMP<sub>α<sub>t</sub></sub> values in sperm from the different ISolate fractions from leukocytospermic samples. (A), Fraction 1 ( $r^2 = 0.71$ ;  $P = .009$ ). (B), Fraction 2 ( $r^2 = 0.79$ ;  $P < .0001$ ). (C), Fraction 3 ( $r^2 = 0.73$ ;  $P = .007$ ). (D), Fraction 4 ( $r^2 = 0.82$ ;  $P = .002$ ).



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## DISCUSSION

In this study, after separation on a density gradient, we analyzed semen samples from normal healthy donors, infertile patients with normal and abnormal semen parameters, and patients with leukocytospermia. Immature germ cells, leukocytes, and morphologically abnormal and immature spermatozoa were separated from mature morphologically normal and highly motile spermatozoa by density gradient centrifugation into four different fractions. Our results show that the percentage of normal forms was significantly lower in sperm from fractions 1, 2, and 3 compared with sperm from fraction 4 ( $P < .01$ ), which is also consistent with previous reports (46). The percentage of normal forms was significantly lower in all fractions from patients with leukocytospermia compared with donors and patients with normal or abnormal semen parameters ( $P < .001$ ).

The ROS production found in leukocytospermic samples

was higher than those reported for semen samples obtained from donors or patients with normal or abnormal semen parameters. Differences in ROS levels in the different fractions in samples from patients with abnormal semen parameters and leukocytospermic samples were only statistically significant for fraction 2 ( $P < .002$ ). In our earlier publication (47), we demonstrated significant cell-to-cell variation in ROS production in subsets of spermatozoa at different stages of maturation. We suggest 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.

The results of this study indicate that there is a significant increase in DNA damage in sperm at different stages of maturation from leukocytospermic samples. In addition, leukocytospermia was directly correlated with an increase in immature germ cell concentration and abnormal sperm mor-



phology and inversely correlated with the recovery of mature sperm. The high levels of ROS production and DNA damage observed in immature spermatozoa may be indicative of derangements in the regulation of spermiogenesis. Also, DNA damage in mature spermatozoa may be the result of oxidative damage by immature spermatozoa producing ROS during sperm migration from the seminiferous tubules to the epididymis (48).

Four main hypotheses can be postulated to explain this observation: [1] DNA damage occurs before spermiation and is the result of alterations in the regulation of spermatogenesis; [2] DNA damage occurs after spermiation and is the result of cross-damage of sperm by leukocytes producing ROS during comigration from the seminiferous tubules to the epididymis; [3] a combination of hypothesis 1 and 2; or [4] DNA damage occurs after ejaculation and is the result of cross-damage of sperm by ROS-producing leukocytes in semen.

The first hypothesis is consistent with our findings that show that DNA damage was observed in subsets of human spermatozoa at different stages of maturation. This hypothesis is also consistent with the increase in immature germ cell concentration and abnormal sperm morphology observed in these samples. The fact that sperm concentration in leukocytospermic samples was within normal limits suggests that a defect in spermiogenesis rather than in the entire process of spermatogenesis may be responsible for the observed increase in DNA fragmentation and abnormal sperm morphology. These findings prompted the following question: what is the relationship between leukocytospermia and abnormal spermiogenesis?

One potential explanation is that leukocytospermia could be a marker for an inflammatory process in the testis and in most cases would be related to a subclinical inflammatory process and not due to an overt epididymo-orchitis. The presence of proinflammatory mediators in the testis could lead to alterations in the regulation of spermiogenesis. In fact, cytokines have been found to interfere with Sertoli cell function leading to abnormal spermiogenesis (19).

This may explain why immature germ cells—including spermatocytes and round spermatids—and mature and immature sperm from leukocytospermic samples have a significant increase in DNA damage compared with those of healthy donors or patients with leukocyte-free samples. Perhaps leukocytospermia may be indicative of an acute or chronic inflammatory process in the testis that would lead to alterations in the regulation of spermatogenesis and to an increase in sperm DNA damage.

The second hypothesis postulates that DNA damage in sperm occurs after spermiation and is the result of ROS-induced cross-damage of sperm by leukocytes during comigration from the seminiferous tubules to the epididymis. This hypothesis would also explain the increase in DNA damage

observed in sperm from all the different ISolate fractions. Although the presence of the blood–testis barrier would preclude leukocytes from entering the lumen of the seminiferous tubules or epididymis, it is still possible that, at least in some cases, damage of this barrier could lead to leakage of leukocytes into the lumen of the seminiferous tubules or epididymis (49).

The third hypothesis postulates that DNA damage is the result of both an alteration in the regulation of spermatogenesis and of cross-damage of sperm by ROS-producing leukocytes during comigration from the seminiferous tubules to the epididymis. This hypothesis would most likely apply to those situations in which there is an acute infectious or inflammatory process in the testis that could damage the blood–testis barrier—resulting in leakage of leukocytes into the seminiferous tubules or epididymis—and at the same time alter the regulation of spermatogenesis.

The fourth hypothesis postulates that DNA damage occurs after ejaculation and is the result of cross-damage of sperm by ROS-producing leukocytes in semen. Because contact time of sperm with leukocytes during ejaculation is very short, it is unlikely that this mechanism would be of any significance *in vivo*. This mechanism could still be of potential significance during processing of semen samples *in vitro*, especially in those samples where there is a decrease in total antioxidant capacity in semen; however, as the age (up to 4 hours) of the semen sample was not correlated with DNA damage, it is highly unlikely that this mechanism would play a role *in vitro*.

It should be pointed out that, as fractions 1 and 2 from leukocytospermic samples contained both sperm and leukocytes,  $COMP\alpha_t$  values in these fractions may reflect ROS-induced sperm DNA damage by leukocytes during sperm isolation. There are several arguments against iatrogenic sperm DNA damage during sperm processing: [1]  $COMP\alpha_t$  values were not correlated with the age (up to 4 hours) of the semen sample, [2]  $COMP\alpha_t$  values in semen from leukocytospermic samples were significantly higher than those found in semen from either donors or patients with normal semen parameters, and [3]  $COMP\alpha_t$  values in the neat semen sample accurately reflected the sum of the fractional  $COMP\alpha_t$  values in the different ISolate fractions.

Another implication of our study is that, should proinflammatory factors in the testis lead to alterations in the regulation of spermiogenesis and to increased sperm DNA damage, males with leukocytospermia might benefit, at least in part, from the use of anti-inflammatory therapy. This is supported by the report of Montag et al. (50), who found that use of anti-inflammatory therapy in a man with nonobstructive azoospermia and leukocytospermia resulted in resumption of sperm production and a significant reduction in leukocyte concentration in the ejaculate. This is consistent with our first hypothesis.

What is the significance of leukocytospermia in the man-

agement of infertility? There have been reports of DNA damage in sperm being an indicator of abnormal spermatogenesis and of poor pregnancy outcome. The COMP $\alpha_t$  values in leukocytospermic semen samples in our study ranged from 8% to 67% with a mean value of 42%. As recently reported, COMP $\alpha_t$  values in semen >30% resulted in delayed pregnancy and/or failure after fertilization in vivo (26) and in vitro (28). Therefore, infertile couples in whom the male has been diagnosed of leukocytospermia should be counseled concerning the potential negative effects of increased sperm DNA damage in their pregnancy outcome.

One fundamental question that remains to be answered is whether there is significant sample-to-sample variation in leukocyte concentration and sperm DNA damage in the samples from males with leukocytospermia. If that were the case, the selection and cryopreservation of semen samples with COMP $\alpha_t$  values <30% should be of potential benefit to these couples.

In summary, the results of this study indicate that there is a significant increase in sperm DNA damage in samples from leukocytospermic men, compared with samples from healthy donors or patients who are leukocyte free. The leukocyte concentration in semen was directly correlated with the immature germ cell concentration and abnormal sperm morphology and was inversely correlated with the recovery of mature sperm. No correlation was found between the age (up to 4 hours) of the semen sample and DNA damage. The results suggest that leukocytospermia may be associated with an inflammatory process in the testis that could lead to alterations in the regulation of spermatogenesis. Studies are underway to determine the sample-to-sample variation in DNA damage in semen from males with leukocytospermia.

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