

Novel associations between specific sperm morphological defects and leukocytospermia

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Objective: To examine the relationship between leukocyte concentrations in semen and sperm morphology in a group of infertile men and healthy fertile donors.

Design: A prospective clinical study.

Setting: Male infertility clinic at a tertiary care teaching hospital and a reproductive medicine unit at a Women's Hospital in the United Kingdom.

Patient(s): Fifty-six infertile men and 13 healthy fertile sperm donors (control).

Intervention(s): Standard semen analysis, seminal leukocyte concentration, and the assessment of sperm morphology and sperm deformity index (SDI), applying Tygerberg's strict criteria.

Main Outcome Measure(s): Granulocyte concentrations in semen, percentages of different sperm morphological abnormalities, and SDI scores.

Result(s): Leukocyte concentrations were statistically significantly and negatively correlated with the proportion of sperm with damaged acrosomes, cytoplasmic droplet, tail defects, and SDI scores with normal and borderline morphology. The percentage sperm motility was significantly and negatively correlated with leukocytic concentration in semen. However, the leukocytic concentration was not significantly correlated with sperm concentration.

Conclusion(s): This is the first study to report a significant positive correlation between leukocytospermia and sperm tail defects, acrosomal damage, and high SDI scores. These observations suggest that leukocytospermia is associated with compromised sperm structural integrity. (*Fertil Steril*® 2004;82:621–7. ©2004 by American Society for Reproductive Medicine.)

Key Words: Semen, spermatozoa, leukocytospermia, morphology, sperm deformity index

Leukocytes are present throughout the male reproductive tract and are found in almost every human ejaculate (1). Physiologically, most leukocytes appear to originate from the epididymis (2) and are thought to play an important role in immunosurveillance (3, 4) and phagocytic clearance of abnormal sperm (5). Granulocytes are the most prevalent type of leukocyte in semen (50% to 60%), followed by macrophages (20% to 30%) and T lymphocytes (2% to 5%) (6). Leukocytospermia is defined by the World Health Organization as the presence of peroxidase-positive leukocytes in concentrations greater than 1×10^6 per mL of semen (6). The origin of the excess leukocytes remains undetermined.

The role of leukocytospermia in the pathogenesis of male infertility remains controversial despite its relatively high incidence (10% to 20%) among infertile men (7–9). Some studies have correlated leukocytospermia with poor sperm quality and defective sperm function, whereas other studies have failed to find such a correlation (2). When sperm morphology is considered, several studies have found that sperm morphology deteriorates as the leukocyte concentration increases (10–15). Other studies have failed to demonstrate an association between increased leukocytic concentrations in semen and increased proportions of morphologically abnormal sperm (16, 17). A third group of studies found an association

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between leukocytospermia and higher proportions of morphological normal sperm in semen, caused by the removal of abnormal sperm by phagocytes (4, 5).

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). One of the mechanisms by which leukocytospermia may lead to sperm dysfunction is related to sperm damage induced by reactive oxygen species (ROS) via activated leukocytes during or after ejaculation (20–23).

Another potential mechanism by which leukocytes could affect sperm function is 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 men with leukocytospermia is still lacking. Whether leukocytospermia plays a role in the pathogenesis of male infertility remains controversial to this date.

Recently, the possible hypotheses to explain the significant production of ROS and DNA damage seen in immature spermatozoa from patients with leukocytospermia were reported (8, 9). Leukocytospermia may be associated with an inflammatory process in the testis that could lead to alterations in the regulation of spermatogenesis. Another explanation for the positive correlation between leukocytospermia, abnormal sperm morphology, and DNA damage may be related to an association between leukocytospermia and defective spermiogenesis. Leukocytospermia is associated with an increased capacity of spermatozoa to generate ROS, and patients with leukocytospermia are at higher risk for leukocyte-mediated oxidative stress.

These conflicting findings could be attributed to the large heterogeneity in the terms used to describe the condition, leukocyte subtypes in semen, and the magnitude of leukocytospermia. In addition, sperm morphology assessment is riddled with subjectivity, and several sperm classification systems and study designs have been used. However, the sophistication of sperm morphological examination has increased in recent years with the inclusion of the strict morphological criteria (24, 25) and sperm deformity index (SDI) (18), which have enhanced this parameter's predictive power and reproducibility. It has been shown that SDI and acrosomal status are accurate measurements of sperm functional competence and are highly correlated with fertilization in vitro (26).

To better understand the relationship between leukocytospermia and male infertility, we examined the SDI and percentages of different sperm morphological abnormalities in semen from infertile men with and without leukocytospermia and compared the results with those from a group of fertile donors.

Semen Samples

The Institutional Review Board of the Cleveland Clinic Foundation approved this study. Both patient enrollment and semen analysis on all subjects, including preparation of semen smears for morphological evaluation, were performed at Cleveland Clinic Foundation only. Semen samples were collected at Cleveland Clinic Foundation from men undergoing infertility screening ($n = 56$) and from normozoospermic healthy donors ($n = 13$) of proven fertility (that is, they had initiated a successful pregnancy within the last 12 months) who served as controls. Samples with a sperm concentration of $<1 \times 10^6$ per milliliter were excluded from this study. All specimens were collected by masturbation at the clinical andrology laboratory after a 48- to 72-hour period of abstinence. After liquefaction, routine semen analysis was performed to measure sperm concentration and percentage motility.

Myeloperoxidase-Staining Test

The presence of peroxidase positive leukocytes (neutrophils and macrophages) in semen was assessed by a myeloperoxidase-staining test (27) at Cleveland Clinic Foundation. A 20- μL volume of liquefied semen specimen was placed in a Corning 2.0-mL cryogenic vial (Corning Costar Corp., Cambridge, MA) with 20 μL of phosphate-buffered saline (pH 7.0) and 40 μL of benzidine solution. The solutions were mixed and allowed to sit at room temperature for 5 minutes. Peroxidase-positive leukocytes stain brown and were counted by a Makler's counting chamber (Sefi Medical, Haifa, Israel) under the bright-field objective (magnification, $\times 20$). The results after correction for dilution were recorded as $\times 10^6$ peroxidase-positive leukocytes per milliliter of semen. A seminal leukocyte concentration of $\leq 1 \times 10^6$ per milliliter of semen was considered normal, and leukocytospermia was defined as the presence of $>1 \times 10^6$ white blood cells per milliliter of semen (6).

Assessment of Sperm Morphology

Thin smears of the well-mixed semen were prepared in duplicate by placing 2- to 5- μL drops (depending on the sperm concentration) on clean poly-L-lysine-coated slides. Thin semen smears facilitated sperm morphology assessment by avoiding sperm cell overlap and ensuring that the sperm were scattered at the same focal depth. After the slides were air-dried, they were stained with the Dif-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL) for assessment of sperm morphology.

Slides of seminal smears for morphological examination were shipped to Liverpool Women's Hospital. One observer (N.A.) at Liverpool Women's Hospital scored these slides by a technique described previously (26). Briefly, a total of 100 spermatozoa were scored per slide by using bright-field illumination and an oil immersion objective with a total magnification of $\times 2,000$. At least 10 high-power fields were

TABLE 1

Seminal leukocytic concentration and sperm abnormalities in seminal ejaculates of fertile donors and infertile men with and without leukocytospermia.

Sperm parameters	Fertile donors (n = 13)	Nonleukocytospermic (n = 36)	Leukocytospermic (n = 20)	P ^a	P value ^b		
					A	B	C
Concentration ($\times 10^6$ /mL)	64 (39, 93)	36 (19, 64)	27 (12, 67)	.027	.012	<.01	.8
Motility (%)	69 (59, 76)	49 (33, 62)	37 (26, 52)	<.0001	.0003	<.0001	.03
Seminal leukocytes ($\times 10^6$ /mL)	0.1 (0.0, 0.2)	0.1 (0.0, 0.2)	2.4 (2, 7)	<.0001	.8	<.0001	<.0001
Normal morphology (%)	10 (8, 18)	5 (2, 12)	3 (0, 8)	.005	.0095	.001	.2
Borderline morphology (%)	16 (10, 21)	10 (5, 14)	4 (3, 10)	.0009	.004	.0001	.08
Amorphous (%)	38 (34, 46)	45 (40, 60)	37 (23, 50)	.09	.1	.03	.3
Acrosomal damage (%)	19 (14, 29)	26 (20, 36)	36 (30, 56)	.0004	.07	<.0001	.002
Nuclear abnormalities (%)	2 (1, 6)	7 (4, 13)	10 (5, 12)	.003	.002	.0009	.5
Midpiece abnormalities (%)	19 (9, 22)	21 (15, 28)	26 (20, 30)	.01	.026	.003	.2
Cytoplasmic droplet (%)	4 (0, 7)	6 (4, 11)	11 (6, 19)	.002	.03	.0003	.026
Tail deformity (%)	4 (2, 6)	7 (4, 12)	17 (7, 28)	.0009	.02	.0001	.02
Sperm deformity index	1.5 (1.4, 1.6)	1.7 (1.6, 2)	1.9 (1.7, 2.3)	.0004	.005	<.0001	.03

Note: Results are presented as median (25th, 75th percentiles). $P < .05$ was considered significant.

^a Univariate comparison of variables among groups was performed with Kruskal-Wallis test.

^b Simultaneous pairwise comparisons among groups were performed with the Convar-Inman test. A = fertile donors vs. nonleukocytospermic infertile patients; B = donors vs. leukocytospermic patients; C = nonleukocytospermic infertile patients vs. leukocytospermic patients.

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selected at random from different areas of the slide and examined. A calibrated micrometer on the eyepiece of the light microscope was used to measure sperm dimensions when there was doubt over sperm classification. All slides were assessed by using a morphological classification based on a modification of the method of Eliasson (28) and the strict criteria for normal sperm morphology (24, 25). A multiple-entry scoring technique was adopted in which an abnormal sperm was classified more than once if more than one deformity was observed.

The SDI was calculated by dividing the total number of deformities observed by the number of sperm that were randomly selected and evaluated, irrespective of their morphological normality. Borderline forms that were considered abnormal included [1] spermatozoa with slightly elongated head with loss of its oval shape, [2] those with rounded heads and intact acrosome, and [3] those with normal heads and a thickened midpiece. Strict quality control was maintained: each slide was in duplicate and coded. The scorer was blinded to the category that each slide had been assigned. The code was broken once the results were mailed back to Cleveland Clinic Foundation. Quality control assessment of sperm morphology slides revealed no significant difference in repeated estimation of different sperm morphological forms.

Statistical Analysis

Data were analyzed by using inbuilt functions within the Statistical Package for Social Science (SPSS UK Ltd., Chertsey, Surrey, UK). Summary statistics are presented as me-

dian and interquartile range (25th and 75th percentiles). Univariate comparison of continuous variables among the groups was performed with the Kruskal-Wallis test. Simultaneous multiple pairwise comparisons among groups were performed with the Conover-Inman test, which is simply Fisher's least significance difference method performed on ranks. Spearman's rank correlation test was used to provide a distribution-free test of independence between leukocyte concentration and sperm attributes. All hypothesis testing was two-tailed; $P < .05$ was considered statistically significant.

RESULTS

Semen specimens from 56 infertile men and 13 healthy donors were studied. All fertile donors had 1 seminal leukocyte concentrations of $\leq 1 \times 10^6$ (group 1). Of the 56 infertile patients, 36 were nonleukocytospermic (group 2), and 20 had leukocytospermia (group 3). Patients in group 3 had significantly higher seminal leukocyte concentrations compared with both patients in group 1 and 2 (Table 1).

Leukocytospermia and Sperm Concentration and Motility

Median and interquartile values (25th, 75th percentiles) of sperm concentration and motility in the 3 study groups are shown in Table 1. The healthy donors had significantly higher sperm concentrations and a higher percentage of sperm motility than the 2 groups of infertile patients. Sperm concentration was similar between the 2 groups of infertile patients. However, the nonleukocytospermic patients had a

significantly higher sperm motility percentage than did the patients with leukocytospermia.

When the 69 study participants were considered collectively, there was no significant correlation between leukocytic concentrations and sperm concentrations ($r = -0.13$; $P = .28$). However, the percentage sperm motility was significantly and negatively correlated with the leukocytic concentration in semen ($r = -0.28$, $P = .02$). The SDI scores were negatively correlated with sperm concentration ($r = -0.31$; $P = .01$) and sperm motility ($r = -0.35$; $P = .005$).

Sperm Morphology and Leukocytospermia

Median and interquartile values (25th, 75th percentiles) of the different morphological subsets of sperm and the SDI scores in the 3 study groups are shown in Table 1.

Pairwise comparison revealed that the healthy donors' semen had a higher proportion of sperm with normal and borderline morphology and significantly lower proportions of sperm with acrosomal damage, nuclear abnormalities, cytoplasmic droplet, midpiece defects, tail deformities, and lower SDI scores compared with the nonleukocytospermic and leukocytospermic groups (Table 1). Comparison of the nonleukocytospermic and leukocytospermic groups, on the other hand, revealed that the latter had significantly higher proportions of sperm with acrosomal damage, cytoplasmic droplet, tail deformities, and higher SDI scores. The proportions of sperm with normal, borderline, and midpiece defect morphologies were comparable in both infertile patient groups. The proportions of sperm with other morphological abnormalities (large head, small head, tapered head, pyriform, and double-headed sperm) were similar among the three groups (data not shown).

When the 69 study participants were considered collectively, leukocyte concentrations in semen were positively correlated with the SDI scores ($r = 0.32$; $P = .008$) and the proportion of sperm with acrosomal damage ($r = 0.36$; $P = .003$), tail deformities ($r = 0.33$; $P = .005$), cytoplasmic droplet ($r = 0.31$; $P = .01$), and midpiece defects ($r = 0.27$; $P = .026$; Fig. 1). On the other hand, leukocyte concentrations in semen were inversely correlated with the proportion of sperm with normal morphology ($r = -0.3$; $P = .013$) and borderline morphology ($r = -0.38$; $P = .0014$; Fig. 1).

DISCUSSION

To the best of our knowledge, this is the first study to assess the relationship between SDI scores applying the Tygerberg strict criteria of sperm morphology and leukocytospermia in a wide spectrum of individuals with varying fertility potential. Our study demonstrated that leukocytospermia was positively correlated with the percentage of sperm with acrosomal damage, cytoplasmic droplet, midpiece and tail defects, and SDI scores. When the whole study population was considered, the proportion of sperm with normal morphology was inversely correlated with leukocytic

concentration in semen. However, there was no evidence of a statistical difference in sperm with normal morphology between the nonleukocytospermic and leukocytospermic infertile men. On the other hand, the average number of abnormalities per sperm was higher in the leukocytospermia patients, which resulted in significantly higher SDI scores in this group of patients compared with in the fertile donors and the nonleukocytospermic infertile men.

Only two studies have reported full assessment of sperm morphology applying the strict criteria for sperm morphology in leukocytospermic patients (14, 15). One study investigated the influence of urogenital infections in 150 consecutive semen samples, as indicated by leukocytospermia (diagnosed cytologically and by means of a leukocyte peroxidase test) on human sperm morphology (15). Leukocytospermia diagnosed cytologically was associated with statistically poorer sperm morphology characteristics, which involved a significant reduction in the proportion of sperm with normal morphology and an increase in the proportion of taper-headed sperm. This statistical relationship was not found in the peroxidase-diagnosed leukocytospermia group (15).

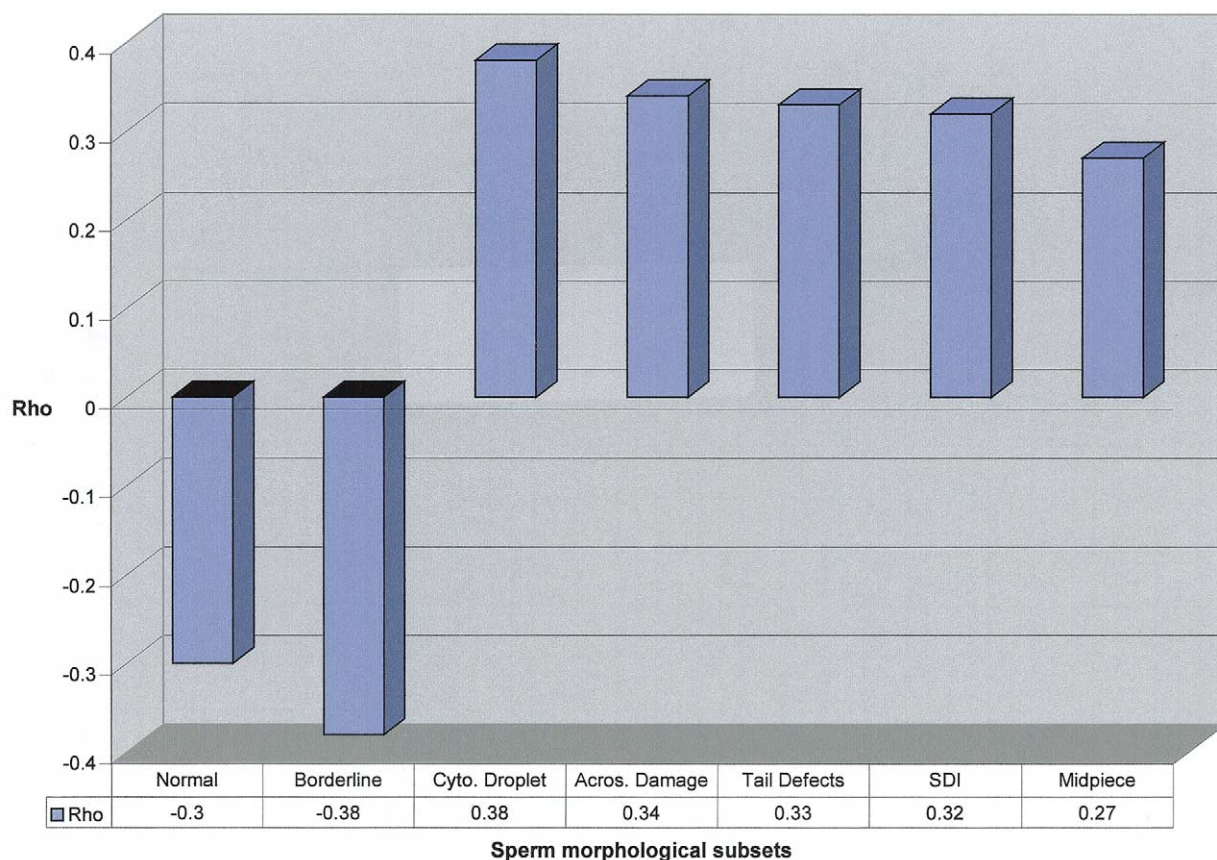
The second study assessed the association of varying concentrations of leukocytes (assessed by an Endtz test) with sperm morphology (evaluated by strict criteria) in semen from 79 infertile patients (14). At two different cutoff points of seminal leukocytic concentration (0.5×10^6 and 1×10^6 leukocytes per milliliter), the patient group with lower mean leukocytic concentration had a significantly higher proportion of sperm with normal morphology compared with patients who had a higher mean leukocytic concentration. There was also a significant positive correlation between leukocytic concentration and midpiece defects. No other correlation was detected.

When compared, the findings that are reported in these two studies are clearly distinct. Moreover, our study demonstrates a correlation between leukocytospermia and certain deformities of different anatomical regions of sperm that has not been observed in the previous two studies. This discrepancy may, at least in part, be attributed to the design of our study, which allowed the analyzed semen samples from a spectrum of individuals (fertile donors and infertile men with and without leukocytospermia) to fully assess the true impact of leukocytospermia on sperm morphology. In view of these discrepancies, additional studies are required to substantiate our finding.

Different hypotheses have been explored recently to explain the relationship between leukocytospermia and sperm structural damage (8). The hypotheses that sperm structural damage occurs during spermatogenesis is consistent with our findings that showed that infertile men with and without leukocytospermia had similar sperm concentrations in semen. Low sperm concentrations would have been expected if the leukocytic-induced damage occurred during spermatocyt-

FIGURE 1

Correlation of the proportions of different sperm morphological forms with leukocyte concentration in semen. Columns represent the value of Spearman correlation. Only statistically significant correlation ($P < .05$) presented in the graph. Cyto. Droplet = cytoplasmic droplet; Acros. Damage = acrosomal damage; SDI = sperm deformity index.



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togenesis (proliferative phase). The mechanism through which leukocytospermia may induce the alteration in sperm structure is not clear. However, 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 epididymoorchitis. 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).

In addition, our finding of significantly higher proportions of sperm with cytoplasmic droplet in the leukocytospermic patient group could be due to defective Sertoli cell function and disorganized spermiation. Alternatively, it could be due to alteration in the maturation process that the sperm undergo while transient in the epididymis. Alteration in the epididymal sperm maturation would also explain why leu-

kocytospermic men have significantly lower sperm motility. Thus, leukocytospermia-induced sperm damage may commence during spermiogenesis and continues through spermiation and epididymal migration.

A second potential mechanism through which leukocytospermia may induce alteration in sperm structure is excessive ROS production by activated granulocytes. It has been argued that oxidative stress may induce alterations in the regulation of spermatogenesis, resulting in structural defects of the sperm (8, 29, 30). On the subcellular level, it has been shown that leukocytospermia and excessive ROS levels are associated with an increase in chromatin alterations and DNA damage in sperm, as defined by the sperm chromatin structure assay (8). On a different level, it has been shown that a high incidence of sperm tail defects is associated with sperm chromosomal abnormalities (31, 32). It is thus conceivable that peroxidative genetic damage may have led to the significant increase in the proportion of sperm with tail

defects observed in our leukocytospermic study population. From this it is possible to speculate that sperm structural defects in leukocytospermic men may have been acquired at different stages of spermiogenesis, spermiation, and/or the epididymis as a result of proinflammatory mediators or peroxidative genetic alterations. Nevertheless, definite evidence for the presence of significant numbers of leukocytes in the seminiferous tubules or epididymis from males with leukocytospermia is still lacking.

In our study, oxidative stress in patients with leukocytospermia may have been intensified as a result of the observed association between leukocytospermia and the increase in the proportion of sperm with cytoplasmic droplet. The retention of residual cytoplasm in the sperm midpiece after spermiation has been associated with excessive production of ROS by spermatozoa (33). Moreover, independent reports have demonstrated that biochemical markers of the cytoplasmic space, such as creatine kinase, are positively correlated with the induction of peroxidative damage (33, 34). It has been hypothesized that the reason that cells with an excess of residual cytoplasm exhibit high rates of ROS generation is related to the enhanced presence of another cytoplasmic enzyme, glucose-6-phosphate dehydrogenase. This enzyme fuels the generation of NADPH, which in turn stimulates the production of ROS (35, 36).

The results of our study clearly demonstrate that there is a significant increase in the frequency of sperm morphological defects in nonleukocytospermic infertile men compared with the case of healthy donors. A genetic constitution or detrimental environmental factors may be the underlying cause for poor sperm morphology in this group of patients. The significantly higher frequency of sperm morphological defects in leukocytospermic infertile men compared with nonleukocytospermic patients may suggest that leukocytospermia is associated with the worst detrimental effect on sperm morphology—especially if we consider it as the only underlying factor for this negative influence.

In an ideal world, a possible causal relationship between leukocytospermia and sperm morphology is confirmed when there is an improvement in sperm morphology after successful treatment of leukocytospermia. This would represent a logical follow-up to our study. However, such study may be difficult to conduct because treatment of leukocytospermia remains controversial. Moreover, functional and anatomical damage acquired as a result of infection is often permanent and not reversible by (antibiotic) treatment (37).

In conclusion, this is the first study to assess the relationship between SDI scores, applying the strict criteria of sperm morphology and leukocytospermia in a wide spectrum of individuals with varying fertility potential. Our study suggests that leukocytospermia is positively correlated with the percentage of sperm with acrosomal damage, cytoplasmic droplet, midpiece and tail defects, and the SDI scores. It is possible that sperm structural defects in the men with leu-

kocytospermia may have been acquired at different stages of spermiogenesis, at spermiation, and/or in the epididymis as a result of proinflammatory mediators or sperm genetic alterations. In view of the fact that our findings have not been observed in previously reported studies employing the strict criteria of normal sperm morphology, further studies are required to confirm our finding.

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