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Effect of Leukocytospermia on Sperm DNA Integrity: A Negative Effect in Abnormal Semen Samples

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ABSTRACT: Controversy exists over levels of DNA integrity in the sperm of fertile and infertile men. In addition, the effect of leukocytospermia on sperm DNA in these 2 groups is unclear. We decided to address these questions by collecting semen samples from men known or presumed to be fertile and men from infertile couples. Samples were analyzed and assessed for sperm concentration, motility, and morphology. Samples failing to meet World Health Organization (WHO) standards in one or more of these parameters were judged abnormal. Samples were then arbitrarily assigned normalized scores in each of the above parameters, and scores were summed to give a normalized value for overall sperm quality. DNA abnormality was determined by an in situ DNA denaturation test with acridine orange and expressed as a percentage of cells with abnormal DNA integrity (ADI). Assessment of 187 samples revealed a moderate inverse correlation between ADI and sperm quality ($r = .58$), although a large degree of ADI dispersion was observed in abnormal semen samples. The average ADI for normal and abnormal semen samples was 18% ± 2.8% and 36% ± 5.8%, respectively, with the threshold of 95% probability set at 30%. When sorted for leukocytospermia, the difference in ADI between normal and abnormal semen groups without leukocytospermia was much smaller (17% ± 2.2% and 22% ± 4.6%; $P = .023$). Leukocytospermia had no significant effect on ADI in the normal semen group ($P = .46$); however, ADI was more than double the ADI in the abnormal semen group (18% ± 2.4% and 50% ± 11%; $P < .001$). The results of our analysis show that at least 3 factors affect net DNA integrity in leukocytospermic samples that fail to meet WHO standards: 1) primary DNA damage, which is moderately inverse to sperm quality, in particular to sperm concentration; 2) effect of leukocytes increasing primary or provoking potential DNA damage in a cascade-like manner, particularly in sperm with poor morphology and motility; and 3) a decreasing proportion of cells with damaged DNA in semen with the worst quality.

Key words: Sperm quality, DNA integrity, leukocytes, oxidative stress.

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Sperm DNA integrity is essential for accurate transmission of genetic information. This parameter has been shown to be of paramount importance for fertilization and normal embryo development (Ibrahim et al, 1988; Vorobjeva et al, 1998; Evenson et al, 1999). Impaired DNA integrity can be detected using the in situ DNA denaturation test with acridine orange (AO; Darzynkiewicz et al, 1975; Darzynkiewicz, 1994) because damaged DNA is more prone to denaturation by heat or low pH than undamaged DNA. This test has been applied to assess sperm DNA integrity using flow cytometry in the sperm chromatin structural assay (SCSA; Evenson et al, 1980), and the same principle has been used in microscopic methods (Tejada et al, 1984; Erenpreiss et al, 2001). Impaired sperm DNA integrity (displaying red fluorescence in the in situ DNA denaturation test) has been highly positively correlated with the number of DNA strand breaks (Bianchi et al, 1993; Gorczyca et al, 1993), as detected by enzyme linked immunosorbent assay (ELISA), single-cell gel electrophoresis assay (COMET), in situ nick translation, and terminal deoxynucleotidyl transferase-mediated nick end labeling.

Controversial reports have detailed the relationship between DNA integrity and conventional semen parameters, and whether measures of DNA integrity can differentiate the sperm of fertile and infertile men. Evenson and coworkers (1999), using SCSA on a large number of samples, have established a threshold prognosis infertility value of greater than 30% of sperm cells with impaired DNA integrity (DNA abnormality). In addition, an association was found between levels of DNA abnormality and the amount of time to achieve pregnancy in fertile couples (Evenson et al, 1999). The authors also report that although high values of DNA abnormality predict infertility, conversely lower rates do not predict fertility. Recent research undertaken by Larson et al (2000) on a group of 24 men has shown that no pregnancies resulted from intracytoplasmic sperm injection when the proportion of sperm with impaired DNA integrity was more than or equal to 27%. These authors concluded that a weak association exists between SCSA parameters with conventional semen parameters, or that there may be no association whatsoever. Hughes et al (1996, 1999), using sen-

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sitive COMET and ELISA, could not find a difference in baseline DNA damage between normozoospermic fertile and asthenozoospermic infertile men, which they determined to be around 20%. Contrary to this, the correlation between poor quality semen and higher levels of DNA damage detected as DNA fragmentation using the COMET assay, has been recently reported by Irvine et al (2000).

Therefore, whether the widely used conventional tests for DNA abnormality are sensitive enough to reveal differences in baseline DNA integrity in fertile and infertile semen, and whether such a difference is actually present, remains unclear. In addition, the designation of fertility versus subfertility or infertility in cases of secondary infertility may be differently interpreted in these comparative studies. Therefore, careful analysis and objective parameters of semen are needed.

The effect of leukocytospermia on sperm DNA integrity is similarly unclear. Aitken et al (1989a) have found that human spermatozoa produce reactive oxygen species (ROS). Physiological concentrations of ROS likely promote sperm capacitation and the acrosome reaction (De Lamirande and Gagnon, 1995). However, particularly high levels of ROS were found in about 40%–50% of cases of oligoasthenozoospermia (Aitken et al, 1989b; Iwasaki and Gagnon, 1992) and were proposed as a central cause of DNA damage in sperm cells that were unable to repair it. Indeed, ROS produce both plasma membrane and dose-dependent DNA damage (Twigg et al, 1998; Barroso et al, 2000; Krzyzosiak et al, 2000). Kodama et al (1997) found a 1.5-fold increase in the level of oxidated DNA derivatives in infertile men compared with fertile men. However, Aitken and West (1990) and others (Sukcharoen et al, 1995; Sharma et al, 2001) have suggested that the main source of sperm ROS is provided not by sperm cells themselves, but by activated polymorphonuclear leukocytes in cases of leukocytospermia. In these cases, ROS concentration appears to exhibit a powerful negative effect on fertilization rate.

In an ongoing study, we have found that 17% of young Latvian army recruits have leukocytospermia (n = 103; unpublished results). However, in sterile Latvian couples (n = 579) studied from 1998 to 2000, 78% of men had abnormal semen parameters and 43% had leukocytospermia (Erenpreiss et al, 2002). A high prevalence of leukocytospermia in infertile men has also been reported in several countries, although the values are different. It is interesting that data from Kuwait (Omu et al, 1999) are similar to those of Latvia.

Therefore, the aims of this study were 1) to evaluate the effect of standard measures of sperm quality on DNA integrity, and 2) to evaluate the effect of leukocytospermia on sperm DNA integrity in normal and abnormal semen samples.

Materials and Methods

Approval for the study was obtained from our institutional review board. Human sperm samples from 187 men (mostly from infertile couples, as well as young army recruits who served as donors and volunteers) were analyzed in the first part of the study. All samples were evaluated for standard semen parameters according to criteria of the World Health Organization (WHO; 1999). These values were entered into a spreadsheet (Excel 97; Microsoft, Redmond, WA) and ranged according to their value (for this set, only fast progressive or A-group motility according WHO guidelines was considered). These values were then normalized and given an arbitrary value from 0 to 10. The sum of these 3 normalized parameters was used to generate a normalized sperm quality index according to the formula:

$$\textit{Sperm quality} = \text{Conc} \times 10/\text{Conc}_{max} + \text{Motil} \times 10/\text{Motil}_{max} + \text{Nmorph} \times 10/\text{Nmorph}_{max}$$

where Conc is sperm concentration in millions, Motil is motility A in %, and Nmorph is the percentage of cells with normal morphology. As such, the sum of these values should, ideally, be 30 arbitrary units.

All reagents were purchased from Sigma Chemical Company (St. Louis, Mo). Sperm DNA integrity was detected by an in situ DNA denaturation test using AO staining on slides as detailed previously (Erenpreiss et al, 2001), and which has been previously applied to detect apoptosis in somatic cells (Erenpreisa et al, 1997). After semen liquefaction at 37°C for 30 minutes, samples were pelleted by low-speed centrifugation (1000 $\times$ *g* for 5 minutes) and resuspended in a smaller portion of supernatant at approximately 5×10^8 cells/mL. Cell smears were prepared and air-dried for 1 hour. Cells were then fixed in freshly made ethanol:acetone (1:1) at 4°C for 0.5–24 hours. Fixed samples were not air-dried before staining, but were rehydrated in 96°C ethanol for 5 minutes, 70°C ethanol for 5 minutes, and 30°C ethanol for 3 minutes, all at room temperature. Washing for 5 minutes in phosphate-buffered saline was followed by treatment with 1 N HCl at 60°C for 1 minute. Slides were then rinsed 3 times in distilled water for 2 minutes and dipped in McIlvain phosphate-citrate buffer (pH 4) for 5 minutes before staining with AO (10^{-4} M) in the same buffer for 15 minutes. The AO working solution was freshly prepared from a 7.6 mg/mL of AO stock stored in the dark at 4°C. Slides were then rinsed 3 times for 5 minutes in the same McIlvain buffer containing freshly diluted AO (10^{-6} M). A coverslip was mounted on this AO solution, blotted at the margins, and sealed with nail polish (moviol was not added to the mounting medium). Preparations were kept in the dark at 4°C and were assessed within 24 hours after staining. Using this method, AO fading does not occur, which allows scoring at a later time, as established in our previous study (Erenpreiss et al, 2001).

The percentage of sperm cells with abnormal DNA integrity (ADI) was determined by analyzing 300 cells per sample with a Nikon TE 300 fluorescence microscope (Nikon GmbH, Dusseldorf, Germany), with a mercury lamp (excitation band-pass filter, 420–490 nm; emission was observed through a suppression filter with a 515 nm limit). Sperm cells with normal DNA integrity have green fluorescence, those with abnormal DNA in-

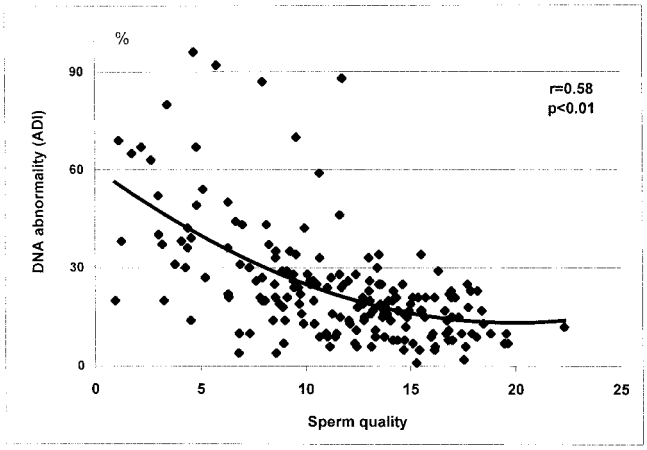

Figure 1. Inverse relationship between DNA abnormality and sperm quality (sum of normalized concentration, A motility, and morphology in arbitrary units) in samples from 187 men, unsorted in relation to leukocytospermia. Note that dispersion of ADI values increases with decreasing sperm quality.

tegrity fluoresce orange-red. Intraassay variation was assessed as 9%, and interassay variation as 11%.

In the second part of the study, 79 normal and 40 abnormal semen samples from the previous set were analyzed for leukocytospermia. This smaller set was chosen because leukocytes had been determined in this set by the same method over a small period of time (4 months). The formula for normalization of sperm quality of this set was the same as described above (with the exception that fast + slow progressive, or group A + group B motility according to WHO guidelines, was considered). In addition, these samples were classified as abnormal if one or more parameters contained the following WHO criteria: sperm concentration, 20 million/mL; motility (A + B), 50%; and normal morphology, 20%. The method of estimating DNA integrity was the same as that described above. Leukocytospermia was considered if leukocytes, as detected by the peroxidase method (WHO, 1999), comprised more than 1 million per milliliter of ejaculate. Leukocyte counts ranged from 2 to 13 (mostly from 2 to 6) million/mL.

Statistical analysis of the results was performed with Microsoft Excel 97 using power analysis and the two-tailed Student's *t*-test.

Results

The relationship between the percentage of sperm cells with ADI and arbitrary sperm quality expressed as a sum of 3 normalized standard parameters is presented in Figure 1. These data are taken from 187 samples unsorted for leukocytospermia. This data set has a moderate inverse correlation ($r = .58$, $P < .01$) between DNA abnormality and normalized sperm quality. In samples of good and average quality (>10 arbitrary units), ADI values are mostly <30%. In samples of poor quality (<10 arbitrary units), ADI values are widely dispersed, showing both low and high values. Although the frequency of high ADI values (40%–96%) is characteristic for poor sperm as such, it also shows a tendency to increase in sperm of poorest quality.

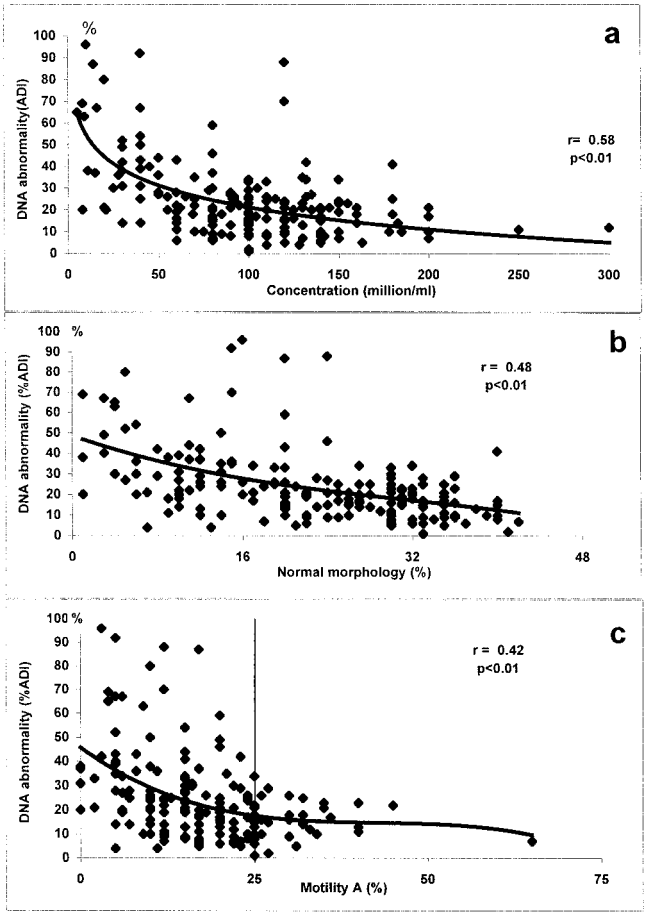

Figure 2. Inverse correlation between DNA abnormality (ADI) and separate standard sperm parameters (in absolute values), in unsorted samples from 187 men. **(a)** The relationship is more significant for sperm concentration, in particular, <50 million/mL; **(b)** it is looser for the percentage of cells with normal morphology; and **(c)** is less dependent on motility A. However, high ADI values occur only in samples possessing motility A under the critical WHO threshold (25%), which is marked by a vertical line.

Analysis of the relationship between DNA abnormality and the 3 individual parameters of semen quality for the 187 samples is presented in Figure 2 (the figure also shows maximum values for this set). Inverse correlation coefficients are demonstrated for the following parameters: sperm concentration, $r = 0.58$; morphology, $r = .48$; and motility A, $r = .42$. These separate graphs also show the same phenomenon of wide dispersion and a tendency for ADI values to increase in the poorest quality sperm. This phenomenon is dependent on lower sperm concentration, particularly <50 million/mL (Figure 2a). In addition, only sperm with critical A motility (<25%) show ADI values higher than 30% (Figure 2c).

*Effect of leukocytospermia on sperm parameters in normal and abnormal semen samples**

Sperm parameters	A. Normal semen without leukocytospermia (n = 33)	B. Normal semen with leukocytospermia (n = 46)	C. Abnormal semen without leukocytospermia (n = 20)	D. Abnormal semen with leukocytospermia (n = 20)
Concentration (million/mL)	115 ± 12.6	117 ± 12.7	66 ± 11.1	47 ± 16.5
Motility (A + B)%	56 ± 2.8	53 ± 4.0	39.3 ± 6	35.8 ± 8.0
Normal morphology (%)	23 ± 2.3	22 ± 2.1	13 ± 2.6	9 ± 2.5
Abnormal DNA integrity (% ADI)	17 ± 2.2	18 ± 2.4	22 ± 4.6	50 ± 10.7

* Probability of ADI difference between groups as determined by two-tail *t*-test: AB ($P = 0.46$); BC ($P = .077$); AC ($P = .023$); BD ($P = .00004$).

A subset of 119 samples designated as normal and abnormal according to WHO criteria were further analyzed for leukocytospermia. The routine sperm parameters of these samples and corresponding ADI data are presented in the Table. Average values of sperm DNA abnormality for normal and abnormal samples in this set, regardless of leukocytospermia, were 18% ± 2.8% and 36% ± 5.8% ($P < .05$), respectively, setting a threshold for possible subfertility or infertility at 30% ADI with 95% confidence.

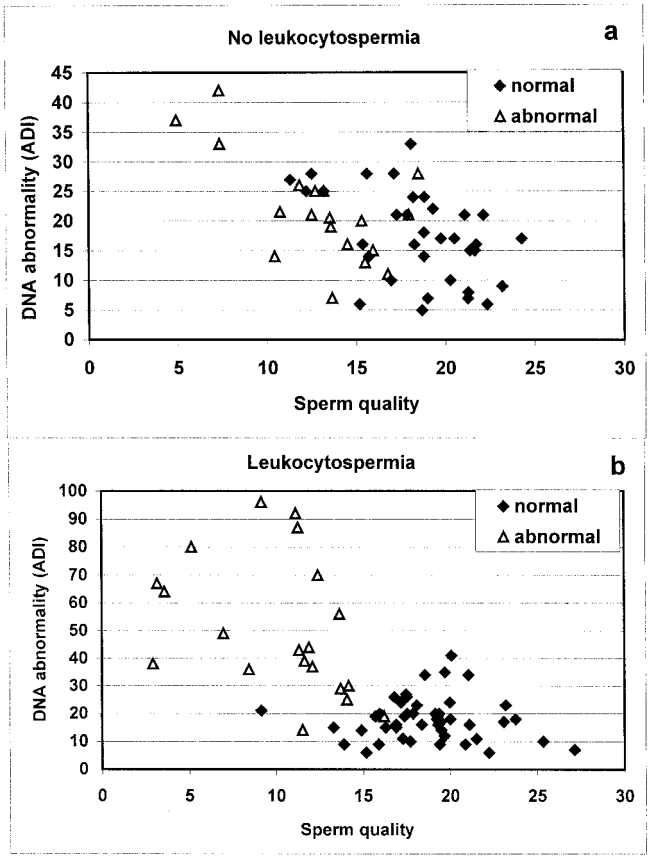

Figure 3. Relationship between DNA abnormality and sperm quality (sum of normalized concentration, A + B motility, and morphology, in arbitrary units) in samples from a subset of 119 men. Samples were sorted in relation to leukocytospermia: **(a)** without leukocytospermia and **(b)** with leukocytospermia. In addition, samples are labeled in **(a)** and **(b)** as normal and abnormal considering WHO criteria (detailed in ''Materials and Methods.'' In **(a)** note the moderate inverse relationship between the parameters of DNA abnormality and sperm quality as well as overlapping ADI values for normal and abnormal semen in the middle quality region (10–20 arbitrary units). In **(b)** note the clear separation of normal and abnormal samples; the latter dramatically react to leukocytospermia by an increase in DNA abnormality; the relationship is close to parabolic.

When samples with leukocytospermia were removed from the analysis, the difference in average ADI values between normal and abnormal semen samples appeared to be much smaller; however, they were statistically significant ($P = .023$), constituting 17% ± 2.4% and 22% ± 4.6%, respectively (see also the Table). Although leukocytospermia did not significantly affect DNA integrity in the normal semen group, it more than doubled the ADI values in the abnormal semen group, with values of 18% ± 2.4% and 50% ± 10.7% ($P < .01$), respectively.

Such a small baseline difference between ADI of normal and abnormal samples, which can presumably categorize fertile and subfertile or infertile sperm, respectively, is at odds with many other reports (some reviewed above). In order to better understand the cause of this discrepancy, we plotted the ADI values against the normalized summed sperm quality values, arranged in a discontinuous row, including both normal and abnormal samples. For this analysis, standard normalized sperm quality was scored in one scale for all groups. Maximum parameter values, which were assigned 10 arbitrary units each, consisted of sperm concentration, 200 million/mL; A + B motility, 84%; and normal morphology, 42%.

The results of such an assessment are presented in Figure 3, which shows that in the set without leukocytospermia (Figure 3a), the majority of samples, both normal and abnormal by WHO criteria, are positioned in the intermediate region of normalized sperm quality (between 10 and 20), and have similar overlapping ADI values, mostly between 10% and 27%. Very good quality samples (with quality consisting of more than 20 arbitrary units) had a lower ADI (mostly under 22%–20%), whereas very poor quality samples (between 0–10 arbitrary units of quality) possessed ADI values above the infertility threshold of 30%–42%. Therefore, in the absence of leukocytospermia, the difference in ADI is significant only between semen of very good and very poor qualities, whereas it is insignificant for samples of intermediate quality, which are either normal or abnormal according

to WHO criteria. The entire relationship is moderately inverse and can be equally well described as linear (r = .58) or as a polynomial of the second power (r = .60), with similar statistical significance ($P < .01$).

For samples with leukocytospermia, the same analytical approach gives quite a different distribution and is presented in Figure 3b. Here, a clear separation of normal and abnormal samples is observed, with DNA integrity greatly impaired in abnormal semen. Moreover, the majority of abnormal samples of intermediate quality (between 10 to 15 arbitrary units), which were indistinguishable from normal samples with respect to ADI without leukocytospermia, now have dramatically elevated ADI. Thus, in Figure 3a, 85% of abnormal samples had ADI values <30%; whereas in Figure 3b, 84% exceed this threshold, and many display very high ADI (>60%). Comparing Figure 3a and b, we see another peculiarity. The distributions of ADI according to sperm quality in Figure 3b seem to have a parabolic form. In cases of leukocytospermia, the peak of ADI for abnormal sperm reaches values as high as 80%–90% or more at 10 arbitrary units of quality, with the tendency for ADI to decrease in the sperm of worst quality.

Therefore, the relationship between sperm quality and ADI in the samples with leukocytospermia are best described by a polynomial of the 4th power (not shown), and this reveals a tighter association between ADI and sperm quality (r = .71, $P < .01$) than any other factor. Additional analysis revealed that this parabolic relationship is more dependent on sperm morphology and motility than on sperm concentration (not shown). ADI was weakly associated with the number of leukocytes in sperm in this set (r = .32; P = .1).

Discussion

It has been demonstrated previously (Evenson et al, 1999; Spano et al, 2000; Erenpreiss et al, 2001) that a statistically significant difference exists in the proportion of sperm cells with impaired DNA integrity in fertile and infertile men. The threshold value of infertility or subfertility at the 95% confidence interval calculated on the population in our studies is around 30%. Our results agree with the data of Evenson et al (1999), which were generated with a flow cytometric SCSA assay, and which demonstrates that our modification of in situ DNA denaturation method is reliable. Our results also support observations of these authors that ADI values below 30% do not necessarily predict fertility; sperm samples with low ADI may also have poor standard quality.

In the 187 samples assessed, a moderate inverse relationship between standard sperm quality and DNA abnormality was found, in particular with sperm concentration. This result is similar to that of Irvine et al (2000), who used the objective parameters of sperm quality and various DNA fragmentation detection methods. Our data are also in accord with the data of Tejada et al (1984), who noted the importance of both DNA normality and sperm concentration for infertility prognosis, and who introduced the integrated parameter of ''effective sperm concentration.'' Some authors (Liu and Baker, 1994; Larson et al, 2000) have concluded that DNA integrity parameters, which are predictive of infertility, are independent of standard sperm quality assessments. The data reporting no baseline difference in sperm DNA integrity between semen groups of fertile and infertile men obtained by Hughes et al (1996, 1999) add yet more confusion to the discussion.

One source of these contradictions can be inferred from Figure 1, where it can be seen that the effect of sperm quality on DNA abnormality is in fact evident only at the poles of sperm quality. In medium-quality sperm, ADI varies very little. In addition, ADI values for poor-quality sperm, as presented in Figure 1, are variable and dispersed around the trend line.

To understand the possible contribution of leukocytospermia to DNA abnormality, a subset of 119 samples sorted for leukocytospermia was analyzed. As seen in Figure 3a, in which normal and abnormal semen samples without leukocytospermia are labeled, both are mostly concentrated in the region of intermediate quality with overlapping ADI values. This explains a small baseline difference of ADI in our normal and abnormal semen sets averaged in the Table. Thus, the average results may be highly dependent on the given data set, sample numbers, etc.

Also in this case, as in Figure 1, ADI values showed a moderate inverse, nearly linear correlation with sperm quality. However, no high dispersion of ADI around the imaginary trend line could be found. On the contrary, in the set of abnormal sperm samples with leukocytospermia, a huge dispersion with high ''jumps'' of ADI were observed (Figure 3b), which accounts for the more than twofold increase in average ADI in this group (Table). Notably, a response to leukocytes provoked potential DNA damage and caused high ADI values in those abnormal samples that possessed moderately poor sperm quality and which, without leukocytospermia, were indistinguishable from normal samples with ADI values under 30%. Thus, the normal and abnormal fractions became clearly separated by leukocytospermia. However, in this case, ADI did not show a linear relationship with sperm quality. Instead, it displayed a clear tendency to dramatically increase with decreasing sperm quality, and with even worse sperm quality, to decrease. This parabolic distribution suggests involvement of 2 different mechanisms

affecting the relationship between sperm quality and DNA integrity in abnormal leukocytospermic semen.

From this analysis, we conclude that high dispersion of ADI values around the trend line in abnormal-quality sperm in our material is mostly due to leukocytospermia. It seems that leukocytospermia may be increasing the ADI of abnormal semen very stringently, in a cascade-like manner, but that it is somehow less effective in the poorest quality semen.

Given that leukocytospermia may affect sperm DNA integrity in a cascade-like manner, the mechanism of the action may occur via oxidative stress. It can therefore be hypothesized that abnormal sperm is susceptible to oxidative stress, which subsequently produces extensive DNA damage, whereas normal sperm is only poorly susceptible to such damage. Oxidative stress is considered an important and key factor in the etiology of male infertility regardless of its origin (Pasqualloto et al, 2000) and its negative effect on fertility is recognized by andrologists (Comhaire et al, 1999).

This hypothesis is likely because leukocytospermia is already known to produce oxidative stress sufficient to damage DNA in sperm cells (Sharma et al, 2001). Furthermore, in our research, the average percentage of cells with impaired DNA was more than doubled in abnormal sperm with leukocytospermia compared with abnormal sperm without leukocytospermia.

An interesting aspect of this phenomenon revealed by our study is the seemingly weaker response of extremely poor quality sperm to DNA damage from leukocytes. These sperm may contain primary DNA damage, and therefore, it may be hypothesized that this paradox is due to the rapid removal of these apoptotic sperm cells by leukocytes. Irrespective of this conjecture, this mechanism is clearly opposing the increase in ADI in abnormal sperm samples, and these conflicting mechanisms result in the great variability of ADI in abnormal leukocytic semen samples.

What is the nature of the susceptibility of abnormal sperm to oxidative stress? It is interesting that weakness of the plasma membrane is a characteristic of abnormal sperm (Barroso et al, 2000) and ROS are known to be detrimental to both plasma membranes and DNA (Twigg et al, 1998; Krzyzosiak et al, 2000). Impairment of the plasma membrane probably decreases the group A motility of sperm with high ADI (under putative oxidative stress) and can be inferred from Figure 2c. In turn, the increased DNA vulnerability in abnormal sperm may, for example, be due to natural alkali-labile DNA sites being unprotected from ROS by poor chromatin packaging in immature sperm.

Regardless of the reason for high susceptibility of abnormal sperm to oxidative stress, one must take account of it when using sperm for assisted conception. Although leukocytospermia may be infrequent in some human populations, when present, it is clearly harmful for subfertile men. In addition, oxidative stress in sperm may be further exacerbated by factors other than leukocytes, either natural or during manipulations with sperm.

Three important clinical implications follow: 1) infections must be removed to prevent damage to DNA integrity in subfertile and infertile men; 2) antioxidants may be useful in decreasing the negative effects of ROS and hence DNA abnormality; and 3) the genetic risk of micro-assisted fertilization using genetic material from infertile men must be considered, and if fertilization is performed, use of antioxidants during manipulations may be of a paramount importance.

In summary, our analysis shows that the relationship between sperm quality and DNA abnormality is complex. Normal semen has low ADI and is resistant to the DNA damage associated with leukocytospermia. However, at least 3 factors affect the net DNA integrity in abnormal quality leukocytospermic samples: 1) primary DNA damage, which is moderately inverse to the sperm quality, in particular to sperm concentration; 2) the effect of leukocytes increasing and provoking potential DNA damage in a cascade-like manner, particularly in sperm of moderately poor morphology and motility; and 3) a decreasing proportion of cells with damaged DNA in semen of the worst quality.

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References

Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod.* 1989a; 40:183–197.

Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS, Wu FCW. Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J Androl.* 1989b;10:214–220.

Aitken RJ, West KM. Analysis of the relationship between reactive oxygen species production and leukocyte infiltration in fractions of human semen separated on Percoll gradients. *Int J Androl.* 1990;13:433–451.

Barroso G, Morshedi M, Oehninger S. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *J Hum Reprod.* 2000;15:1338–1344.

Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas, D. Effect of deoxyribonucleic acid protamination on fluorochrom staining and in situ nick-translation of murine and human spermatozoa. *Biol Reprod.* 1993;49:1083–1088.

Comhaire FH, Mahmoud AMA, Depuydt CE, Zalata AA, Christophe AB. Mechanisms and effects of male genital tract infection on sperm qual-

ity and fertilizing potential: the andrologist' s viewpoint. *Hum Reprod Update.* 1999;5:393–398.

Darzynkiewicz Z. Acid-induced denaturation of DNA in situ as a probe of chromatin structure. *Methods Cell Biol.* 1994;41:527–541.

Darzynkiewicz Z, Traganos F, Sharpless T, Melamed MR. Thermal denaturation of DNA in situ as studied by acridine orange staining and automated cytofluorometry. *Exp Cell Res.* 1975;90:411–428.

De Lamirande E, Gagnon C. Capacitation-associated production of superoxide anion by human spermatozoa. *Free Radic Biol Med.* 1995; 18:487–495.

Erenpreisa J, Freivalds T, Roach H, Alston R. Apoptotic cell nuclei favour aggregation and fluorescence quenching of DNA dyes. *Histochem Cell Biol.* 1997;108:67–75.

Erenpreiss J, Bars J, Lipatnikova V, Erenpreisa J, Zalkalns J. Comparative study of cytochemical tests for sperm chromatin integrity. *J Androl.* 2001;22:45–53.

Erenpreiss J, Hlevicka S, Rauda R. High impact of male infertility in barren couples in Latvia, 1998–2001. *Proc Latv Acad Sci. Ser B* 2002; 56:48–51.

Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm heterogeneity to fertility. *Science.* 1980;240:1131–1133.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in human fertility clinic. *Hum Reprod.* 1999;14:1039–1049.

Gorczyca W, Traganos F, Jesionovska H, Darzynkiewicz Z. Presence of DNA strand breaks and increased sensitivity of DNA to in situ denaturation in abnormal human sperm cells: analogy to apoptosis in somatic cells. *Exp Cell Res.* 1993;207:202–205.

Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified Comet assay. *Mol Hum Reprod.* 1996;2:613–619.

Hughes CM, McKelvey-Martin V, Lewis SEM. Human sperm DNA integrity assessed by the Comet and ELISA assays. *Mutagenesis.* 1999; 14:71–75.

Ibrahim ME, Moussa MAA, Pedersen H, Sperm chromatin heterogeneity as an infertility factor. *Arch Androl.* 1988;21:129–133.

Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA, Aitken RJ. DNA integrity in human spermatozoa: relationships with semen quality. *J Androl.* 2000;21:33–44.

Iwasaki A, Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril.* 1992;57:409–416.

Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril.* 1997;68:519–524.

Krzyzosiak J, Evenson D, Pitt C, Jost L, Molan P, Vishwanath R. Changes in susceptibility of bovine sperm to in situ DNA denaturation during prolonged incubation at ambient temperature under conditions of exposure to reactive oxygen species and nuclease inhibitor. *Reprod Fertil Dev.* 2000;12:251–261.

Larson KL, DeJonge CJ, Barnes AM, Jost LK, Evenson DP. Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum Reprod.* 2000;15: 1717–1722.

Liu DY, Baker HWG. A new test for the assessment of sperm-zona pellucida penetration: relationship with results of other sperm tests and fertilization in vitro. *Hum Reprod.* 1994;9:489–496.

Omu AE, Al-Quattan F, Al-Abdul-Hadi FM, Fatinikun MT, Fernandes S. Seminal immune response in infertile men with leukocytospermia: effect on antioxidant activity. *Eur J Obstet Gynecol Reprod Biol.* 1999;86:195–202.

Pasqualotto FF, Sharma RK, Nelson DR, Thomas A, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. *Fertil Steril.* 2000;73:459–464.

Sharma KR, Pasqualotto F, Nelson D, Thomas AJ Jr, Agarwal A. Relationship between seminal white blood counts and oxidative stress in men treated in infertility clinic. *J Androl.* 2001;22:575–583.

Spano M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E, Leter G. The Danish first pregnancy planner study team. Sperm chromatin damage impairs human fertility. *Fertil Steril.* 2000;73:43–50.

Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Predicting the fertilizing potential of human sperm suspensions in vitro: importance of sperm morphology and leukocyte contamination. *Fertil Steril.* 1995;63: 1293–1300.

Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil Steril.* 1984;42:87–91.

Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. Analysis of the impact of intracellular reactive oxygen species on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum Reprod.* 1998; 13:1429–1436.

Vorobjeva OA, Filatov MV, Leontjeva OA, Semenova EV. Influence of anomalous sperm chromatin organisation on development of human embryos [in Russian]. *Probl Reprod.* 1998;1;14–18.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction.* 4th ed. Cambridge, United Kingdom: Cambridge University Press; 1999.