

Infertile Men With Varicocele Show a High Relative Proportion of Sperm Cells With Intense Nuclear Damage Level, Evidenced by the Sperm Chromatin Dispersion Test

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ABSTRACT: The frequency of sperm cells with fragmented DNA was studied in a group of 18 infertile patients with varicocele and compared with those obtained in a group of 51 normozoospermic patients, 103 patients with abnormal standard semen parameters, and 22 fertile men. The spermatozoa were processed to discriminate different levels of DNA fragmentation using the Halosperm® kit, an improved Sperm Chromatin Dispersion (SCD) test. In this technique, after an acid incubation and subsequent lysis, those sperm cells without DNA fragmentation show big or medium-sized halos of dispersion of DNA loops from the central nuclear core. Otherwise, those spermatozoa containing fragmented DNA either show a small halo, exhibit no halo with solid staining of the core, or show no halo and irregular or faint stain of the remaining core. The latter, that is, degraded type, corresponds to a much higher level of DNA-nuclear damage. The varicocele patients showed $32.4\% \pm 22.3\%$ of spermatozoa with fragmented DNA, significantly different from the group of fertile subjects ($12.6\% \pm 5.0\%$). Nevertheless, this was not different from that of normozoospermic patients ($31.3\% \pm 16.6\%$) (P

$= .83$) and with abnormal semen parameters ($36.6\% \pm 15.5\%$) ($P = .31$). No significant differences were found between the normozoospermic patients and the patients with abnormal semen parameters. Strikingly, the proportion of the degraded cells in the total of sperm cells with fragmented DNA was 1 out of 4.2 ($23.9\% \pm 12.9\%$) in the case of varicocele patients, whereas it was 1 out of 8.2 to 9.7 in the normozoospermic patients ($11.1\% \pm 9.9\%$) in the patients with abnormal sperm parameters ($12.2\% \pm 8.3\%$) and in the fertile group ($10.3\% \pm 7.2\%$). Thus, whereas no differences in the percentage of sperm cells with fragmented DNA were evident with respect to other infertile patients, individuals with varicocele exhibit a higher yield of sperm cells with the greatest nuclear DNA damage level in the population with fragmented DNA. This finding illustrates the value of assessing different patterns of DNA-nuclear damage within each sperm cell and the particular ability of the Halosperm® kit to reveal them.

Key words: Human sperm, DNA fragmentation.

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Varicocele, that is, dilation of the pampiniform venous plexus above and around the testicle, occurs in approximately 15% to 20% of the general male population, especially in adolescents. Varicocele occurs in 19% to 41% of men seeking infertility treatment and in around 80% of men with secondary infertility. Thus, this anatomical abnormality is perhaps one of the most common causes of poor sperm production and decreased semen quality.

Nevertheless, the effects of varicocele on sperm quality and quantity are difficult to define and predict. The mechanisms by which varicoceles may lead to spermatogenic failure are not well recognized (Marmar, 2001). An elevated scrotal temperature caused by impaired circulation appears to be the most reproducible defect. In fact, a varicocele created in an experimental animal led to poor sperm function with elevated intratesticular temperature.

Recently, a higher frequency of sperm cells with fragmented DNA has been reported in the ejaculate of subjects with varicocele, in comparison with fertile donors (Saleh et al, 2003; Chen et al, 2004). This phenomenon may be correlated with an increase of reactive oxygen species (ROS), resulting in oxidative stress (OS), which causes lipid peroxidation of sperm plasma membrane and nuclear DNA damage. Nitric oxide (NO) released by the endothelial cells from the dilated spermatic veins and the

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peroxynitrite generated by its reaction with the superoxide radical is probably an important source of such oxidative damage (Mitropoulos et al, 1996). DNA fragmentation may be a direct expression of this damage or a consequence of triggering of an apoptotic-like process by the ROS overproduction. These seminal abnormalities were also evidenced in experimental varicocele in rats, where enhanced ROS production and an increase in the sperm cells with fragmented DNA was demonstrated.

Enzymatic end-labeling with terminal nucleotidyl transferase (TUNEL) or the sperm chromatin structure assay (SCSA) have been used to determine DNA fragmentation in sperm cells in subjects with varicocele. Recently, we developed a new technique, called sperm chromatin dispersion (SCD) test, as a simple procedure to analyze sperm DNA fragmentation (Fernández et al, 2003). The SCD has been improved and developed as the Halosperm® kit, to be used mainly with the conventional bright-field microscope (Fernández et al, 2005). Using this procedure, sperm cells, immersed on an agarose microgel on a slide, are incubated in an acid solution to transform DNA breaks into single-stranded DNA motifs, and then in a lysing solution to remove membranes and proteins. The resulting partially deproteinized nucleus, that is, the nucleoid, consists of a central core and a halo of dispersion of DNA loops. The sequential DNA breakage detection-fluorescence in situ hybridization (DBD-FISH) demonstrated that those sperm nuclei without fragmented DNA show big or medium-sized halos, whereas those containing DNA fragmentation appear with a small halo, without a halo and with a solid staining of the core, or without halo and irregular or faint staining of the core, that is, degraded. This consecutive pattern reflects a progressive higher yield of DNA-nuclear damage, as evidenced by the improved SCD test. Thus, the degraded-type corresponds to the strongest nuclear damage that also compromises the nuclear matrix, so possibly DNA and the residual proteins from the core result partially removed under the technical conditions.

We have determined the sperm DNA fragmentation, detailing the relative profile of different categories of DNA-nuclear damage resulting after the processing by the Halosperm® kit, in sperm cells from infertile patients with varicocele. The results have been compared with those from fertile men and those with idiopathic infertility, either from normozoospermic patients or from patients with abnormal standard semen parameters.

Materials and Methods

Study Groups

Two hundred men were analyzed and categorized into 4 different groups. First, a group of 18 infertile patients diagnosed with

varicocele by scrotal Doppler ultrasound. The median age of these subjects was 29 (range: 18–37). Ten men presented with grade I, 7 with grade II, and 1 with grade III. Second, a group of 51 normozoospermic patients attending the infertility clinic. Third, a group of 103 patients with abnormal standard semen parameters, also assessed in the infertility clinic. Finally, a group of 22 fertile men who had initiated a normal pregnancy within the past 12 months. Men belonging to the second, third, and fourth groups had normal genital examination. Written consent was obtained from all participants. Semen analysis was performed according to the World Health Organization guidelines (WHO, 1999). Samples with abnormal semen parameters are defined as those having a sperm concentration less than or equal to 20 million/mL and/or a sperm motility less than 50% and/or normal forms less than or equal to 14%. All the varicocele samples had abnormal standard semen parameters.

SCD Test

The SCD test was developed as the Halosperm® kit (INDAS Laboratories, Madrid, Spain). An aliquot of each sperm sample was diluted to 10 million/mL in PBS. Gelled aliquots of low melting-point agarose in Eppendorf tubes are provided in the kit, each 1 to process a semen sample. The Eppendorf tube was placed in a water bath at 90°C to 100°C for 5 minutes to fuse the agarose, and then in a water bath at 37°C. After 5 minutes incubation for temperature equilibration at 37°C, 60 µL of the diluted semen sample was added to the Eppendorf tube and mixed with the fused agarose. Twenty microliters of the semen-agarose mix was pipetted onto an agarose precoated slide, provided in the kit, and covered with a 22- × 22-mm coverslip. The slide was placed on a cold plate in the refrigerator (4°C) for 5 minutes to allow the agarose to produce a microgel with the sperm cells trapped. The coverslip was gently removed and the slide immediately immersed horizontally in an acid solution, previously prepared by mixing 80 µL of HCl from an Eppendorf tube in the kit, with 10 mL of distilled water, and incubated for 7 minutes. The slides were horizontally immersed in 10 mL of the lysing solution for 25 minutes. After washing 5 minutes in a tray with abundant distilled water, the slides were dehydrated in increasing ethanol baths (70%–90%–100%) for 2 minutes each and air dried. The slides may be stored for several months in a tightly closed box in the dark, at room temperature, or immediately stained for fluorescence or bright-field microscopy. For the latter, the slide was horizontally covered with a mix of Wright's solution (Merck, Darmstadt, Germany) and phosphate buffer solution (Merck) (1:1) for 5 to 10 minutes, with continuous airflow. Then the slide was briefly washed in tap water and allowed to dry. Strong staining is preferred to easily visualize the periphery of the dispersed DNA loop halos. A minimum of 500 spermatozoa per sample were scored under the 100× objective of the microscope.

Scoring Criteria

The categorization of the different halo sizes is performed using the minor diameter of the core from the own nucleoid as a reference to which the halo width is compared. Five SCD patterns were established (Fernández et al, 2005): a) Sperm cells with large halos: those whose halo width is similar or higher than the

Sperm chromatin dispersion (SCD) data (mean \pm standard deviation) from semen samples from infertile patients with varicocele, infertile normozoospermic patients, infertile patients with abnormal semen parameters, and fertile subjects

	% Big Halo	% Medium- Size Halo	% Small Halo	% Without Halo	% Degraded	% Fragmented	% Degraded/ Fragmented
Varicocele (n = 18)	57.6 \pm 21.3	9.4 \pm 4.3	12.5 \pm 9.2	11.8 \pm 10.3	8.1 \pm 7.1	32.4 \pm 22.3	23.9 \pm 12.9
Infertile normozoospermic (n = 51)	61.5 \pm 16.7	7.2 \pm 5.5	12.2 \pm 6.6	15.1 \pm 10.2	4.0 \pm 4.8	31.3 \pm 16.6	11.1 \pm 9.9
Infertile with abnormal semen parameters (n = 103)	56.1 \pm 15.6	7.3 \pm 4.4	13.2 \pm 6.2	18.9 \pm 12.2	4.6 \pm 4.3	36.6 \pm 15.5	12.2 \pm 8.3
Fertile (n = 22)	71.0 \pm 8.2	16.5 \pm 6.9	6.5 \pm 3.1	4.7 \pm 1.9	1.4 \pm 1.0	12.6 \pm 5.0	10.3 \pm 7.2

minor diameter of the core. b) Sperm cells with medium-size halos: the halo size is between those with high and with very small halo. c) Sperm cells with very small-size halo: the halo width is similar or smaller than one third of the minor diameter of the core. d) Sperm cells without a halo. e) Sperm cells without a halo-degraded: similar to d but weakly or irregularly stained. Sperm cells with very small halos, without halos, and without halo-degraded contain fragmented DNA. Nucleoids that do not correspond to sperm cells were separately scored.

Statistical Analysis

All data were analyzed by using the SPSS package software. Statistical analysis was carried out using nonparametric Mann-Whitney *U* test ($P < .01$).

Results

The Table shows the distribution of the different categories of halo size in the different groups here examined for sperm DNA fragmentation using the SCD test. The frequency of sperm cells with fragmented DNA was 32.4 ± 22.3 in the patients with varicocele. This was 2.6 times higher than that obtained in the fertile subjects (12.6 ± 5.0) ($P < .0001$). Nevertheless, no significant differences were found in the rate of sperm cells with fragmented DNA when compared with other infertile patients, the normozoospermic patients showing 31.3 ± 16.6 ($P = .96$) and those with abnormal semen parameters 36.6 ± 15.5 ($P = .061$). These 3 groups could not be differentiated by the frequency of spermatozoa containing fragmented DNA.

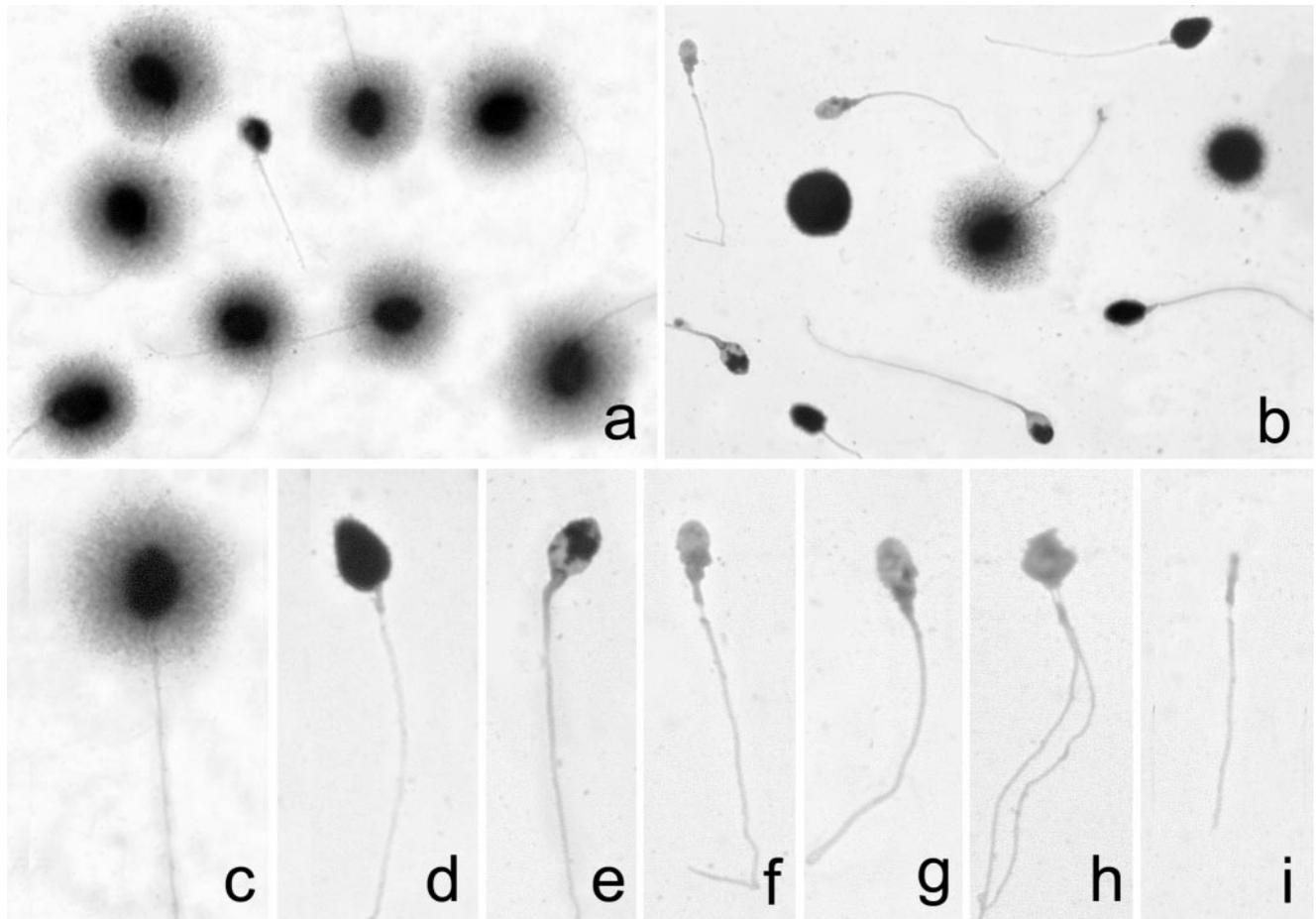
A different situation was revealed when the different patterns of sperm cells with fragmented DNA were evaluated. Specifically, the contribution of the sperm cells with the higher DNA-nuclear damage level, that is, the degraded type, to the whole population of sperm cells with fragmented DNA, was determined (the Table; the Figure). The proportion of the degraded cells in the total of sperm cells with fragmented DNA was 1 out of 4.2 (23.9 ± 12.9) in the case of varicocele patients, whereas it was 1 out of 8.2 to 9.7 in the normozoospermic patients (11.1 ± 9.9) ($P < .0001$), in the patients with abnormal sperm parameters (12.2 ± 8.3) ($P < .0001$), and in the

fertile group (10.3 ± 7.2) ($P = .001$). Thus, this proportion was not statistically significant among the 3 later groups, but significantly higher in the group with varicocele.

Discussion

Recent studies demonstrated that the varicocele samples contain a significantly higher proportion of sperm cells with fragmented DNA than those from fertile men. The mean percentage of spermatozoa with fragmented DNA determined using the SCSA was reported to be 25% (Saleh et al, 2003), close to the 23% obtained with the TUNEL assay (Chen et al, 2004). Our results using the Halosperm[®] kit confirm those findings. Nevertheless, a slightly higher proportion was evidenced in our varicocele samples (32.4%) in relation to the fertile controls (12.6%). This may reflect a slightly higher sensitivity of the SCD procedure to detect sperm cells with fragmented DNA, as reported (Fernández et al, 2005). Because all the varicocele samples showed abnormal standard semen parameters, we compared them with those from idiopathic infertility, either normozoospermic patients and from patients with abnormal semen parameters, all attending the infertility clinic. All the varicocele samples showed abnormal standard semen parameters. No significant differences were found between the 3 groups. In conclusion, the varicocele samples do not have a particular range of frequency of sperm cells with fragmented DNA that may orient to the presence of varicose spermatic veins, especially in cases of difficult clinical diagnosis. The study of Saleh et al (2003) also did not find significant differences in the frequency of sperm cells with fragmented DNA between the varicocele group and an infertile group.

Otherwise, the absence of significant differences in the frequency of sperm cells with fragmented DNA between the normozoospermic patients and the patients with abnormal semen parameters should be considered in relative terms. In a previous report, we found that a group of 23 oligoasthenoteratozoospermic patients showed a mean of 47.3% of DNA fragmentation level (Fernández et al, 2005), significantly higher than that from the normozoospermic



Sperm DNA fragmentation determined with the sperm chromatin dispersion (SCD) test using the Halosperm® kit. **(a)** Sample from a fertile subject, only showing a sperm nucleus with fragmented DNA in the microscopic field, evidenced by the absence of halo. **(b)** Sample from an individual with varicocele, showing 7 sperm cells with fragmented DNA. Four of them correspond to the degraded type, with irregular or lower staining. Moreover, 2 nonsperm cells, with big nucleus and without tail, are evident. Detailed images are presented in **(c–i)**. **(c)** Sperm cell without DNA fragmentation, with big halo. **(d)** Sperm cell with fragmented DNA, without halo. **(e–i)** Selected sperm cells with DNA fragmentation and degraded.

spermic patients. This suggests that the group with abnormal semen parameters is very heterogeneous, and probably those samples with more intense and combined abnormalities could have more frequency of sperm cells with fragmented DNA. Thus, differences in the DNA fragmentation level should be found depending on the relative contribution of certain subgroups to the whole group of patients with abnormal semen parameters.

The discrimination of different degrees of DNA fragmentation is a unique attribute of the SCD test. The sperm cells with fragmented DNA may show small halos of dispersion of DNA loops, exhibit no halo but a solid stained core, or no halo with a faint or irregularly stained core. This gradation pattern reflects a progressively stronger DNA-nuclear damage. The latter, that is, the degraded type, must also compromise the nuclear matrix because, under the initial SCD protocol, they appear pulverized in small fragments (Fernández et al, 2003). This fact, together with the lack of preservation of the tail, sometimes

makes it difficult to accurately discriminate spermatozoa from other cell types. The new SCD protocol developed in the Halosperm® kit is much less aggressive than the previous one, with better chromatin preservation and retention of the sperm tail, for the accurate discrimination of this extreme type of nuclear damage. Within the population with fragmented DNA, the contribution of the sperm cells with small halo and those without halo but with solid staining of the core is similar between each of the 4 groups. Nevertheless, 1 out of 4.2 sperm cells with fragmented DNA belong to the degraded type, in the varicocele samples. This duplicates the proportion of that observed in the normozoospermic patients, the patients with abnormal semen parameters, and in the fertile men. In these groups, it was found that 1 out of 8.2 to 9.7 cells with fragmented DNA had an extreme level of nuclear damage. Overall, our study shows that the mean frequency of sperm cells with fragmented DNA is almost twofold higher in infertile than in fertile men. Nevertheless, the

relative proportion of extremely damaged sperm cells in the whole population containing fragmented DNA seems low and independent of the fertility status, except in the varicocele group. Thus, the determination of the contribution of the degraded type to the population with fragmented DNA could perhaps orient to the presence of a varicocele, as a complementary adjunct test in the diagnostic study, as the proposed determination of ROS and total antioxidant activity in semen (Saleh et al, 2003; Allamaneni et al, 2004). Moreover, it could be of great interest to assess if varicocelectomy diminishes the frequency of sperm cells with fragmented DNA, as recently reported (Zini et al, 2005), as well as the relative contribution of those with extreme nuclear damage to this population.

The nature of the nuclear damage in the degraded type deserves further investigation in our laboratories. It could be related to a strong and prolonged exposure to DNA-nuclear-damaging factors. Not only the DNA, but the proteins, especially those from the nuclear matrix, would be affected, leading to an advanced lytic stage. An enhancement in OS, both due to an increase in ROS production and a decrease in the antioxidant capacity, has been reported in men with varicocele (Barbieri et al, 1999; Hendin et al, 1999). Moreover, the increase in seminal ROS levels seems correlated with varicocele grade (Allamaneni et al, 2004). This is a well-known factor that may induce DNA fragmentation either in vivo or in vitro (Agarwal et al, 2003). NO and peroxynitrite, a potent oxidant ROS, have been demonstrated to be produced in high concentrations in the dilated spermatic veins, so they could be main contributors to the high OS level in varicocele (Mitropoulos et al, 1996; Romeo et al, 2001; Turkyilmaz et al, 2004). Besides the dilated veins, ROS may be released in the seminiferous tubules by the cytoplasmic droplets retained in immature spermatozoa (Ollero et al, 2001), which seem to be frequent in the sperm samples from infertile men with varicocele (Zini et al, 2000). Immaturity is a consequence of defective spermiogenesis that could also lead to differences in disulfide crosslinking and in susceptibility toward DNA fragmentation.

Our results after incubating seminal samples with the NO donor sodium nitroprusside revealed a dose-response effect in the induction of DNA fragmentation, using the Halosperm® kit (Fernández et al, 2005). Nevertheless, this increase was due to those nucleoids with small halo and those without halo and solid staining of the core, those belonging to the degraded type remaining unchanged with respect to the untreated controls. This suggests that the ROS-induced DNA damage does not appreciably affect the architecture of the nuclear matrix. Nevertheless, this is an acute exposure, while the in vivo situation supposes a chronic OS, possibly mixed with cytokines and proteases, that may damage not only the DNA but also the

nuclear proteins in a significant way. If this is the case, the relatively high frequency of sperm nuclei with the strongest nuclear damage level within the population of sperm cells with fragmented DNA may not only result from varicocele. Other pathologies, such as chronic infections or inflammatory processes, should be evaluated for this effect. Another possibility could be that this subgroup of spermatozoa is more susceptible to the ROS-induced nuclear damage, as previously cited. In fact, it has been reported that infertility due to male factor and those presenting varicocele may produce spermatozoa with less condensed chromatin (Molina et al, 2001), so ROS could have a better accessibility to the inside of these nuclei, producing a higher damage level. Independent of the mechanisms, our results indicate that the assessment of the relative profile of the different patterns of nuclear damage level, allowed by the SCD test, may be of great value in sperm analysis.

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