Published-Ahead-of-Print August 9, 2006, DOI:10.2164/jandrol.106.000067
Journal of Andrology, Vol. 28, No. 1, January/February 2007

DOI: 10.2164/jandrol.106.000067

Increased Aneuploidy Rate in Sperm With Fragmented DNA as Determined by the Sperm Chromatin Dispersion (SCD) Test and FISH Analysis

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Received for publication December 22, 2005; accepted for publication August 8, 2006.

Abstract

Previous studies suggest that sperm DNA fragmentation may be associated with aneuploidy. However, currently available tests have not made it possible to simultaneously perform DNA fragmentation and chromosomal analyses on the same sperm cell. The recently introduced sperm chromatin dispersion (SCD) test allows users to determine this relationship. Semen samples from 16 males, including 4 fertile donors, 7 normozoospermic, 3 teratozoospermic, 1 asthenozoospermic, and 1 oligoasthenoteratozoospermic, were processed for DNA fragmentation analysis by the SCD test using the Halosperm kit. Three-color fluorescence in situ hybridization (FISH) was performed on SCD-processed slides to determine aneuploidy for chromosomes X, Y, and 18. Spermatozoa with DNA fragmentation showed a 4.4 ± 1.9-fold increase in diploidy rate and a 5.9 ± 3.5-fold increase in disomy rate compared to spermatozoa without DNA fragmentation. The overall aneuploidy rate was 4.6 ± 2.0-fold higher in sperm with fragmented DNA (Wilcoxon rank test: $P < .001$ in the 3 comparisons). A higher frequency of DNA fragmentation was found in sperm cells containing sex chromosome aneuploidies originated in both first and second meiotic divisions. The observed increase in aneuploidy rate in sperm with fragmented DNA may suggest that the occurrence of aneuploidy during sperm maturation may lead to sperm DNA fragmentation as

part of a genomic screening mechanism developed to genetically inactivate sperm with a defective genomic makeup.

Key words: Human, fragmentation

The presence of spermatozoa with extensive DNA breaks is a well-established observation in human ejaculates. A number of studies have demonstrated that the proportion of sperm with fragmented DNA appears to be higher in infertile males compared to fertile controls (Ollero et al, 2001; Agarwal and Said, 2003). Moreover, men with abnormal semen parameters are more likely to show a higher percentage of sperm nuclear DNA damage than men with normal semen parameters (Lopes et al, 1998; Irvine et al, 2000; Ollero et al, 2001; Sakkas et al, 2003). Defects in chromatin and DNA structure are important parameters to evaluate in order to assess sperm quality. Accumulating evidence shows that they could be indicative of male subfertility regardless of sperm concentration, percent motility, and morphology (Evenson et al, 1999, 2002). The determination of sperm DNA fragmentation, using a variety of assays, could be of great value in the assessment of the fertility potential of spermatozoa in vivo (Evenson et al, 1999; Spano et al, 2000), after intrauterine insemination (IUI; Duran et al, 2002; Bungum et al, 2004), in vitro fertilization (IVF; Sun et al, 1997; Henkel et al, 2004), or intracytoplasmatic sperm injection (ICSI) (Lopes et al, 1998; Virro et al, 2004).

Although the significance of sperm DNA fragmentation in human reproduction is well established, the underlying mechanisms responsible for the induction of DNA fragmentation in sperm are poorly understood. Several mechanisms have been proposed to explain the presence of sperm with fragmented DNA in the ejaculate (Agarwal and Said, 2003). Sperm DNA breaks could be result of defective spermiogenesis, due to unrepaired DNA breaks generated during the process of chromatin remodeling (Manicardi et al, 1995; McPherson and Longo, 1992). Another mechanism could be DNA damage induced by oxidative stress. This could be the result of exposure of mature spermatozoa to excessive levels of reactive oxygen species (ROS) produced by immature sperm during comigration from the seminiferous tubules to the epididymis (Aitken et al, 1998,Aitken et al, 1998; Ollero et al, 2001; Agarwal et al, 2003). Finally, DNA fragmentation could be caused by an apoptotic DNA degradation process (Gorczyca et al, 1993) resembling that observed in somatic cells.

Sperm may also be genetically defective at the chromosomal level, containing numerical and/or structural chromosomal aberrations (Egozcue et al, 2000). The use of the interspecific in vitro fertilization system between human sperm and golden hamster oocytes has allowed the study of sperm-derived chromosomes. These sperm karyotyping studies have demonstrated that human spermatozoa contain higher baseline numerical and structural chromosome aberrations compared to somatic cells, as well as a higher incidence of chromosome aberrations after in vitro and in vivo exposure to different mutagens (Martin et al, 1989; Genesca et al, 1990; Kamiguchi and Tateno, 2002). More recently, the use of fluorescence in situ hybridization (FISH) has demonstrated a higher rate of sperm chromosome aneuploidies in infertile men compared with fertile men (Levron et al, 2001; Ohasi et al, 2001). Furthermore, the frequency of aneuploidies appears to be higher in semen samples of poor quality (eg, from oligoasthenoteratozoospermic patients) (Vegetti et al, 2000; Rubio et al, 2001). The higher incidence of chromosome anomalies in sperm from infertile men and from males with poor sperm quality is correlated with higher levels of sperm DNA fragmentation. Furthermore, it has been shown that sperm with a higher aneuploidy rate have lower pregnancy and implantation rates after ICSI (Rubio et al, 2001), similarly to those samples with higher levels of DNA fragmentation (Lopes et al, 1998,Lopes et al, 1998; Virro et al, 2004).

We have recently developed a new test for the determination of DNA fragmentation in human sperm (Fernández et al, 2003). Sperm are immersed in an agarose matrix on a slide, treated with an acid solution to denature DNA strands with DNA breaks, and then lysed to remove the membranes and proteins. Removal of nuclear proteins results in nucleoids with a core and with a peripheral halo of dispersion of DNA loops. Recognition/detection of DNA breaks with the DNA breakage fluorescence in situ hybridization (DBD-FISH) procedure (Fernández and Gosálvez, 2002) demonstrated that those sperm nuclei with DNA fragmentation do not produce halos of dispersion of DNA loops or produce very small halos, whereas those without DNA fragmentation release their DNA loops forming large halos. This relatively simple technique has been designated the sperm chromatin dispersion (SCD) test, and has been recently modified and improved (Halosperm kit; INDAS Laboratories, Madrid, Spain) (Fernández et al, 2005).

The SCD test is the only DNA fragmentation assay currently available that allows the simultaneous determination of sperm DNA fragmentation and chromosomal analysis by FISH on the same sperm cell. Although FISH analysis could be performed on the comets (Santos et al, 1997), those comets with fragmented DNA show extremely diffused fragments, thus making discrimination of the signals difficult. In contrast, these DNA fragments remain close together when the SCD test is performed. In addition, the use of the Halosperm kit further facilitates FISH analysis compared to the original SCD test protocol (Fernández et al, 2003). In the latter protocol (Fernández et al, 2005), the lysis is considerably more aggressive, resulting in 1) removal of the sperm tails and 2) removal of the chromatin from the halos after FISH denaturation, incubation, and washing steps. In contrast, the newly improved SCD protocol, using the Halosperm kit, allows better preservation of both the flagellum and nuclear chromatin. Therefore, sperm cells can be better discriminated from other cell types, and the chromatin is more resistant to the denaturation and washing steps used in FISH. This advantage was exploited in the present study to determine, for the first time, the incidence of aneuploidies in spermatozoa with fragmented DNA compared to spermatozoa without DNA fragmentation.

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Semen Samples

Semen samples from 16 males, 4 fertile and the rest from couples attending an infertility clinic, were used in this study. Seven of these samples were normozoospermic, 3 teratozoospermic, 1 asthenozoospermic, and 1 was oligoasthenoteratozoospermic. All of them except the oligoasthenoteratozoospermic patient resulted in pregnancy using assisted reproduction procedures. Semen analysis was performed according to the World Health Organization (1999) guidelines.

SCD Test

The newly modified and improved version of the SCD test (Halosperm kit; INDAS Laboratories) was used in this study. An aliquot of each semen sample was diluted to a concentration of 10 million spermatozoa/mL in phosphate-based saline (PBS) medium. Eppendorf tubes containing gelled aliquots of low-melting point agarose are provided with the kit to process 1 semen sample. The Eppendorf tube was placed in a water bath at 90 °C to 100 °C for 5 minutes to melt the agarose, and then placed in a water bath at 37 °C. After 5 minutes incubation, to allow for equilibration to 37 °C, 60 µL of the diluted semen sample was added to the Eppendorf tube and mixed with the fused agarose. Aliquots of 20 µL of the semen-agarose mixture were pipetted onto an agarose precoated slide, provided with the kit, and covered with a 22 x 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 trapped sperm

cells inside. 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 provided with the kit, with 10 mL of distilled water, and incubated for 7 minutes at room temperature (22 °C). The slide was horizontally immersed in 10 mL of the lysing solution for 25 minutes. After washing 5 minutes in a tray with abundant distilled water, the slide was dehydrated in increasing ethanol baths (70%—90%—100%) for 2 minutes each, air-dried, and stored in a tightly closed box in the dark at room temperature.

FISH Analysis

FISH analysis was performed on the sperm cells processed for the SCD test immersed in the dried microgel. It should be pointed out that the agarose microfilm is very delicate, so the typical DNA denaturation with 70% formamide/2xSSC at 70 °C may disrupt it. The dried slides were incubated with 10% formaldehyde in phosphate buffer for 12 minutes, washed in excess of phosphate buffer for 1 minute, and denatured by incubation in NaOH 0.05N/50% ethanol for 15 seconds. Then they were dehydrated in solutions of increasing ethanol concentration (70%—90%—100%) for 2 minutes each, air-dried, and incubated with with a mixture of denatured DNA probes for the alphoid centromeric regions of X chromosome (DXZ1 Locus, SpectrumGreen; Vysis, Inc, Izasa, Spain), Y chromosome (DYZ3 locus, SpectrumOrange; Vysis), and chromosome 18 (D18Z1 locus, SpectrumAqua; Vysis). After overnight incubation at 37 °C, the slides were washed in 50% formamide/2xSSC, pH 7, during 8 minutes, and in 2xSSC, pH 7, for 5 minutes, both at 44 °C. Cells were counterstained with DAPI (2 µg/mL; Roche Diagnostics, Barcelona, Spain) in Vectashield (Vector Laboratories, Burlingame, Calif).

FISH analysis was also performed on conventional sperm spreads. The sperm cells were centrifuged and resuspended in 4 mL of methanol:acetic acid (3:1) at 4 °C. After an additional centrifugation step, the sperm cells were resuspended in methanol:acetic acid, spread onto glass slides, and air-dried. To allow the DNA probes to access the chromatin, the sperm nuclei were partially decondensed by incubation in the lysing solution from the Halosperm kit, which had been previously diluted in distilled water (1:3), for 12 minutes. Then they were washed in abundant distilled water, incubated in increasing ethanol baths (70%—90%—100%) for 2 minutes each, and air-dried. The slides were denatured in 70% formamide/2xSSC, pH 7, for 2 minutes, dehydrated in solutions of increasing ethanol concentrations (70%—90%—100%), air-dried, and incubated with a mixture of denatured DNA probes, as described before.

In order to rule out the potential occurrence of mechanical breaks during the procedure, 2 SCD processed samples were denatured and hybridized with a commercial MALT1 dual color probe mix (Vysis). This consists of a 460-kb probe labeled in SpectrumOrange that flanks the 5' side of the MALT1 gene and a 660-kb probe labeled in SpectrumGreen that flanks the 3' side of the MALT1 gene, in the 18q21.31 region. Both probes appear with a fused yellow signal or with a contiguous orange-green signal, unless a DNA break occurred within the target, with subsequent chromatin redistribution. This target is in the range of size of the alphoid sequences employed for the aneuploidy study.

Fluorescence Microscopy and Scoring Criteria

The slides were examined with a Nikon fluorescence microscope, equipped with a triple-band pass filter and with monochrome filters for DAPI, SpectrumGreen, SpectrumOrange, and SpectrumAqua for improved signal resolution. Once again, it should be pointed out that the halos of the nucleoids in the agarose microgels are very delicate, so that denaturation and washing steps tend to affect their preservation. Therefore, only those slides with well-preserved halos were analyzed. A total of about 3000 to 6000 spermatozoa were scored per sample. Those sperm nuclei that overlapped or showed nullisomy were not directly scored. The presence of sperm tails was confirmed under the SpectrumAqua

filter set of the microscope, under which it can be clearly visualized. A sperm nucleus was considered disomic when it showed 2 fluorescent domains of the same chromosome, comparable in size and brightness and separated by at least one-half diameter of the domain of 1 signal in nucleoids with big and medium halo size (ie, those without DNA fragmentation) or by a distance of at least 1 domain in those sperm nucleoids with small halo or without halo (ie, those with DNA fragmentation). This is a conservative criterion for comparison. Diploidy was established when 2 distinct chromosome 18 signals and also 2-signals for X and/or Y chromosomes were present in the same sperm nucleus. Although FISH signals in sperm nucleoids with halos may be spread, their dispersion starts from a restricted location from the core. Moreover, this origin from which the DNA fiber spread usually has a stronger intensity than that of the diffused fibers (Klaus et al, 2001). This may help overcome possible questions that may arise in a very few cases. Thus, images were taken with a high-sensitivity CCD camera at high resolution to perform an electronic manipulation of the grey levels. Selecting those pixels with higher fluorescence intensity made it possible to eliminate the dispersed signals, so an accurate discrimination of the number of signals per nucleus was achieved.

In the case of FISH analysis on conventional sperm spreads, sperm were considered disomic when showed duplicated domains with similar size, shape and intensity, being separated by a distance of at least 1 domain. Sperm nuclei were scored only if they were intact, nonoverlapped, had a clearly defined border, and had not been decondensed to more than twice the size of a nondecondensed sperm head.

Statistical Analysis

All data were analyzed using the SPSS 12.5 package software (SPSS Inc, Chicago, Ill). Wilcoxon rank test was employed for statistical contrast. Differences within each sample were analysed using Pearson's χ^2 test ($P < .05$). Associations were determined with Spearman's rank correlation.

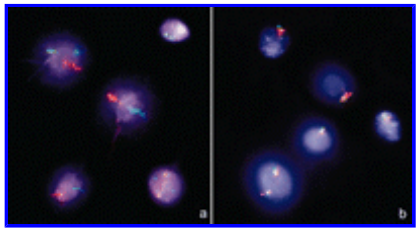

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Figure 1. Sequential FISH on SCD-processed spermatozoa. **(A)** The cores and halos of dispersion of DNA loops are presented in grey-blue, whereas the hybridized alphoid sequences from the X chromosome are shown in green, those from the Y chromosome in red, and those from chromosome 18 in blue. **Upper left**: sperm cell without DNA fragmentation, that is, with a big halo, showing an XY chromosome disomy (18, X, Y). **Upper right**: spermatozoon with fragmented DNA, that is, without halo, and without aneuploidy for the analyzed chromosomes (18, X). **Center**: sperm cell without DNA fragmentation and without aneuploidy (18, Y). **Lower left**: spermatozoon without DNA fragmentation and without aneuploidy (18, Y). The halo size is actually wider than that seen in the picture. **Lower right**: sperm cell with DNA fragmentation and diploid (18, 18, Y, Y). Note its higher core size. All of these cells had a tail, 2 in the sperm cell above left, as demonstrated under the SpectrumAqua filter set of the microscope. The nuclei shown were selected in order to show the different halo patterns of sperm DNA fragmentation and are not to be taken as representative of the true frequency of aneuploidy in sperm with DNA fragmentation. **(B)** SCD processed sperm cells hybridized with the MALT1 dual color probe. One continuous or overlapped green-red signal is observed in each sperm cell, except that in the lower left, which reveals 2 signals.

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Sperm nuclei containing DNA fragmentation were visualized as nucleoids without halo or with very small halo of dispersion of DNA loops, whereas those without DNA fragmentation exhibited large and medium-sized halos under the DAPI filter of the fluorescence microscope (Fernández et al, 2005). FISH analysis was performed on the nucleoids, so diploidies, disomies, and DNA fragmentation were determined simultaneously on the same sperm cell. Total preservation of the halos after FISH processing was obtained in 65% of the cases using the Halosperm kit and the protocol of denaturation described in "Materials and Methods." However, the preservation of the halos was very poor when the sperm cells were processed using the old SCD protocol (Fernández et al, 2003). This is probably due to the more aggressive conditions used in the original protocol. The experiments carried out in this study were only performed in slides where the chromatin from the halos remained totally preserved after the FISH procedure. Furthermore, the percentage of sperm cells with DNA fragmentation obtained by the SCD test was similar either in the FISH processed or in the nondenatured slides. Spermatozoa with fragmented DNA displayed spotted FISH signals, while these FISH signals tend to spread from the core to the halo in the nucleoids of spermatozoa without DNA fragmentation (Figure 1A).

Table 1 shows the percent values of diploidies, disomies, and overall aneuploidy rate in sperm with and without DNA fragmentation. The percentage of diploidies in cells with fragmented DNA was 2.19 (1.80–4.52) (median and Q1: 25% percentile; Q3: 75% percentile), and 0.51 (0.39–0.92) in sperm cells without DNA fragmentation. In case of disomies, the percentage was 0.88 (0.48–1.46) in sperm cells containing fragmented DNA and 0.15 (0.09–0.31) in sperm cells without DNA fragmentation. Concerning aneuploidies (diploidies and disomies), the percentage was 3.03 (2.00–5.17) in spermatozoa with DNA fragmentation and 0.66 (0.48–1.36) in those without DNA fragmentation (Figure 2). In conclusion, sperm nuclei with DNA fragmentation showed a 4.4 ± 1.9-fold increase in diploidy rate and a 5.9 ± 3.5-fold increase in disomy rate compared to those without DNA fragmentation. The overall aneuploidy rate was 4.6 ± 2.0-fold higher in sperm containing fragmented DNA (Wilcoxon rank test: $P < .001$ in the 3 comparisons). When analysing sample by sample, the differences were statistically significant in all samples except 1 in the case of diploidies, and in all except 4 subjects in the case of disomies. Nevertheless, all samples gave significant differences when considering aneuploidies as a whole (χ^2 test, $P < .05$, Table 1). No significant differences were evident between fertile and infertile subjects or between normozoospermic subjects and those with abnormal semen parameters. It is noteworthy that the higher the frequency of global diploidies, disomies, or aneuploidies in the sample, the higher the difference in the frequency of diploidies, disomies, or aneuploidies, respectively, in the fraction of sperm cells with fragmented DNA compared to the fraction without DNA fragmentation (diploidies: rho = 0.63; $P = .009$; disomies: rho = 0.66; $P = .006$; aneuploidies: rho = 0.76; $P = .001$) (Figure 3).

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Table 1. *Results of FISH analysis for chromosomes X, Y, and 18 in sperm processed by the SCD test (**IF SCD**: percentage of sperm cells with fragmented DNA; **F**: fragmented; **NF**: nonfragmented). Asterisks identify those samples without significant differences between sperm cells with and without fragmented DNA (χ^2 test; P <.05). Subjects 1-4: fertile donors; subjects 5-11: normozoospermic patients; subject 12: asthenozoospermic patient; subjects 13-15: teratozoospermic patients; subject 16: oligoasthenoteratozoospermic patient. Subjects 1, 4, 5, 6, 7, and 16 were also processed by FISH on conventional spreads (below)*

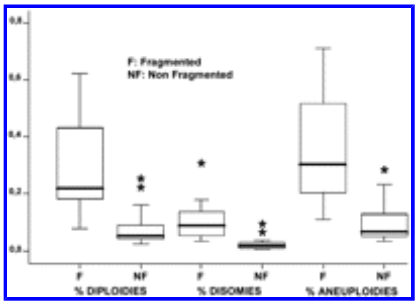

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Figure 2. Median (Q1–Q3) of the frequencies of diploidies, disomies, and aneuploidies in sperm cells with and without DNA fragmentation.

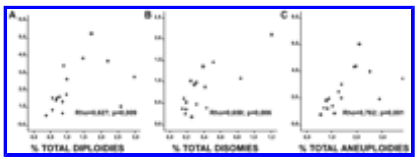

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Figure 3. Difference level in the frequency of diploidies, disomies, or aneuploidies in the fraction of sperm cells with fragmented DNA compared to the fraction without DNA fragmentation, in relation to the global frequency of diploidies, disomies, or aneuploidies, respectively. **(A)** Difference of percentage of diploidies between sperm cells with fragmented and without fragmented DNA. **(B)** Difference of percentage of disomies between sperm cells with fragmented and without fragmented DNA. **(C)** Difference of percentage of aneuploidies between sperm cells with fragmented and without fragmented DNA.

FISH analysis was also performed on conventional sperm spreads in 6 subjects (Table 1). The main objective of this experiment was to compare the overall aneuploidy rate and the diploidy and disomy rates obtained using both FISH protocols. No statistically significant differences were found in diploidy (P = .36) and disomy (P = .35) rate on either SCD-processed or conventionally processed slides. Moreover, no significant differences were found for the different types of diploidies and disomies in the 6 samples analysed with both protocols. Furthermore, the potential occurrence of mechanical breaks during the procedure was assessed hybridizing 2 SCD processed samples with the MALT1 dual color probe mix (Figure 1B). In a total of 9000 cells, no orange-green separation signal was observed in either sperm cell type, with or without DNA fragmentation. All these results rule out technical and scoring artifacts.

The distribution of diploidies and disomies for the different chromosomes analyzed are shown in Tables 2 and 3 and Figures 4 and 5. The percentage of diploidies was significantly higher in sperm cells containing fragmented DNA, either for XY type: 0.88 (0.49–2.06) vs 0.22 (0.13–0.38), P = .001; for XX type: 0.64 (0.38–1.26) vs 0.19 (0.12–0.41), P < .001; or for YY type: 0.55 (0.32–1.11) vs 0.13 (0.10–0.19), P = .001. This was also the case for XY disomies: 0.22 (0.13–0.42) vs 0.06 (0.03–0.11), P = .002. For XX disomies: 0.19 (0.00–0.33) vs 0.03 (0.00–0.07), P = .011, and for YY disomies: 0.02 (0.00–0.15) vs 0.00 (0.00–0.03), P = .041, the differences were not statistically significant at the 1% level, presumably due to the low number recorded. Overall, it was obvious that DNA fragmentation was increased in sperm cells containing diploidies and disomies originated in both the first (XY disomies and diploidies) and in the second (XX and YY diploidies and disomies) meiotic divisions.

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Table 2. *Diploidy frequencies for chromosomes X, Y, and 18, specifying the chromosomes, in sperm cells processed by the SCD test (**F**: fragmented; **NF**: nonfragmented). Subjects 1-4: fertile donors; subjects 5-11: normozoospermic patients; subject 12: asthenozoospermic patient; subjects 13-15: teratozoospermic patients; subject 16: oligoasthenoteratozoospermic patient. Subjects 1, 4, 5, 6, 7, and 16 were also processed by FISH on conventional spreads (below)*

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Table 3. *Disomy frequencies for chromosomes X, Y, and 18, specifying the chromosomes, in sperm cells processed by the SCD test (**F**: fragmented; **NF**: nonfragmented). Subjects 1-4: fertile donors; subjects 5-11: normozoospermic patients; subject 12: asthenozoospermic patient; subjects 13-15: teratozoospermic patients; subject 16: oligoasthenoteratozoospermic patient. Subjects 1, 4, 5, 6, 7, and 16 were also processed by FISH on conventional spreads (below)*

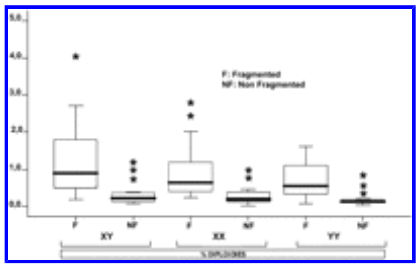

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Figure 4. Median (Q1–Q3) of the frequencies of diploidies, specifying the chromosomes, in sperm cells with and without DNA fragmentation.

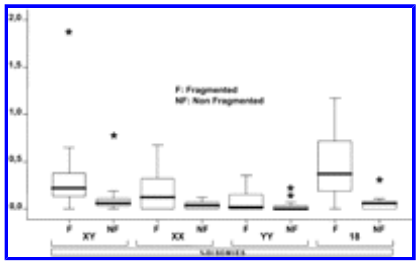

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Figure 5. Median (Q1–Q3) of the frequencies of disomies, specifying the chromosomes, in sperm cells with and without DNA fragmentation.

Discussion

The main finding emerging from this study is the significant increase in aneuploidy observed in sperm with fragmented DNA. The analysis of chromosomes X, Y, and 18 showed that aneuploidy was not randomly distributed in the sperm population. Sperm cells with fragmented DNA had on average a 4.6-fold higher level of aneuploidy compared to sperm cells with nonfragmented DNA, with an

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average 4.4- and 5.9-fold increase in diploidy and disomy rates, respectively. Although it could be argued that in sperm without DNA fragmentation 2 FISH signals from the same chromosome could be overlapped by their tendency to spread, resulting in an underestimation of the aneuploidy rate, this is a very rare event and unlikely to occur, given the scoring criteria used in this study. Nevertheless, in order to rule out this possibility, FISH analysis was also performed on conventional spreads of sperm cells from 6 subjects (Table 1). Comparison of the overall disomy and diploidy rates obtained by SCD-FISH and conventional FISH analysis did not show statistically significant differences, thus ruling out the presence of technical and scoring artifacts in SCD-processed cells. Moreover, the potential occurrence of mechanical breaks during the procedure was ruled out by hybridizing a MALT1 dual color probe mix.

The mechanisms responsible for spontaneous DNA fragmentation in spermatozoa are not well known. Three main hypotheses have been proposed. The first hypothesis postulates that DNA fragmentation is the result of changes in DNA topology during the replacement of histones by protamines that take place during mid-spermiogenesis. As a result of nucleosomal removal, a high number of unconstrained supercoils would be present in the haploid genome of the spermatid. Nevertheless, to eliminate the torsional stress and facilitate protamine deposition, an induction and subsequent repair of nicks arises in the DNA of the elongating spermatids of mouse, and possibly in the round spermatids of man (McPherson and Longo, 1992; Marcon and Boissonneault, 2004). Abnormalities during spermiogenesis could lead to an incomplete chromatin maturation process, resulting in DNA breaks that would persist unrepaired in differentiated sperm cells.

The second hypothesis postulates that DNA fragmentation is caused by oxidative stress in the male reproductive tract. High levels of reactive oxygen species (ROS) may be produced by 1) activated resident leukocytes and/or macrophages in the testis, 2) as a result of inflammatory/infectious processes, 3) by ROS-producing immature spermatozoa with excessive cytoplasmic retention (1), and 4) by nitric oxide synthase-expressing epithelial cells in the epididymis (Wiszniewska et al, 1997). In fact, in vitro exposure of sperm to high levels of ROS from chemical donors results in significant DNA damage (Aitken et al, 1998,Aitken et al, 1998; Lopes et al, 1998,Lopes et al, 1998). Moreover, spermatozoa from patients with leukocytospermia have higher levels of DNA fragmentation (Saleh et al, 2002).

The third hypothesis postulates that sperm DNA fragmentation is the result of apoptosis-induced DNA strand breaks, similar to what occurs in somatic cells (18). Programmed cell death has been shown to occur in all 3 phases of spermatogenesis (Braun, 1998). Proliferation of the diploid spermatogonia may be blocked in order to maintain a normal ratio of developing germ cells to Sertoli cells. Germ cells earmarked for apoptosis express the mediator protein Fas on their surface, which is subsequently activated by the Fas ligand (FasL) expressed by the Sertoli cell. Meiotic spermatocytes with asynapsed chromosomes may also be removed through apoptosis (Odorisio et al, 1998). Finally, the haploid spermatids may also have an inducible death pathway used in quality control. The products from gene Bclw, belonging to the Bcl2 anti-apoptotic family, may be involved in this late apoptotic pathway (Ross et al, 1998). Sperm cells with fragmented DNA could correspond to apoptotic spermatocytes that have completed the processes of meiosis and spermiogenesis. Nevertheless, it appears quite unlikely that spermatocytes with extensive DNA breaks could undergo the profound structural and functional chromatin changes that take place during the process of meiosis and spermiogenesis. Most likely, DNA fragmentation would occur during spermiogenesis (Rodriguez et al, 2005). Some studies have suggested that the classical apoptotic pathway may be at play in sperm cells. Certain caspases, like caspase 3, have been detected in the cytoplasmic droplets of immature spermatozoa, but were absent in mature sperm cells (De Vries et al, 2003). Nevertheless, other groups reported the presence of activated caspases 8, 1, and 3 in the postacrosomal region and

caspase 9 in the midpiece (Paash et al, 2004) of sperm. The externalization of phosphatidylserine in the cytoplasmic membrane is an initial event of apoptosis in most cells, being detected by annexin-V binding. In spermatozoa the presence of activated caspases appears to be associated with annexin-V binding (Paash et al, 2004), but sperm capacitation induced by bicarbonate also triggers the scrambling of phosphatidylserine (De Vries et al, 2003). The presence of mature sperm cells with apoptotic markers, like Fas, Bcl-x, p53, or annexin-V binding in the sperm membrane, especially in some infertile men, has suggested an abortive-apoptotic-like mechanism, where some germ cells, earmarked for elimination, escape the removal mechanism. Nevertheless, no correlation has been found between the presence of these apoptotic markers and DNA fragmentation (Sakkas et al, 2002; Henkel et al, 2004; Moustafa et al, 2004). That is, the presence of markers that in other cell types would be indicative of apoptosis and DNA fragmentation appears to be dissociated in the case of sperm cells. This suggests that the process of sperm DNA fragmentation, if induced via apoptosis, could be mediated by a different mechanism than that observed in other cell types.

Kovanci et al (2001) found a close relationship between the proportion of immature spermatozoa and disomies, although this did not apply to diploidies. Immature spermatozoa with excess cytoplasmic retention had a 1.5- to 4-fold higher rate of chromosomal abnormalities than mature spermatozoa, based on the analysis of chromosomes X, Y, and 17. Moreover, the frequency of aneuploidies, especially disomies, was significantly lower in sperm from the 80% Percoll fraction, that is, enriched in mature spermatozoa compared to the unprocessed semen sample. Immature spermatozoa also show higher levels of DNA fragmentation, as determined by either the sperm chromatin structure assay (Ollero et al, 2001) or the SCD test (Fernández et al, 2003). Therefore, aneuploidies and DNA fragmentation appear to preferentially occur in immature arrested sperm cells. Accordingly, it has been reported that the percentage of aneuploid sperm is associated with the percentage of apoptotic sperm, lending further support to the hypothesis of a relationship between DNA fragmentation and aneuploidy in sperm (Carrell et al, 2003; Schmid et al, 2003; Liu et al, 2004). Although it cannot be ruled out that this could be an epiphenomenon, it seems reasonable to assume that there is a causal relationship. In fact, it has been proposed that missegregated chromosomes themselves may trigger apoptotic cell death (Dobles et al, 2000), which could possibly be related to a polyploidy checkpoint (Castedo et al, 2004).

In different cell types, apoptotic DNA fragmentation by an endogenous nuclease is triggered through checkpoint pathways when DNA damage has been originated (Envan and Littlewood, 1998). Thus, in these situations, apoptosis could be related to a quality control mechanism to maintain the integrity of DNA in order to avoid the production of abnormal or unstable genomes that could promote neoplastic growth. In our case, aneuploidy could trigger DNA fragmentation, resembling an apoptotic-like process mediated by endogenous nucleases, as part of a mechanism designed to genetically inactivate a sperm nucleus with an abnormal genomic constitution. This could contribute to arresting the sperm cell at an immature stage and unable to fertilize the egg. Since mature protaminated chromatin is not very sensitive to nuclease digestion (Sakkas et al, 1995), it is more likely that this putative nuclease would fragment the DNA during chromatin maturation, since at this stage the chromatin is not highly packed. In fact, it is known that active gap filling repair occurs in the late elongating spermatid from mice, when the DNA is being tightly packed, as long as the DNA is still accessible (McMurray and Kortum, 2003). Another possibility could be that the occurrence of DNA fragmentation is a passive process and secondary to partial arrest at the nuclear level during chromatin remodeling. In this case, after detection of chromosome anomalies, the genomic surveillance mechanisms would retain the ability to repair DNA breaks originated to remove the DNA super-coiling in the process of the exchange of histones by protamines. The persistence of the DNA nicks would not prevent protamination and disulfide bonding. Further support is derived from the fact that knockout mice for telomerase, with critically short telomeres, show a 6-fold increase in the percentage of

sperm cells with fragmented DNA (Rodriguez et al, 2005). In this case, dysfunctional telomeres, or the subsequent derived chromosomal abnormalities, may be detected, triggering DNA fragmentation.

DNA breaks as a result of apoptotic DNA degradation or persistence of unrepaired DNA nicks should correspond to a massive DNA damage level. Although sperm with fragmented DNA can penetrate the oocyte (Henkel et al, 2004), embryo development would be greatly compromised, since the DNA repair capacity of the oocyte is very limited (Twigg et al, 1998; Ahmadi and Ng, 1999), both in rate and in fidelity. This might lead to sperm chromatin decondensation failure (Sakkas et al, 1996) or result in cell arrest during initial embryonic development (Henkel et al, 2004; Seli et al, 2004). In fact, although there is some controversy (Gandini et al, 2004), pregnancy rates, either natural or using IUI, IVF, and ICSI procedures, tend to be lower in patients with higher levels of sperm DNA fragmentation (Lopes et al, 1998; Evenson et al, 1999; Spano et al, 2000; Duran et al, 2002; Bungum et al, 2004; Sun et al, 1997; Henkel et al, 2004; Virro et al, 2004). Therefore, aneuploid sperm cells with fragmented DNA would not fertilize the oocyte. Even if this occurs, it would produce a nonviable embryo, thus preventing the development of an offspring with an abnormal genetic makeup. Perhaps this could be the case not only with numerical anomalies but also when certain structural chromosomal aberrations are present in the sperm nucleus.

Recently, pregnancy rates have been reported to be higher after ICSI compared to conventional IVF when sperm samples with high DNA fragmentation values were used for insemination (Host et al, 2000; Bungum et al, 2004). If confirmed, this would suggest that perhaps ICSI bypasses a screening mechanism that precludes the fertilization of oocytes by spermatozoa with fragmented DNA. Although fertilization does occur under these conditions, the fertilization of an oocyte by a spermatozoon with fragmented DNA could result in defective embryo and/or fetal development. This is consistent with studies that show that sperm DNA fragmentation could be associated with recurrent abortion (Carrell et al, 2003).

Finally, if this putative genomic surveillance mechanism failed to be triggered, this could result in the production of aneuploid spermatozoa with intact DNA, thus increasing the probability of obtaining an aneuploid conceptus. Therefore, aneuploid sperm without fragmented DNA would be even more harmful than sperm with fragmented DNA. Perhaps FISH analysis in sperm with nonfragmented DNA could be more informative of the true risk of chromosomal abnormalities in the embryo or fetus, especially when using ICSI for the treatment of couples with severe male factor infertility.

In conclusion, our results support the hypothesis that aneuploidy during sperm maturation may lead to sperm DNA fragmentation as part of a genomic surveillance mechanism developed to genetically inactivate sperm with a defective genomic makeup. Further studies are required to assess the incidence of sperm aneuploidies in sperm with intact DNA. The possible association between DNA fragmentation and aneuploidy could perhaps be dependent on the origin of the infertility. It may be speculated that those patients with spermatogenic problems could have a differential aneuploidy rate, with different levels of association depending on the infertility subgroup. Otherwise, such association could be masked in those with high oxidative damage that is produced after spermiation, that is, with sperm DNA fragmentation being produced in the genital tract through exogenous damaging agents. More extensive work should be performed to address these possibilities.

Footnotes

Supported by the Xunta de Galicia (PGIDIT 05SAN43PR).

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