human reproduction

ORIGINAL ARTICLE *Reproductive genetics*

Increased numbers of DNA-damaged spermatozoa in samples presenting an elevated rate of numerical chromosome abnormalities

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STUDY QUESTION: Is there a relationship between DNA damage and numerical chromosome abnormalities in the sperm of infertile patients?

SUMMARY ANSWER: A strong link between DNA fragmentation and the presence of numerical chromosome abnormalities was detected in human sperm. Chromosomally abnormal spermatozoa were more likely to be affected by DNA fragmentation than those that were chromosomally normal.

WHAT IS KNOWN ALREADY: Several studies have described the presence of elevated levels of DNA damage or chromosome defects in the sperm of infertile or subfertile men. However, the nature of the relationship between sperm DNA damage and chromosome abnormalities is poorly understood. The fact that some assisted reproductive techniques have the potential to allow abnormal spermatozoa to achieve oocyte fertilization has led to concerns that pregnancies achieved using such methods may be at elevated risk of genetic anomalies.

STUDY DESIGN, SIZE, DURATION: For this prospective study, semen samples were collected from 45 infertile men.

PARTICIPANTS, SETTING, METHODS: Samples were assessed for DNA fragmentation using the Sperm Chromatin Dispersion Test (SCDt) and for chromosome abnormalities using multi-colour fluorescence *in situ* hybridization (FISH) with probes specific to chromosomes 13, 16, 18, 21, 22, X and Y. Additionally, both parameters were assessed simultaneously in 10 of the samples using a protocol combining SCDt and FISH.

MAIN RESULTS AND THE ROLE OF CHANCE: A significant correlation between the proportion of sperm with a numerical chromosome abnormality and the level of DNA fragmentation was observed ($P < 0.05$). Data from individual spermatozoa subjected to combined chromosome and DNA fragmentation analysis indicated that chromosomally abnormal sperm cells were more likely to display DNA damage than those that were normal for the chromosomes tested ($P < 0.05$). Not only was this association detected in samples with elevated levels of numerical chromosome abnormalities, but it was also evident in samples with chromosome abnormality rates in the normal range.

LIMITATIONS, REASONS FOR CAUTION: The inability to assess the entire chromosome complement is the main limitation of all studies aimed at assessing numerical chromosome abnormalities in sperm samples. As a result, some of the sperm classified as 'chromosomally normal' may be aneuploid for chromosomes that were not tested.

WIDER IMPLICATIONS OF THE FINDINGS: During spermatogenesis, apoptosis (a process that involves active DNA degradation) acts to eliminate abnormal sperm. Failure to complete apoptosis may explain the coincident detection of aneuploidy and DNA fragmentation in some spermatozoa. In addition to shedding light on the biological mechanisms involved in the processing of defective sperm, this finding may also be of clinical relevance for the identification of patients at increased risk of miscarriage or chromosomally abnormal pregnancy. In some instances, detection of elevated sperm DNA fragmentation may indicate the presence of chromosomal abnormalities. It may be worth considering preimplantation genetic screening (PGS) of embryos produced using such samples in order to minimize the risk of aneuploidy.

STUDY FUNDING AND COMPETING INTERESTS: This work was supported by funding from Oxford NIHR Biomedical Research Centre programme, the Spanish Ministerio de Educación Cultura y Deporte and a Grant for Fertility Innovation (Merck Serono). The views expressed are those of the authors. No competing interests are declared.

Key words: sperm DNA damage / aneuploidy / chromosome abnormalities

Introduction

Infertility affects 8–12% of the population worldwide (WHO, 1991). It is estimated that 50–80 million people are unable to conceive after 12 months of unprotected regular sexual intercourse. Among these couples, male factor infertility accounts for (or is a significant contributory factor in) ∼50% of the cases (McLachlan and de Kretser, 2001). Male infertility is a multifactorial problem encompassing a wide range of disorders that can be due to a variety of factors (Irvine, 1998).

In some cases, the cause of male infertility is clear, resulting in an obvious defect in sperm concentration, morphology, motility or function. However, for a substantial proportion of affected men, the basis of their infertility remains unknown. There is evidence that genetic factors may be responsible for some instances of male infertility (Ferlin *et al.*, 2006). For example, there is a well-established link between male infertility and constitutional chromosomal abnormalities (Harton and Tempest, 2012). While the incidence of chromosomal rearrangements and other karyotype defects in the general population is only ∼0.6% (Berger, 1975), the incidence in males presenting with infertility is more than three times higher, between 2 and 14% (Shi and Martin, 2000). This includes sex chromosome alterations, Robertsonian or reciprocal translocations and Y microdeletions (Ferlin *et al.*, 2007). In addition to chromosome rearrangements, chromosome imbalance (i.e. aneuploidy) has also been implicated in infertility of chromosomally normal men. Several studies have suggested that aneuploidy rates in the sperm of karyotypically normal infertile men are elevated, such that a 3-fold increase in sperm aneuploidy levels compared with fertile men has been reported (Shi and Martin, 2000, 2001; Tempest and Griffin, 2004). Although aneuploid sperm have an altered genetic content, in most cases they are capable of fertilizing an oocyte and hence transmitting genetic abnormalities to the resulting embryo. To date, increases in sperm aneuploidy have been reported in samples from men presenting with a variety of abnormal semen parameters, including oligozoospermia, asthenozoospermia and teratozoospermia (Colombero *et al.*, 1999; Pang *et al.*, 1999; Pfeffer *et al.*, 1999; Calogero *et al.*, 2001). Sperm aneuploidy levels have also been reported to be strongly correlated with the severity of the infertility (Tempest and Griffin, 2004).

In addition to abnormalities affecting the chromosome copy number, another possible genetic cause of (or contributor to) male infertility is sperm DNA fragmentation (SDF). DNA integrity has emerged in recent years as a new parameter of semen quality and a potential fertility predictor (Zini and Sigman, 2009; Barratt and De Jonge, 2010; Barratt *et al.*, 2010). Several studies have reported that semen from infertile men presents a greater rate of DNA damage compared with semen from fertile donors (Sun *et al.*, 1997; Lopes *et al.*, 1998; Sergerie *et al.*, 2005). The presence of high levels of DNA damage in sperm has been proved to have adverse effects on reproductive outcomes. Fertilization, embryo development and pregnancy rates are negatively affected by SDF (Larson *et al.*, 2000; Morris *et al.*, 2002; Borini *et al.*, 2006; Benchaib *et al.*, 2007). A positive correlation between DNA damage and risk of pregnancy loss following assisted reproductive techniques (ARTs) has also been reported (Zini *et al.*, 2005, 2008; Zini and Sigman, 2009).

It is likely that some cases of male infertility may be explained by the presence of chromosomal abnormalities, DNA damage or a combination of both. A number of publications have reported elevated levels of both DNA damage and aneuploidy in the sperm of infertile or subfertile men. For example, an increased incidence of these phenomena has been described in patients with recurrent pregnancy loss (Carrell *et al.*, 2003): men with globozoospermia (Brahem *et al.*, 2011) or carriers of a constitutional chromosomal abnormality (Brugnon *et al.*, 2006; Perrin *et al.*, 2011).

Since the birth of the first baby conceived through *in vitro* fertilization over 30 years ago, the use of assisted reproduction has continued to transform the treatment of infertility. Patients with poor semen quality parameters can now utilize ARTs to father children. Assisted reproductive treatments, intracytoplasmic sperm injection (ICSI) in particular, bypass natural barriers that might normally prevent fertilization with abnormal spermatozoa. The incidence of *de novo* chromosomal abnormalities has been reported to be higher in ICSI conceptions compared with natural conceptions (Lam *et al.*, 2001; Bonduelle *et al.*, 2002; Gjerris *et al.*, 2008). This finding together with the fact that fertilization with DNA-damaged sperm can be achieved with the use of ICSI (Twigg *et al.*, 1998; Gandini *et al.*, 2004) has led to some concerns that pregnancies conceived using such methods might be at elevated risk of genetic anomalies.

The purpose of the present study is to explore the relationship between sperm DNA integrity and aneuploidy in infertile patients with normal and abnormal traditional semen quality parameters. We aimed to determine whether or not DNA fragmentation and numerical chromosome abnormality are truly independent variables and to shed light on the cellular response to chromosome imbalances in sperm.

Materials and Methods

Semen samples were collected from 45 infertile men seeking assisted reproduction treatment and two separate aliquots were taken. One aliquot was assessed using the Sperm Chromatin Dispersion Test (SCDt) for SDF analysis, while the other was used for the analysis of chromosome numerical abnormalities, employing multi-colour fluorescence *in situ* hybridization (FISH) with probes specific to chromosomes 13, 16, 18, 21, 22, X and Y. Additionally, both parameters were assessed simultaneously in 10 of the samples, using a modified protocol that combines SCDt and FISH.

Patient selection

A cohort of 45 men with a normal karyotype and undergoing assisted conception cycles between July 2011 and April 2012 were included in the study. A fertile control population including samples from 40 men of proven fertility, having conventional semen quality parameters (concentration, motility and morphology) within the normal range defined by the World Health Organization (WHO, 2010), was used for the verification of the aneuploidy levels typical of fertile males. The study was approved by the institutional ethics committee and a written informed consent form was signed by all the participants involved in the study.

Sample collection and preparation

Samples were obtained by masturbation after 48 h of sexual abstinence. Conventional semen quality parameters such as concentration, motility and morphology were assessed immediately after collection following the procedures described elsewhere (WHO: World Health Organization, 2010). The samples were classified as normozoospermic and non-normozoospermic according to the WHO criteria established in 2010.

An aliquot of 500 µl of each semen sample was used for SDF and/or chromosome analyses.

SDF analysis

For SDF analysis, the SCDt was performed using the Halosperm® kit (Halotech DNA, Madrid, Spain). Briefly, 25 µl of spermatozoa, diluted to a concentration of 1×10^7 spermatozoa/ml, were added to a vial with low-melting-point agarose and mixed. Provided agarose-coated slides were placed horizontally onto a metallic plate previously cooled at 4°C and a drop of the cell suspension was deposited onto the treated face of the slide, covered with a glass coverslip and allowed to solidify for 5 min at 4°C. The coverslip was smoothly removed and the slide was placed horizontally in 10 ml of the lysing solution provided in the kit. Finally, the slides were washed in distilled water, dehydrated in sequential 70, 90 and 100% ethanol baths and stained with DAPI (2 µg/ml; Roche, Basel, Sweden). The samples could be immediately analysed or stored at room temperature in the dark until needed. The sperm DNA fragmentation index (SDFI) was established as the percentage of fragmented sperm cells in a semen sample following the criteria established by Fernández et al. (2003) (Fig. 1). The sperm DNA degradation index (SDDI) was established as the percentage of degraded sperm cells in a semen sample (Enciso et al., 2006). Both SDFI and SDDI were calculated by assessing at least 500 spermatozoa per slide. For the SDFI, a threshold of 30% was used to discriminate between samples with normal and elevated levels of DNA-damaged spermatozoa (Chohan et al., 2006).

Sperm chromosome analysis

Semen samples were washed in phosphate-buffered saline (PBS, pH 7.2, Fischer Scientific International, Pittsburgh, PA, USA) at 400g for 5 min, the supernatant was discarded and the pellet treated with a hypotonic solution [0.56% KCl (w/v), Sigma, St Louis, MO, USA] for 20 min at 37°C. The samples were then centrifuged at 400g for 5 min, resuspended in cold methanol: acetic acid (3:1; Sigma, St Louis, MO, USA) and eventually stored at −20°C until further processing.

The fixed sperm were spread on a slide by applying 7−8 drops per sample and air-drying. The slides were washed twice in 2× saline sodium citrate (SSC, Fischer Scientific International, Pittsburgh, PA, USA) and dehydrated in an increasing ethanol series (70, 85 and 100%,

Figure 1 SDF determined with the SCDt. Human semen sample processed with the SCDt and showing two sperm cells with fragmented DNA, evidenced by the absence of a halo (red arrows), a sperm cell with degraded DNA, shown by weakly or irregularly staining and the absence of a halo (white arrow), and six sperm cells with intact DNA and a halo of dispersed DNA loops.

Sigma, St Louis, MO, USA). Sperm nuclei were decondensed using a 10 min incubation in fresh lysing solution (5 mM DTT, 0.05 M Tris Base, pH 7.4, Sigma, St Louis, MO, USA). Multi-colour FISH was used to diagnose each sperm cell for chromosomes X, Y, 13, 16, 18, 21 and 22 (Abbott Molecular Inc., Des Plaines, IL, USA). Sperm nuclei were denaturated at 72°C for 5 min in fresh 70% Formamide/2× SSC. The slides were then washed twice in 2× SSC, dehydrated in an ethanol series (70, 85 and 100%) and air-dried. Next, 3 µl of probe mixture, previously denatured at 75°C for 5 min, was added to the slide. Hybridization was performed overnight in a humid chamber at 37°C followed by washing at 71°C in 0.7× SSC/0.3% NP40 and at RT in 0.7× SSC. The slides were then counterstained in antifade solution (Vectashield, Vector Laboratories, Burlingame, CA) and were analysed using a digital image-analysis platform based on a Olympus BX 61 fluorescence microscope (Olympus, Tokyo, Japan) equipped with single-band pass fluorescence filters for the probe fluorophores used (red, green, blue, gold, aqua and DAPI). Images were captured as tiff files using an Olympus digital camera and processed with the Cytovision software (Genetix Ltd., Hampshire, UK). The sperm were scored according to previously described criteria (Blanco et al., 1996). Briefly, sperm were diagnosed as disomic if they presented two or more fluorescent signals for the same chromosome with a size and intensity similar to those detected in normal nuclei; sperm were defined as diploid by the presence of two signals for each of the studied chromosomes in the presence of the sperm tail and an oval head shape; nullisomic sperm were defined by no fluorescent signal being detected for a given chromosome. All signals were separated from each other by at least a single domain. Chromosome abnormality rates were calculated by assessing at least 500 spermatozoa per sample.

Once scored, the numerical chromosome abnormality rate of each of the patient samples analysed was statistically compared (Chi squared test) with those established in the fertile samples used as controls during each FISH experiment. The analysis of significant differences allowed the classification of the patient samples into two subgroups: those with a normal rate of numerical abnormalities and those with an elevated rate of numerical chromosome abnormalities.

Combined SDF and chromosome analysis

Ten out of the 45 semen samples analysed in this study were processed following the protocol described by Muriel et al. (2007). For reliable comparison of the results obtained, the samples were coded so that specific samples could not be identified by the scorer. Details about the 10 samples selected were as follows: five normozoospermic samples according to the World Health Organization criteria (2010) with a normal rate of numerical chromosome abnormalities and five non-normozoospermic samples (WHO criteria 2010) with an elevated rate of chromosomal abnormalities as assessed by the standard chromosome analysis protocol described above. FISH analysis was performed on the sperm cells previously processed for the SCD test using the Halosperm® kit. Briefly, the slides were incubated with 10% formaldehyde in phosphate-buffered saline for 12 min, washed in phosphate-buffered saline for 1 min and denatured by incubation in NaOH 0.05N/50% ethanol for 15 s. The slides were then dehydrated in increasing ethanol solutions (70−90−100%) for 2 min each, air-dried and incubated overnight at 37°C with a denatured probe mixture containing probes specific to chromosomes 13, 16, 18, 21 and 22 (Abbott Molecular Inc., Des Plaines, IL, USA). The slides were then washed in 50% formamide/2× SSC, pH 7, for 8 min, and in 2× SSC, pH 7, for 5 min, both at 44°C. Finally, sperm cells were counterstained with DAPI (2 mg/ml; Roche Diagnostics, Barcelona, Spain) in Vectashield (Vector Laboratories, Burlingame, CA, USA) and immediately analysed. Sperm nuclei were scored according to previously described criteria (Muriel et al., 2007). A sperm nucleus was diagnosed as disomic if it
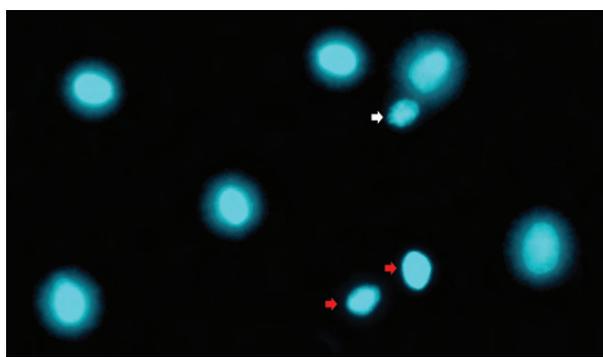
presented two or more fluorescent domains for the same chromosome, comparable in size and intensity, and separated by at least one domain in those nuclei with a big or medium halo size (i.e. DNA-intact sperm nuclei) or in those sperm nuclei with small halo or without a halo (i.e. DNA-fragmented sperm nuclei). Sperm were defined as diploid by the presence of two signals for each of the studied chromosomes in the presence of a sperm tail and/or an oval nucleoid core shape. Nullisomic sperm were defined by no fluorescent signal being detected for a given chromosome. FISH signals in sperm nuclei may be spread but their dispersion starts from a restricted location in the core, where usually the signal intensity is stronger. This may help overcome the few possibly unclear cases.

Statistical analyses

Data analyses were performed using the Statistical Package for the Social Sciences v14. (SPSS Inc., Chicago, IL, USA) and a *P*-value of 0.05 was considered significant. Differences between groups were examined using one-way ANOVA, Chi squared or Mann−Whitney *U*-test, as appropriate. Relationships between semen quality parameters were studied using Pearson's correlation coefficient. This correlation coefficient was also used to assess the concordance of the results obtained by independent FISH and SCDt assays and the combined SCD-FISH protocol. Differences between independent and combined SCD and FISH protocols were examined using Paired samples student's *t*-test.

Results

Patient characteristics and semen quality parameters are shown in Table I.

The results indicate a positive and significant correlation between the numerical chromosome abnormality rate and the SDFI (Pearson correlation, $R = 0.511$, $P < 0.05$).

Out of the 45 patients analysed, 11 showed significantly increased sperm aneuploidy rates compared with results obtained from fertile controls (Chi squared, $P < 0.05$) 6.46 ± 0.33 versus 3.67 ± 0.17,

respectively. Those samples defined as having an elevated rate had significantly higher levels of DNA-damaged spermatozoa in comparison with both fertile samples (49.52 ± 6.23 versus 24.06 ± 2.35, One-way ANOVA, $P < 0.05$) and infertile samples with normal rates of numerical chromosome defects (49.52 ± 6.23 versus 31.50 ± 2.54, One-way ANOVA, $P < 0.05$) (Table II).

Results from the simultaneous analysis of sperm chromosomes and DNA fragmentation indicated a strong and significant positive correlation between the results obtained using the individual or combined tests (Pearson correlation, $R = 0.912$, $P < 0.05$). No significant differences in the rates of sperm chromosomal abnormalities or of DNA fragmentation were observed when the FISH and SCD results from independent and combined assays were compared (Paired samples *t*-test, $P > 0.05$).

Results from individual spermatozoa subjected to combined chromosome and DNA fragmentation analysis suggested that chromosomally abnormal sperm cells were more likely to display DNA damage than those from the same sample with a normal karyotype (Fig. 2, Table III). Similarly, the incidence of chromosomal abnormalities in DNA-damaged sperm cells was shown to be significantly higher than in the case of DNA-intact spermatozoa (Mann−Whitney *U*-test, $P < 0.05$) (Table IV).

A significant and positive correlation was also found between the sperm chromosome abnormality rate and the proportion of sperm with highly degraded DNA (SDDI) (Pearson correlation, $R = 0.453$, $P < 0.05$). On average, samples defined as having an elevated rate of numerical chromosome defects after clinical testing had significantly higher levels of DNA-degraded spermatozoa compared with samples with a normal rate of chromosome abnormalities (12.35 ± 3.38 versus 7.10 ± 0.84, One-way ANOVA, $P < 0.05$) (Table II).

With respect to the other semen quality parameters analysed, motility and sperm count, the percentage of chromosomally abnormal sperm was found to significantly correlate with sperm motility (Pearson correlation, $R = -0.430$, $P < 0.05$). However, no significant

Table I Patient and fertile group characteristics and their semen quality parameters. Details of the characteristics of the group of patients with normal or abnormal traditional semen quality parameters according to World Health Organization criteria (WHO, 2010) are also included.

	Patient group	Fertile group	Patient group	
			Non-normozoospermic	Normozoospermic
Number of patients	45	40	18	27
Age	39.20 (27–56 years)	37.50 (27–46 years)	37.89 ± 1.26	40.86 ± 1.41
Sperm count ($\times 10^6$/ml)	30.22 ± 4.86	58.16 ± 3.97	9.88 ± 1.38	46.86 ± 7.02
Sperm motility (%)	52.74 ± 4.20	70.96 ± 0.98	46.75 ± 6.97	59.27 ± 3.84
Aneuploid sperm (%)	4.93 ± 0.21	3.67 ± 0.17	5.45 ± 0.40	4.63 ± 0.24
Sperm disomies (%)	3.54 ± 0.24	2.86 ± 0.21	3.47 ± 0.36	3.42 ± 0.38
Sperm nullisomies (%)	3.54 ± 0.27	2.74 ± 0.28	3.93 ± 0.52	3.47 ± 0.29
Diploid sperm (%)	0.15 ± 0.02	0.12 ± 0.02	0.18 ± 0.04	0.12 ± 0.02
SDFI (%)	35.91 ± 2.68	24.06 ± 2.35	40.36 ± 5.30	33.04 ± 3.16
SDDI (%)	8.38 ± 1.07	4.75 ± 1.53	10.82 ± 2.21	6.33 ± 1.06

Age values are mean (range).
All other values are mean $\pm$ SEM.
SDFI, sperm DNA fragmentation index.
SDDI, sperm DNA degradation index.

Table II Patient characteristics and semen quality parameters of patients with normal and elevated numerical chromosome abnormality rates.

	Numerical chromosome abnormality rate		P-value*
	Normal range	**Elevated**	
Number of patients	34	11	–
Age	38.88 (27–56 years)	40.18 (28–47 years)	NS (0.529)
Sperm count ($\times 10^6$/ml)	33.76 ± 6.17	19.60 ± 4.97	NS (0.211)
Sperm motility (%)	56.24 ± 4.83	42.83 ± 7.73	NS (0.166)
Aneuploid sperm (%)	4.44 ± 0.19	6.46 ± 0.33	<0.001
Sperm disomies (%)	3.19 ± 0.25	4.62 ± 0.52	0.010
Sperm nullisomies (%)	3.22 ± 0.24	4.50 ± 0.75	0.038
Diploid sperm (%)	0.14 ± 0.02	0.18 ± 0.04	NS (0.503)
SDFI (%)	31.50 ± 2.54	49.52 ± 6.23	0.003
SDDI (%)	7.10 ± 0.84	12.35 ± 3.38	0.034

Age values are mean (range).
All other values are mean ± SEM.
SDFI, sperm DNA fragmentation index.
SDDI, sperm DNA degradation index.
*One-way ANOVA test, $P < 0.05$.

correlation between the percentage of chromosomally abnormal cells and sperm count was observed. None of the sperm DNA damage indexes measured, SDFI or SDDI, correlated with any of the conventional semen quality parameters studied, i.e. count or motility. When the samples were divided into groups according to the presence or absence of normozoospermia following the World Health Organization criteria (WHO, 2010), no significant differences were observed, neither in the percentages of chromosomally abnormal sperm nor in the frequency of DNA-damaged spermatozoa (Table I).

Discussion

Results from the present study indicate a significant association between numerical chromosome abnormalities and DNA fragmentation in sperm samples from infertile patients undergoing assisted reproductive treatment. Such an association has been described previously in carriers of a constitutional chromosomal abnormality (Perrin et al., 2011) and patients with unexplained recurrent pregnancy loss (Carrell et al., 2003), globozoospermia (Brahem et al., 2011), teratozoospermia or oligozoospermia (Liu et al., 2004), but our results suggest that the association is not limited to those patients.

Using a combined method, we were able to investigate DNA damage and chromosome defects in individual cells. Chromosomally abnormal spermatozoa were more likely to be affected by DNA fragmentation than those that were normal for the five chromosomes tested (13, 16, 18, 21 and 22). This finding is consistent with that of a previous study by Muriel et al. (2007), who analysed chromosomes 18, X and Y in sperm samples from men with a variety of different semen characteristics (fertile donors, normozoospermic, teratozoospermic, asthenozoospermic and oligoasthenoteratozoospermic), and reported a 4-fold increase in the incidence of aneuploidy for sperm with fragmented DNA compared with those with intact DNA. The current study, which involved analysis of a larger number of chromosomes in each cell, presumably providing more sensitive aneuploidy detection, found a 3-fold increase in the likelihood of aneuploidy among spermatozoa displaying DNA fragmentation. The association between chromosome abnormality and DNA damage was seen in samples that had chromosome defects rates in the normal range and also in sperm samples with elevated levels of chromosome abnormalities.

Another recent study involving simultaneous assessment of chromosomal abnormalities and DNA fragmentation investigated the spermatozoa of four carriers of a balanced chromosomal abnormality and reported similar findings (Perrin et al., 2011). Specifically, the proportion of spermatozoa with an unbalanced chromosomal content and damaged DNA was significantly increased in comparison with those that had a normal/balanced content. However, a study carried out by Balasuriya et al. (2011) found no significant correlation between DNA fragmentation and aneuploidy in individual cells. It is possible that the lack of an association in that investigation was due to the fact that a smaller number of chromosomes were assessed (X, Y and 18), which may have made it more difficult to identify aneuploid spermatozoa. The inability to assess the entire chromosome complement is a limitation shared by all studies aimed at assessing aneuploidy in sperm samples, but the problem can be reduced by increasing the number of chromosomes tested (seven in the current study). Another reason for an apparent lack of correlation between aneuploidy and DNA fragmentation in individual sperm might be related to difficulties combining the two methods. For a given sperm sample, the results obtained from a combined SCD-FISH protocol should be essentially identical to those obtained when FISH and SCDt assays are carried out separately. Reassuringly, in the current study, the results were not affected by combining the methods together. However, the previous study that found no association between DNA fragmentation and aneuploidy displayed a significant alteration in the rates of sperm chromosomal abnormalities and of DNA fragmentation when the tests were combined (Balasuriya et al., 2011).

In contrast to the results we present here, a recent study by Bronet et al. (2012), conducted in patients with recurrent miscarriage or implantation failure, found no correlation between SDFI and sperm aneuploidy levels. Neither did they find a relationship between SDFI and embryo aneuploidy rate. In their study, poor semen quality samples were excluded, only samples with a sperm count over 15×10^6/ml and 50% motility and no abnormally high aneuploidy rates were considered. These criteria of patient selection may have introduced a significant bias in the results obtained and may explain some of the differences between the results of their study and ours. Moreover, instead of total aneuploidy rates, disomy rates for individual chromosomes and diploidy rates based on the analysis of two chromosomes (sex diploidy and 13/21 diploidy rates) were used to perform the correlation analyses described in the Bronet et al. (2012) study. In our study, results from all the three types of numerical chromosome aberrations (nullisomies, disomies and diploidies) were considered in order to explore the correlation between the sperm chromosome abnormalities rate and DNA damage. Variation in the
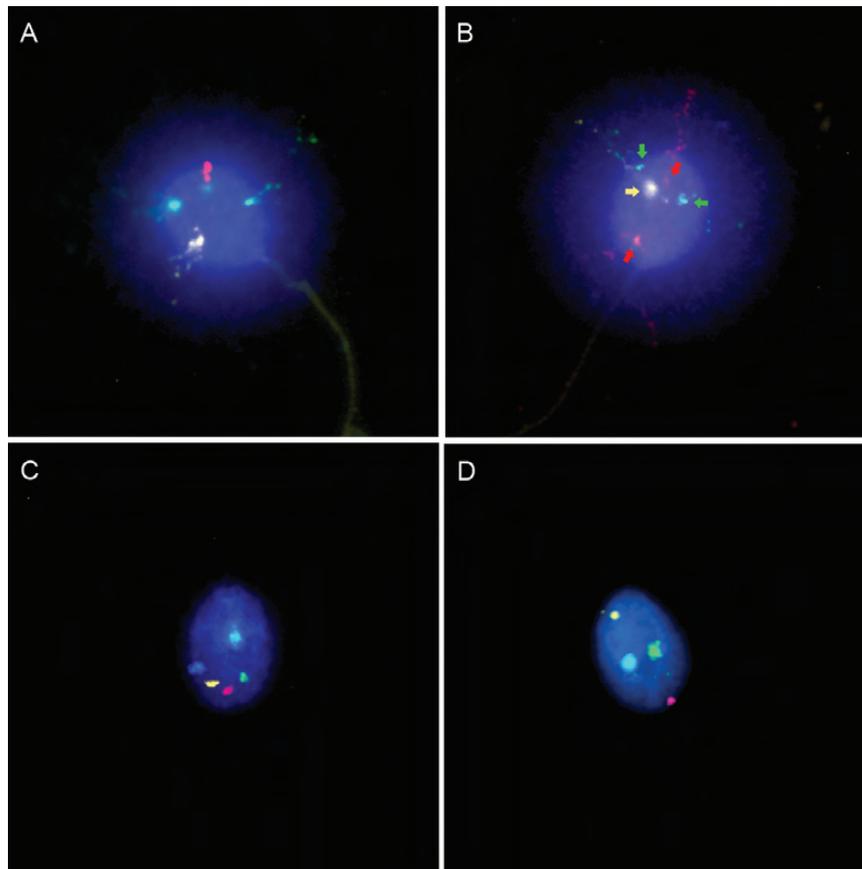
Figure 2 SDF and chromosome analysis using a combined SCDt and FISH protocol. Sperm nuclei counterstained with DAPI are presented in dark blue, and centromeric probes for chromosomes 13, 16, 18, 21 and 22 are shown in red, aqua, blue, green and gold, respectively. (**A**) Spermatozoon with intact DNA and a correct number of the chromosomes analysed; (**B**) Spermatozoon with intact DNA and an abnormal number of chromosomes: two copies of chromosome 13 (red arrows), two copies of chromosome 21 (green arrows), a copy of chromosome 22 (gold arrow) and no copies of chromosomes 16 and 18; (**C**) Spermatozoon with fragmented DNA and a correct number of the analysed chromosomes; (**D**) Spermatozoon with fragmented DNA and an abnormal number of chromosomes: a copy each of chromosomes 13 (red), 16 (aqua), 21 (green) and 22 (gold) and no copy of chromosome 18.

Table III Level of DNA damage present in spermatozoa with numerical chromosome abnormalities (*n* = 10 semen samples, 1000 sperm per sample).

	Aneuploid sperm	Sperm with disomies	Sperm with nullisomies	Diploid sperm
Sperm with damaged DNA (%)	78.11 ± 1.14	72.22 ± 1.99	80.34 ± 1.52	73.33 ± 9.92
Sperm with intact DNA (%)	21.89 ± 1.14	27.78 ± 1.99	19.66 ± 1.52	26.67 ± 9.92

Values are mean ± SEM.

technique used for sperm DNA damage analysis may also have influenced some of the differences between studies. The combined technique (SCD-FISH) used in our analysis is indeed a more powerful tool to explore the relationship between chromosome content and DNA damage in individual cells. The results obtained do not rely only on a correlation analysis of two variables independently measured in different cells, as is the case of the TUNEL and FISH assays performed by Bronet *et al.* (2012), but on the simultaneous evaluation of both parameters in the same cell.

The fact that chromosomal defects and DNA fragmentation coincided in the same spermatozoon more often than would be expected by random chance confirms a direct link between the two phenomena. During spermatogenesis, meiotic checkpoints regulate the process of gamete formation, inducing apoptosis and associated DNA fragmentation when errors occur (Eaker *et al.*, 2001; Handel, 2001). This process presumably acts to control cell proliferation and eliminate genetically abnormal sperm (Sakkas *et al.*, 1999). We hypothesize that during the process of spermatogenesis, chromosomally

Table IV Numerical chromosome abnormalities present in spermatozoa with and without DNA damage (*n* = 10 semen samples, 1000 sperm per sample).

	DNA-intact sperm	DNA-damaged sperm	P-value[*]
Number of sperm cells analysed	500	500	–
Sperm disomies (%)	1.36 ± 0.14	3.68 ± 0.33	<0.001
Sperm nullisomies (%)	1.96 ± 0.191	7.18 ± 0.39	<0.001
Diploid sperm (%)	0.12 ± 0.04	0.46 ± 0.09	0.007

Values are mean ± SEM.
*Mann–Whitney *U*-test, *P* < 0.05.

abnormal gametes are 'marked' for apoptosis. However, some of these gametes escape from the process of cellular death, and as a result, they are found in the ejaculate. This hypothesis involving abortive apoptosis is in agreement with that proposed by Perrin *et al.* (2011) and with research evaluating apoptotic markers (e.g. phosphatidyl serine externalization and DNA fragmentation) reported by Brugnon *et al.* (2006, 2010).

In the current study, assessing men with a normal karyotype, approximately one quarter of the infertile patients analysed presented numerical chromosome abnormality rates significantly higher than those of fertile controls. We have acknowledged that the baseline aneuploidy rate in our normal controls is higher than reported in some studies, perhaps due to technical differences or other study–study differences. However, it is interesting to note that these samples with elevated rates of numerical chromosomal defects almost always displayed an increased rate of DNA fragmentation [9 of 11 samples with excessive aneuploidy/diploidy had a high SDFI (>30%), and the other two samples had intermediate SDFI scores (22 and 27%)]. Multiple forms of meiotic or spermiogenic defect could conceivably induce apoptosis, so it is not surprising that some of the sperm displaying DNA fragmentation appeared to be chromosomally normal. Furthermore, less than a quarter of the chromosomes were tested in each cell, and consequently, some of the 'normal' sperm may have been aneuploid for chromosomes that were not tested. Combined analysis of aneuploidy and DNA integrity in individual sperm revealed a 3.3% aneuploidy rate among sperm with intact DNA, but a 3-fold increase (10.9%) in sperm displaying with DNA fragmentation. Of those spermatozoa affected by aneuploidy, more than 78% contained fragmented DNA. This high level of DNA damage was equally associated with all forms of aneuploidy in spermatozoa (nullisomy and disomy) and also with the other group of chromosomal abnormalities studied, i.e. diploid sperm.

The use of ARTs has provided an opportunity for men with poor semen quality parameters to father children. Some of the techniques used (i.e. ICSI) may allow abnormal spermatozoa to bypass natural barriers that would normally prevent them from fertilizing the oocyte. This has led to concerns that patients utilizing these techniques could be at an elevated risk of conceiving offspring with genetic anomalies (Lam *et al.*, 2001; Bonduelle *et al.*, 2002; Gjerris *et al.*, 2008). The current study shows that sperm samples with a raised incidence of chromosome abnormalities also display a significant increase in DNA fragmentation. DNA damage may, therefore, in some cases, be an indicator of the presence of chromosomal defects in male gametes and hence assist in the identification of patients at an increased risk of producing aneuploid embryos. Given the association of DNA fragmentation with chromosome abnormalities, it might be worth patients with a high SDFI considering preimplantation genetic screening (PGS) to help avoid transfer of chromosomally abnormal embryos. However, much more work needs to be done to establish relative risk rates before such a clinical strategy can be recommended.

In conclusion, this study confirms a link between SDF and numerical chromosome abnormalities in infertile patients undergoing ART. The association was seen in samples that had chromosomal abnormality rates in the normal range and also in sperm samples with elevated levels of chromosome defects. In addition to shedding light on the biological mechanisms involved in the processing of defective sperm, this finding may also be of clinical relevance for the identification of patients at an increased risk of miscarriage, chromosomally abnormal pregnancies and/or transmission of genetic defects to the offspring.

Acknowledgements

Authors' roles

M.E. participated in the design of the study, collected and analysed the data and drafted the manuscript. S.A. collected the aneuploidy data and contributed to manuscript preparation. D.W. participated in the design of the study, supervised the data analysis and was involved in manuscript preparation.

Funding

Conflict of interest

None declared.

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