Human Reproduction, Vol.27, No.7 pp. 1922–1929, 2012
Advanced Access publication on April 26, 2012 doi:10.1093/humrep/des148

human reproduction

ORIGINAL ARTICLE ***Andrology***

Sperm DNA fragmentation index does not correlate with the sperm or embryo aneuploidy rate in recurrent miscarriage or implantation failure patients

F. Bronet*, E. Martínez, M. Gaytán, A. Liñán, D. Cernuda, M. Ariza, M. Nogales, A. Pacheco, M. San Celestino, and J.A. Garcia-Velasco

Departamento de Diagnostico Genetico Preimplantacional, IVI Madrid, Madrid 28023, Spain

*Correspondence address. E-mail: fernando.bronet@ivi.es

Submitted on December 13, 2011; resubmitted on February 29, 2012; accepted on April 2, 2012

BACKGROUND: The aneuploidy rate is higher in poor-quality sperm samples, which also have higher DNA fragmentation index values. The aim of this study was to assess the relationship between sperm DNA fragmentation in samples from infertile men belonging to couples with recurrent miscarriage or implantation failure and the aneuploidy rate in spermatozoa as well as in embryos from patients.

METHODS: This prospective study evaluated DNA damage and the aneuploidy rate in fresh and processed (density gradient centrifugation) ejaculated sperm as well as the aneuploidy rate in biopsied embryos from fertility cycles. Fluorescence *in situ* hybridization was used for the aneuploidy analysis. Results were compared using linear regression and analysis of variance.

RESULTS: A total of 154 embryos were evaluated from 38 patients undergoing PGD cycles; 35.2% of the embryos were chromosomally normal. Analysis of the same sperm samples showed an increased DNA fragmentation after sperm preparation in 76% of the patients. There was no correlation between DNA fragmentation and the aneuploidy rate in embryos or in fresh or processed sperm samples.

CONCLUSIONS: Sperm DNA fragmentation is not related to chromosomal anomalies in embryos from patients with recurrent miscarriage or implantation failure. However, we cannot rule out the possibility that a relationship between DNA fragmentation and aneuploidy exists for other causes of infertility. Furthermore, the different methods used to evaluate DNA fragmentation may produce different results.

Key words: aneuploidy / DNA fragmentation / fluorescence *in situ* hybridization / terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling

Introduction

Semen quality is frequently used as an indirect measure of male infertility. The parameters that have been used historically as indicators of male fertility potential include sperm count, motility and morphology, all of which are evaluated in fertility clinics as a part of routine semen analyses (WHO, 2010).

Recently, there has been growing interest in the use of DNA integrity as a marker of male infertility. Men with abnormal semen parameters, especially sperm motility and morphology parameters (Zini *et al.*, 2001), have an increased risk of high levels of DNA fragmentation (Evenson *et al.*, 1980; Irvine *et al.*, 2000). However, most fertility clinics do not routinely evaluate sperm DNA fragmentation before choosing an appropriate fertility treatment.

Sperm DNA integrity is associated with male fertility potential *in vivo* and *in vitro*. There are increased levels of fragmented sperm DNA in a high percentage (≤40%) of men presenting clinically for subfertility (Lopes *et al.*, 1998). Notably, semen with a high percentage of damaged spermatozoa (e.g. denatured DNA) has very low potential for natural fertility (Lopes *et al.*, 1998; Evenson *et al.*, 1999; Spano *et al.*, 2000; Virro *et al.*, 2004). However, DNA damage in sperm does not preclude IVF, as there is still a chance that samples in which sperm have damaged DNA can be used to achieve a pregnancy (Twigg *et al.*, 1998).

Sperm DNA fragmentation can be caused by internal factors, such as apoptosis in the epithelium of the seminiferous tubules, defects in chromatin remodelling during the process of spermatogenesis and the production of free radicals during sperm migration from the seminiferous tubules to the epididymis. External factors, such as leukocytes, can also cause DNA fragmentation (Manicardi *et al.*, 1995; Sakkas *et al.*, 1999; Aitken and Krausz, 2001; Zini *et al.*, 2001; Alvarez, 2005).

In assisted reproduction technology, the reproductive parameters that could be affected by the integrity of the DNA in ejaculated spermatozoa include fertilization, blastocyst development and pregnancy rates; a correlation between DNA integrity and fertilization rates has been described (Evenson *et al.*, 1999, 2002; Virro *et al.*, 2004; Borini *et al.*, 2006). In fact, pregnancy rates using conventional IVF and ICSI treatments are significantly reduced in couples with a high percentage of sperm with DNA damage (Høst *et al.*, 2000; Bungum *et al.*, 2004).

Furthermore, aneuploidy can trigger DNA fragmentation (Muriel *et al.*, 2007). These findings, along with the observation that the aneuploidy rate is higher in poor-quality sperm samples (Vegetti *et al.*, 2000; Rubio *et al.*, 2001), led us to speculate that high DNA fragmentation index (DFI) values could correlate with aneuploidy rate in embryos or sperm samples, based on the available evidence that supports a link between repeated miscarriage and implantation failure with embryo chromosomal abnormalities and the need for further investigation into a paternal origin for these abnormalities.

The aim of this study was to assess the relationship between DNA fragmentation, as evaluated by the terminal (deoxynucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) assay, and the aneuploidy rate in embryos and sperm from patients with recurrent miscarriage or implantation failure.

Materials and Methods

Study population

This study was conducted at the Instituto Valenciano de Infertilidad (IVI) in Madrid, Spain. The procedures and protocols were approved by the Institutional Review Board, which regulates and approves database analysis and clinical IVF procedures for research at IVI. Informed consent was obtained from all participants. A total of 38 IVF cycles were included from January 2010 to December 2010. All patients were undergoing our preimplantation genetic screening (PGS) owing to recurrent pregnancy loss (RPL) ($n = 30$), defined as two or more miscarriages before 20 weeks of pregnancy or repeated implantation failure (RIF) ($n = 8$), defined as the failure of a couple to conceive after the transfer of 10 or more good quality embryos or after three IVF cycles (ESHRE PGD Consortium Steering Committee, 2002). Women over 39-year-old and men with a sperm count $<15 \times 10^6$/ml and 50% motility were excluded.

Four aliquots were extracted from each sperm sample, two for DNA fragmentation testing using the TUNEL assay and two for fluorescence *in situ* hybridization (FISH) analysis. Both DNA fragmentation and FISH analysis were performed on fresh and processed sperm samples on the day of oocyte retrieval. Patients who showed abnormal FISH results in the sperm analysis were excluded from the study in order to have clear results regarding the correlation between aneuploidy rate in embryos and DFI. The study design is shown in Fig. 1.

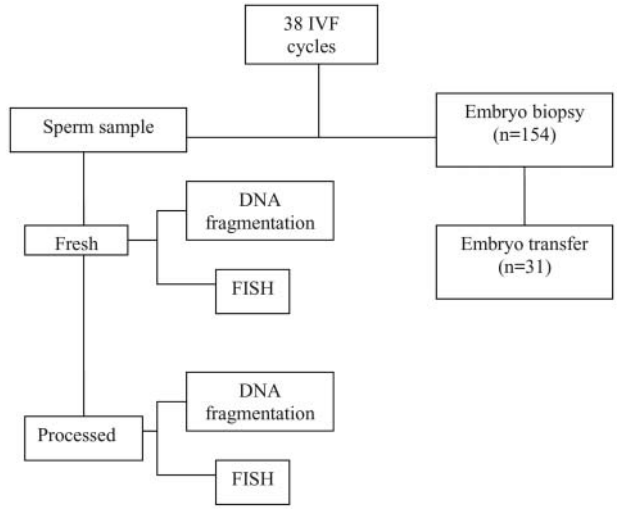

Figure 1 Study design.

Ovarian stimulation and oocyte retrieval

For ovarian stimulation and oocyte retrieval, the patients were treated as described previously (Garcia-Velasco *et al.*, 2011). Briefly, the women received a starting dose of recombinant FSH (Puregon®, Organon; Gonal F®, Serono) ranging from 150 to 225 IU (maximum) and 0.25 mg of the GnRH antagonist, ganirelix (Orgalutran®, Organon) daily, starting on Day 5 or 6 after FSH administration. The patient's cycle was monitored according to the individual policy of the clinic. Recombinant hCG (Ovitrelle®, Serono) was administered as soon as two leading follicles reached a mean diameter $\geq$17 mm, and oocyte retrieval was performed 36 h later. IVF or ICSI was used as appropriate for each couple.

Semen collection and preparation

Semen samples were obtained by masturbation after 3 days of abstinence. Spermatozoa were prepared using density gradient centrifugation (90–45%) with SpermGrad (Vitrolife, Scandinavian IVF). The samples were centrifuged at 400*g* for 15 min at room temperature, and the pellet was washed with 1 ml of IVF medium (Vitrolife, Scandinavian IVF) by centrifugation at 2700*g* for 10 min. A single spermatozoon that appeared to be morphologically normal was selected for ICSI.

Embryo culture evaluation and embryo biopsy

Fertilization was confirmed 16–20 h after insemination by the presence of two pronuclei and extrusion of the second polar body. Normal fertilized oocytes were cultured in a microdroplet of culture media (Vitrolife, Scandinavian IVF) until the day of blastomere biopsy. Embryos were evaluated on Days 2 and 3. Cell number, fragmentation pattern (defined as the embryonic volume occupied by the enucleated cytoplasm and expressed as a percentage), symmetry and multinucleation were recorded.

Embryo biopsy was performed on Day 3 in embryos with more than 5 cells and <25% fragmentation. The zona pellucida was perforated using laser technology (OCTAX, Herbron, Germany) (Rubio *et al.*, 2003). In all cases, one cell was removed and all blastomeres were fixed individually (to minimize signal overlap and loss of micronuclei) under an inverted microscope using a slightly modified version of Tarkowski's protocol,

without hypotonic pretreatment (Tarkowski, 1966). Subsequent FISH analysis was performed. The biopsied embryos were cultured until they reach the blastocyst stage (Day 5). Embryo transfer was performed on Day 5 when a normal embryo was available. The maximum number of embryos transferred was 2.

FISH analysis

For blastomere analysis, nine chromosomes were analysed: 13, 15, 16, 17, 18, 21, 22, X and Y. The FISH procedure included two consecutive hybridization rounds. In the first round, a cocktail (MultiVysion PB; Vysis Inc., Downers Grove, IL, USA) that included probes for the following chromosomes were used: 13 (LSI 13 region 13q14; locus RB, spectrum red), 16 (CEP16, satellite II, region 16p11.1-q11.1; locus D16Z3, spectrum aqua), 18 (CEP 18; alpha satellite, region 18p11-q11; locus D18Z1, spectrum blue), 21 (LSI 21; region 21q22.13-q22.2; loci D21S341, D21S342, spectrum green) and 22 (LSI 22; region 22q11.2; locus BCR, spectrum gold). In a second round, and after signal elimination (Vidal et al., 1998), nuclei were hybridized with another cocktail of probes (4CC; Vysis Inc.) that included centromeric probes for chromosomes X (CEP X, alpha satellite, region Xp11-q11; locus DXZ1, spectrum green), Y (CEP Y, satellite III, region Yq12; locus DYZ1, spectrum blue), 17 (CEP 17, alpha satellite, region 17p11.1-q11.1, locus D17Z1, spectrum aqua) and 15 (CEP 15, alpha satellite, region 15p11.1-q11.1, locus D15Z4, spectrum orange). For embryos with ambiguous signals in the previous rounds, a third hybridization round was performed with subtelomeric DNA probes to decrease the risk of false monosomies and non-informative embryos.

For sperm analysis, numerical abnormalities on chromosomes 13, 18, 21, X and Y were evaluated on different slides (each with material from the same sample), using triple colour FISH for chromosomes 18, X and Y and dual colour for chromosomes 13 and 21. A commercial kit was used for this analysis (AneuVysion; Vysis Inc.) that included in the first vial chromosome 18 (CEP 18; alpha satellite, region 18p11.1-q11.1; locus D18Z1, spectrum aqua), X (CEP X, alpha satellite, region Xp11-q11; locus DXZ1, spectrum green) and Y (CEP Y, satellite III, region Yq12; locus DYZ1, spectrum blue) and in the second vial chromosome 13 (LSI 13 region 13q14; locus RB, spectrum green) and 21 (LSI 21; region 21q22.13-q22.2; loci D21S341, D21S342, spectrum orange). Hybridization and detection were performed according to the methods of Blanco et al. (1996). Each sample was evaluated by three experienced specialists.

Data of the study population were compared with a historical control group, comprising ejaculated spermatozoa from normozoospermic fertile donors that were previously described by our group (Rodrigo et al., 2004).

Evaluation of DNA damage

The integrity of sperm DNA was evaluated in an aliquot of semen by the TUNEL test using a commercial kit (Roche Diagnostic, Lewes, UK) and following the manufacturer's recommendations. Briefly, cells were washed twice in phosphate-buffered saline (PBS; Sigma-Aldrich, Gillingham, UK) and adjusted to a concentration of 2×10^7 cells/ml in PBS. The cell suspension was subsequently fixed in PBS containing 2% formaldehyde (Sigma-Aldrich) for 60 min at room temperature. Cells were then washed twice with PBS, centrifuged at 300*g* for 5 min and incubated with a permeabilization buffer (0.1% Triton X-100, and 0.1% sodium citrate in PBS) for 2 min at 4°C. Permeabilized cells were then washed with PBS, resuspended in 50 µl of TdT buffer containing 50 µl of TdT enzyme plus labelled nucleotides and incubated at 37°C for 60 min. For the negative controls, we omitted the terminal transferase enzyme from the reaction mixture. For the positive controls, we treated the samples with 1 IU/µl DNase I (Roche Diagnostic) for 15 min at room temperature before incubation with the TdT buffer. Finally, the cell sample was washed twice with PBS, centrifuged at 300*g* and resuspended in 300 µl of PBS.

We immediately analysed the samples using an FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) with an argon laser operating at 488 nm. The flow rate during analysis was controlled at 100 events/s, and we analysed a minimum of 10 000 events in each sample. We measured the green fluorescence with the FL1 detector at 525 nm and identified the proportion of labelled sperm in the sample. Data were then recorded and processed with a Becton Dickinson program (Cell Quest, Becton Dickinson).

Statistical analysis

The distribution of the variables was examined; when necessary, variables were normalized by log or square root transformation. Differences between groups were examined using the Student's *t*-test. In order to determine whether the DFI was related to the aneuploidy rate, these two parameters were compared using the Pearson's correlation coefficient. Analysis based on quartiles was not possible owing to the small sample sizes. A *P*-value ≤ 0.05 was considered statistically significant. Data were analysed with the Statistical Package for the Social Sciences software program (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

In the study population, 30 of 38 patients (78.9%) were RPL, and the other 8 (21.1%) were RIF. Mean and SD maternal and paternal ages were 36.9 ± 2.1 and 39.2 ± 4.0 years, respectively. The mean number of miscarriages was 2.5 ± 0.7, and the mean number of unsuccessful cycles in RIF patients was 3.6. The mean sperm concentration was $62.3 \pm 40.2 \times 10^6$/ml, and 48.5 ± 15.6% were progressively motile. The mean number of mature metaphase II oocytes obtained was 11.2 ± 4.2. A total of 154 embryos were biopsied (an average of 4.1 ± 0.4 embryos per patient). A total of 27 (71.1%) embryo transfers were performed and 16 (59.3%) patients achieved a pregnancy. The sample size is too small to investigate a correlation between DFI and the pregnancy rate.

Aneuploidy analysis

In the sperm study, an average of 3356 ± 331 spermatozoa were evaluated per patient for chromosomes 18, X and Y, and an average of 3010 ± 462 were evaluated for chromosomes 13 and 21. Tables I and II show the results for meiotic segregation for chromosomes 13, 18, 21 X and Y.

Four patients (10.5%) showed significant increases in sperm aneuploidy rate versus the historical control group ($P < 0.001$). These four patients, who all showed a significant increase in the aneuploidy rate of the sperm, were excluded from the embryo aneuploidy study.

Blastomeres from 154 embryos were screened for aneuploidy, 99.4% of the embryos were analysed and the rate of euploid embryos was 35.2%. All the embryos were euploid only in 3 patients (7.9%), whereas 10 patients (23.3%) showed abnormal results in all of their embryos. The results obtained for each patient are shown in Table III.

Table I Aneuploidy rates of chromosomes 18, X and Y in fresh sperm samples.

Patient	Analysed gametes (*n*)	Disomy (%)		Diploidy (%)
		Sex chromosomes	18	
1	4519	0.3	0.07	0.22
2	4235	0.28	0.023	0.02
3	3346	0.18	0.03	0
4	3300	0.18	0.06	0.18
5	3377	0.18	0	0.15
6	3372	0.15	0	0.03
7	3282	0	0	0.18
8	3256	0.4	0	0.06
9	3209	0.16	0	0.03
10	3231	0.15	0	0.12
11	3209	0.19	0	0.09
12	3326	0.33	0	0.03
13	3336	0.36	0	0.06
14	3199	0.19	0	0.16
15	3304	0.97*	0.09	0.3
16	3288	0.48	0	0
17	3432	0.21	0	0
18	3213	0.31	0.03	0.9*
19	3342	0.09	0	0.12
20	3451	0.61*	0	0.23
21	3342	0	0	0
22	3333	0.3	0	0.09
23	3448	0.12	0	0.2
24	3440	0.03	0	0.06
25	3400	0.18	0.03	0.06
26	3255	0.19	0.03	0.12
27	3338	0.87*	0	0
28	2132	0.19	0	0.05
29	3297	0.18	0	0
30	3279	0.06	0	0.09
31	3260	0.12	0	0.18
32	3409	0.03	0	0.2
33	3297	0.06	0	0.09
34	3021	0.23	0	0.12
35	3603	0.08	0	0.03
36	3432	0.35	0	0.17
37	3569	0.17	0	0.06
38	3472	0.14	0	0.03

*$P < 0.05$ compared with a control group.

Table II Aneuploidy rates of chromosomes 13 and 21 in fresh sperm samples.

Patient	Analysed gametes (*n*)	Disomy (%)		Diploidy (%)
		13	21	
1	4267	0.02	0.09	0.05
2	4094	0.17	0.22	0
3	3031	0.03	0.099	0
4	3006	0.09	0.03	0.066
5	3115	0.06	0	0.03
6	3053	0.07	0.03	0.03
7	3020	0	0.13	0.067
8	3025	0.23	0.3	0.066
9	3009	0.033	0.066	0.13
10	3017	0.1	0	0.17
11	3000	0	0.03	0
12	3003	0.03	0	0.06
13	3003	0	0.03	0.06
14	3006	0	0.13	0.067
15	3011	0.03	0.06	0.27
16	3012	0.1	0.2	0.13
17	3000	0.25	0.25	0
18	3027	0	0.13	0.76*
19	3013	0.03	0.07	0.1
20	3019	0.13	0.13	0.36
21	2006	0	0	0
22	3006	0.1	0	0.03
23	3055	0	0	0.13
24	3020	0.06	0.06	0.06
25	3020	0	0	0.03
26	3000	0	0.07	0
27	3009	0.033	0.17	0.1
28	1018	0	0.3	0.2
29	3003	0.03	0.06	0
30	3003	0	0	0.1
31	3042	0.07	0	0.03
32	3023	0	0.07	0.07
33	3007	0.03	0	0.1
34	3427	0.03	0.06	0.165
35	3004	0.03	0.03	0.07
36	3002	0	0	0.07
37	3003	0	0.03	0.06
38	3002	0	0.03	0.03

*$P < 0.05$ compared with a control group.

Evaluation of DNA damage

Evaluation of DNA fragmentation showed an average of 11.5% in the fresh sperm sample, whereas in the processed sperm samples this increased to 38.9%. The results obtained for each patient are shown in Table III. An increase in DNA fragmentation was observed after sperm processing in 29 of 38 (76%) of the patients. There was no correlation between DNA fragmentation and the embryo aneuploidy rate ($R^2 = 0.0215$, $P = 0.37$; $R^2 = 0.0373$, $P = 0.18$, for fresh and processed sperm samples respectively, Fig. 2). We also found

Table III Percentage of embryos with normal chromosomes and DNA fragmentation index (DFI) values of fresh and processed sperm samples in each patient.

Patient	Maternal age (years)	Embryos biopsied (*n*)	Normal embryos (%)	DFI fresh sperm (%)	DFI-processed sperm (%)
1	38	1	0	19.6	47.9
2	38	7	100	5.4	47.4
3	38	1	100	8.7	54.5
4	35	1	0	13.0	97.9
5	37	9	22.2	4.5	88.9
6	38	6	33.3	5.4	9.4
7	38	2	0	14.9	36.0
8	38	5	40	26.8	16.9
9	39	4	0	0.5	3.2
10	34	2	50	15.6	55.1
11	37	3	33.3	27.1	40.5
12	37	4	25	6.0	67.0
13	35	6	33.3	7.4	18.1
14	33	7	42.8	21.6	86.7
16	38	1	0	5.2	66.0
17	33	2	0	18.3	2.8
19	37	5	50	10.3	6.9
21	33	2	0	2.0	88.0
22	37	7	14.2	6.0	67.0
23	38	3	66.7	2.0	42.0
24	35	6	33.3	10.3	6.9
25	38	3	0	9.0	12.5
26	39	5	20	30.2	38.5
28	36	3	33.3	9.4	7.6
29	38	5	60	4.05	14.3
30	30	10	70	4.8	16.5
31	39	6	40	17.3	23.4
32	39	3	0	17.8	84.2
33	36	2	50	16.8	79.7
34	38	5	40	25.0	72.5
35	36	3	0	2.5	10.4
36	37	6	33.3	6.8	14.7
37	39	4	100	12.0	31.0
38	39	4	25	4.0	18.0

there was no correlation between DNA fragmentation and aneuploidy in fresh or processed sperm samples (Table IV).

Discussion

The aim of this study was to assess the relationship between DNA fragmentation and aneuploidy rate in embryos from patients with RPL or RIF. The main finding from this study was that there was no relationship between sperm DNA fragmentation from RPL or RIF couples and the aneuploidy rate in embryos or in spermatozoa. Our findings are only applicable to our specific group of patients, and their generalization to other infertile males where partners do not have RPL or RIF is inappropriate at this time. However, we cannot rule out the possibility that a relationship between DNA fragmentation and aneuploidy exists for other causes of infertility. There are three earlier studies focused on the relationship between DFI and the sperm aneuploidy rate. Two studies found a positive correlation between these two parameters (Liu *et al.*, 2004; Muriel *et al.*, 2007), while the third and most recent study failed to find a correlation (Balasuriya *et al.*, 2011). None of these studies evaluated the embryo aneuploidy rate.

In order to study the relationship between DFI and the aneuploidy rate, sperm DNA damage in fresh and processed sperm samples was evaluated using the TUNEL assay, while aneuploidy rates were determined in the same samples as well as in embryos using FISH. The difference between this and other recent studies is that we

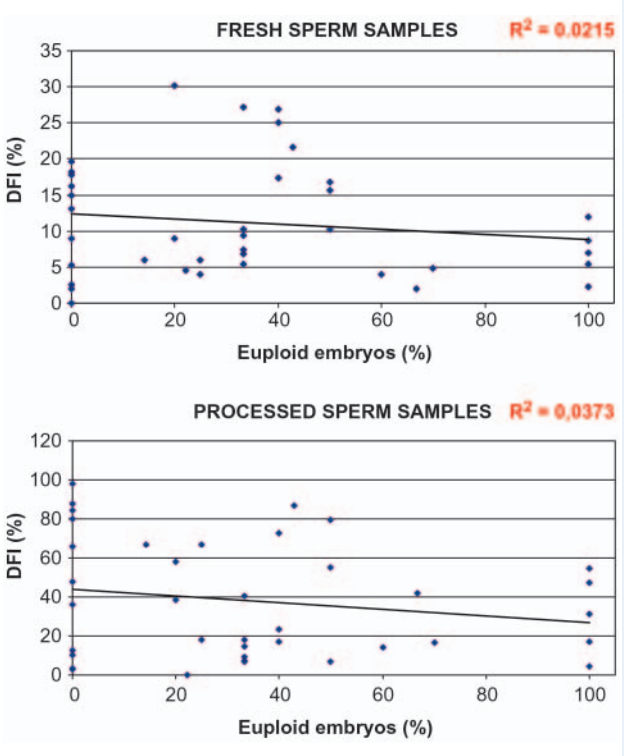

Figure 2 Correlation between DNA fragmentation index (DFI) of fresh and processed sperm samples and the embryo aneuploidy rate.

Table IV Correlation between DFI and sperm aneuploidy in fresh and processed sperm samples.

	Fresh sperm		Processed sperm	
	R^2	*P*-value	R^2	*P*-value
Sex disomies	0.0003	0.91	0.0145	0.75
18 disomy	0.0446	0.20	0.1308	0.10
Sex diploidy	0.0133	0.49	0.0024	0.56
13 disomy	0.0217	0.38	0.1254	0.85
21 disomy	0.0397	0.23	0.0564	0.17
13/21 diploidy	0.0077	0.60	0.0013	0.86

tested the aneuploidy rate in embryos fertilized by males with and without elevated sperm DFI values. To the best of our knowledge, this is the first study to look for a correlation of the embryo aneuploidy rate and the sperm DFI.

Tang *et al.* (2010) found that spermatozoa from teratozoospermic males showed higher rates of chromosomal abnormalities and higher DFI values compared with fertile men. Perrin *et al.* (2009) found an increase in the DFI in the sperm of male carriers of a structural chromosomal abnormality, compared with non-carriers. Finally, Muriel *et al.* (2007) reported a higher aneuploidy rate in spermatozoa with fragmented DNA than in spermatozoa with no fragmentation.

Liu *et al.* (2004) found an increase in the aneuploidy rate and DNA fragmentation in spermatozoa from men with oligoasthenoteratozoospermia compared with normozoospermic men. However, DFI did not correlate with the sperm aneuploidy rate.

Muriel *et al.* (2007) evaluated the DFI and the sperm aneuploidy rate using a modified version of the sperm chromatin dispersion (SCD) test (Halosperm kit; INDAS Laboratories), and found an average 4.4-fold increase in aneuploidy in men with fragmented DNA in sperm compared with sperm with non-fragmented DNA. They concluded that aneuploidy could trigger DNA fragmentation via an apoptotic-like process mediated by endogenous nucleases.

On the contrary, Balasuriya *et al.* (2011) concluded that there was no significant correlation between DNA fragmentation and aneuploidy, in agreement with our findings. Balasuriya used four different tests (SCD, SCD-FISH, Halosperm and sperm chromatin structure assay), as well as FISH, to analyse sperm. They found a significant difference between the FISH and SCD-FISH results in terms of detecting aneuploidy and concluded that SCD-FISH can overestimate aneuploidy. Based on their results, SCD-FISH should not be used to measure the sperm aneuploidy rate.

The present study included a larger sample size, 38 patients, whereas the previous two studies that found a correlation between DNA fragmentation and aneuploidy included only 20 patients (Liu *et al.*, 2004) and 16 patients (Muriel *et al.*, 2007). Another technical difference is that we analysed five chromosomes compared with three chromosomes in those two studies. With our larger sample size and analysis of five chromosomes, we found that the copy number of chromosomes in embryos and spermatozoa was not related to sperm DFI.

It has been described by many authors that sperm quality contributes to recurrent miscarriage and RIF (Vanderzwalmen *et al.*, 1991; Perinaud *et al.*, 1993; Janny and Menezo, 1994; Hammadeh *et al.*, 1996; Sánchez *et al.*, 1996; Shoukir *et al.*, 1998; Rubio *et al.*, 1999; Carrell *et al.*, 2003; Douglas *et al.*, 2003; Saleh *et al.*, 2003; Lin *et al.*, 2005, 2008; Borini *et al.*, 2006; Weissman *et al.*, 2008; Zini *et al.*, 2008; Kennedy *et al.*, 2011). The meta-analysis carried out by Zini *et al.* (2008) concluded that spontaneous pregnancy loss is associated with sperm DFI. Many studies have shown an increase in the aneuploidy rate in embryos from patients with RPL and demonstrated that PGD-aneuploidy screening (PGS) may improve implantation, pregnancy and miscarriage rates (Rubio *et al.*, 2003, 2005, 2009; Munné *et al.*, 2005; Platteau *et al.*, 2005; ; Blockeel *et al.*, 2008; Garrisi *et al.*, 2008; Lathi *et al.*, 2008). However, we did not find a correlation between sperm DFI and the aneuploidy rate in our series. A possible explanation might be that the sperm samples included in this study were far from being abnormal, which may have biased the results. In fact, Bellver *et al.* (2010) showed significant differences in DFI among oligozoospermic patients, fertile sperm donors and couples who had experienced iodiopathic RPL, but failed to find DFI as a cause of miscarriage in patients with RPL.

In contrast to previous publications (Meseguer *et al.*, 2011), we found an increased DNA fragmentation after sperm processing. However, different protocols were used to process the sperm samples, and it may be that density gradients affect sperm cells differently, in terms of DNA fragmentation, from swim-up processing.

Given our finding of the lack of a correlation between DNA fragmentation and aneuploidy, it may not be useful to perform PGS

cycle in patients in which the eggs were fertilized by sperm with elevated DNA fragmentation, at least when the sperm sample is normal, in terms of motility, concentration or morphology.

In conclusion, our results showed no correlation between the sperm DFI and the sperm or embryo aneuploidy rate in patients with RPL or RIF. However, we cannot rule out the possibility that a relationship between DNA fragmentation and aneuploidy exists for other causes of infertility.

Authors' roles

F.B. and E.M.: study design; J.A.G.-V.: ovarian stimulation; F.B., E.M., M.A. and M.N.: embryo culture and biopsy; A.P. and M.S.C.: sperm DNA fragmentation analysis; F.B., M.G., A.L. and D.C.: embryo and sperm FISH analysis; F.B., E.M. and J.A.G.-V.: statistical analysis; F.B. and E.M.: manuscript drafting; F.B., E.M. and J.A.G.-V.: manuscript review.

Funding

No external funding was received for this study.

Conflict of interest

None declared.

References

Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 2001;**122**:497–506.

Alvarez JG. The predictive value of sperm chromatin structure assay. *Hum Reprod* 2005;**28**:2365–2367.

Balasuriya A, Speyer B, Serhal P, Doshi A, Harper JC. Sperm chromatin dispersion test in the assessment of DNA fragmentation and aneuploidy in human spermatozoa. *Reprod Biomed Online* 2011; **22**:428–436.

Bellver J, Meseguer M, Muriel L, García-Herrero S, Barreto MA, Garda AL, Remohí J, Pellicer A, Garrido N. Y chromosome microdeletions, sperm DNA fragmentation and sperm oxidative stress as causes of recurrent spontaneous abortion of unknown etiology. *Hum Reprod* 2010; **25**:1713–1721.

Blanco J, Egozque J, Vidal F. Incidence of chromosome 21 disomy in human spermatozoa as determined by fluorescent in-situ hybridization. *Hum Reprod* 1996;**4**:722–726.

Blockeel C, Schutyser V, Vos A, Verpoest W, Vos M, Staessen C, Haentjens P, Elst J, Devroey P, Prospectively randomized controlled trial of PGS in IVF/ICSI patients with poor implantation. *Reprod Biomed Online* 2008;**17**:848–854.

Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, Coticchio G. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. *Hum Reprod* 2006;**21**:2876–2881.

Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod* 2004;**19**:1401–1408.

Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, Campbell B. Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl* 2003;**49**:49–55.

Douglas T, Wilcox AC, Lowy L, Peterson M, Jones K, Erickson L, Campbell B, Branch DW, Hatasaka H. Elevated sperm chromosome aneuploidy and apoptosis in patients with unexplained recurrent pregnancy loss. *Am J Obstet Gynecol* 2003;**101**:1129–1235.

ESHRE PGD Consortium Steering Committee. ESHRE Preimplantation Genetic Diagnosis Consortium: data collection III (May 2001). *Hum Reprod* 2002;**17**:233–246.

Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 1980;**210**: 1131–1133.

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

Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl* 2002;**23**:25–43.

Garcia-Velasco JA, Bermejo A, Ruiz F, Martinez-Salazar J, Requena A, Pellicer A. Cycle scheduling with oral contraceptive pills in the GnRH antagonist protocol vs the long protocol: a randomized, controlled trial. *Fertil Steril* 2011;**96**:590–593.

Garrisi J, Ferry KM, Ketterson K, Esprit-Ngatchou N, Cekleniak NA, Cohen J. Single blastomere biopsy of slow cleaving embryos can support subsequent development and implantation. *Fertil Steril* 2008; **90-S**:S307–S308.

Hammadeh ME, Hasani SA1, Stieber M, Rosenbaum P, Kiipker D, Diedrich K, Schmidt W. The effect of chromatin condensation (Aniline Blue staining) and morphology (strict criteria) of human spermatozoa on fertilization, cleavage and pregnancy rates in an intracytoplasmic sperm injection programme. *Hum Reprod* 1996;**11**:2468–2471.

Høst E, Lindenberg S, Smidt-Jensen S. The role of DNA strand breaks in human spermatozoa used for IVF and ICSI. *Acta Obstet Gynecol Scand* 2000;**79**:189–193.

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

Janny L, Menezo YJR. Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. *Mol Reprod Dev* 1994;**38**:36–42.

Kennedy C, Ahlering P, Rodriguez H, Levy S, Suovsky P. Sperm chromatin structure correlates with spontaneous abortion and multiple pregnancy rates inassisted reproduction. *Reprod Biomed Online* 2011;**22**:272–276.

Lathi RB, Westphal LM, Milki AA. Aneuploidy in the miscarriages of infertile women and the potential benefit of preimplanation genetic diagnosis. *Fertil Steril* 2008;**89**:353–357.

Lin YM, Kuo PL, Lin YH, Teng YN, Nan Lin JS. Messenger RNA transcripts of the meiotic regulator BOULE in the testis of azoospermic men and their application in predicting the success of sperm retrieval. *Hum Reprod* 2005;**20**:782–788.

Lin MH, Kuo-Kuang LR, Li SH, Lu CH, Sun FJ, Hwu YM. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. *Fertil Steril* 2008;**90**:352–359.

Liu CH, Tsao HM, Cheng TC, Wu HM, Huang CC, Chen CI, Lin DP, Lee MS. DNA fragmentation, mitochondrial dysfunction and chromosomal aneuploidy in the spermatozoa of oligoasthenoteratozoospermic males. *J Assist Reprod Genet* 2004;**21**:119–126.

Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil Steril* 1998;**69**:528–532.

Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U *et al*. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biol Reprod* 1995;**52**:864–867.

Meseguer M, Santiso R, Garrido N, García-Herrero S, Remohí J, Fernandez JL. Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. *Fertil Steril* 2011;**95**:124–128.

Munné S, Chen S, Fischer J, Colls P, Zheng X, Stevens J, Escudero T, Oter M, Schoolcraft B, Simpson JL *et al*. Preimplantation genetic diagnosis reduces pregnancy loss in women aged 35 years and older with a history of recurrent miscarriages. *Fertil Steril* 2005;**84**:331–335.

Muriel L, Goyanes V, Segrelles E, Gosálvez J, Alvarez JG, Fernández JL. Increased Aneuploidy rate in sperm with fragmented DNA as determined by the sperm chromatin dispersion (SCD) test and fish analysis. *J Androl* 2007;**28**:38–49.

Perinaud J, Mieusset R, Vieitez G, Labal R, Richoilley G. Influence of sperm parameters on embryo quality. *Fertil Steril* 1993;**60**:888–892.

Perrin A, Caer E, Oliver-Bonet M, Navarro J, Benet J, Amice V, De Braekeleer M, Morel F. DNA fragmentation and meiotic segregation in sperm of carriers of a chromosomal structural abnormality. *Fertil Steril* 2009;**92**:583–589.

Platteau P, Staessen C, Michiels A, Steirteghem A, Liebaers I, Devroey P. Preimplantation genetic diagnosis for aneuploidy screening in patients with unexplained recurrent miscarriages. *Fertil Steril* 2005;**83**:393–397.

Rodrigo L, Rubio C, Mateu E, Simón C, Remohí J, Pellicer A, Gil-Salom M. Analysis of chromosomal abnormalities in testicular and epididymal spermatozoa from azoospermic ICSI patients by fluorescence in situ hybridization. *Hum Reprod* 2004;**19**:118–123.

Rubio C, Simón C, Blanco V, Vidal F, Minguez Y, Egozcue J, Crespo J, Remohí J, Pellicer A. Implications of sperm chromosome abnormalities in recurrent miscarriage. *J Assist Reprod Genet* 1999;**16**:253–258.

Rubio C, Gil- Salom M, Simon C, Vidal F, Rodrigo L, Mínguez Y, Remohí J, Pellicer A. Incidence of sperm chromosomal abnormalities in a risk population: relationship with sperm quality and ICSI outcome. *Hum Reprod* 2001;**16**:2084–2092.

Rubio C, Simón C, Vidal F, Rodrigo L, Pehlivan T, Remohí J, Pellicer A. Chromosomal abnormalities and embryo development in recurrent miscarriage couples. *Hum Reprod* 2003;**18**:182–188.

Rubio C, Pehlivan T, Rodrigo L, Simón C, Remohí J, Pellicer A. Embryo aneuploidy screening for unexplained recurrent miscarriage: a mini review. *Am J Reprod Immunol* 2005;**53**:159–165.

Rubio C, Buendía P, Rodrigo L, Mercader A, Mateu E, Peinado V, Delgado A, Milán M, Mir P, Simón C *et al*. Remohí J, Pellicer A.< Prognostic factors for preimplantation genetic screening in repeated pregnancy loss. *Reprod Biomed Online* 2009;**18**:687–693.

Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod* 1999;**4**:31–37.

Saleh RA, Agarwal A, Sharma RK, Said TM, Sikka SC, Thomas AJ Jr. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. *Fertil Steril* 2003;**80**:1431–1436.

Sánchez R, Stalf T, Khanaga O, Turley H, Gips H, Schill WB. Sperm selection methods for intracytoplasmic sperm injection (ICSI) and andrological patients. *J Assist Reprod Genet* 1996;**13**:228–233.

Shoukir Y, Chardonnens D, Campana A, Sakkas D. Blastocyst development from supernumerary embryos after intracytoplamic sperm injection: a paternal influence? *Hum Reprod* 1998;**13**:1632–1637.

Spano M, Bonde JP, Hjøllund HI, Kolstad HA, Cordelli E, Leter G. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. *Fertil Steril* 2000;**73**:43–50.

Tang SS, Gao H, Zhao Y, Ma S. Aneuploidy and DNA fragmentation in morphologically abnormal sperm. *Int J Androl* 2010;**33**:163–179.

Tarkowski AK. An air drying method for chromosome preparations from mouse eggs. *Cytogenetics* 1966;**5**:394–400.

Twigg JP, Irvine DS, Aitken RJ. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum Reprod* 1998;**13**:1864–1871.

Vanderzwalmen P, Berin-Segal G, Geetz L, Debauche C, Schoysman R. Sperm morphology and IVF pregnancy rate: comparison between Percoll gradient centrifugation and swim-up procedures. *Hum Reprod* 1991;**6**:581–588.

Vegetti W, Van Assche E, Frias A, Verheyen G, Bianchi MM, Bonduelle M, Liebaers I, Van Steirteghem A. Correlation between semen parameters and sperm aneuploidy rates investigated by fluorescence in-situ hybridization in infertile men. *Hum Reprod* 2000;**15**:351–365.

Vidal F, Giménez C, Rubio C, Simón C, Pellicer A, Santaló J, Egozcue J. FISH preimplantation diagnosis of chromosome aneuploidy in recurrent pregnancy wastage. *J Assist Reprod Genet* 1998;**15**:310–313.

Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril* 2004;**81**:1289–1295.

Weissman A, Horowitz E, Ravhon A, Nahum H, Golan A, Levran D. Pregnancies and live births following ICSI with testicular spermatozoa after repeated implantation failure using ejaculated spermatozoa. *Reprod Biomed Online* 2008;**17**:220–228.

World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. Cambridge: Cambridge University, 2010.

Zini A, Bielecki R, Phang D, Zenzes MT. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril* 2001; **75**:674–677.

Zini A, Boman JM, Belzile E, Ciampi A. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. *Hum Reprod* 2008;**23**:2663–2668.