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Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups

J. Ribas-Maynou[1,2], A. García-Peiró[1,2], C. Abad[3], M.J. Amengual[4], J. Navarro[1,2], and J. Benet[1,2,*]

[1]Càtedra de Recerca Eugin-UAB, Universitat Autònoma de Barcelona, Bellaterra, Spain [2]Departament de Biologia Cel·lular, Fisiologia i Immunologia, Universitat Autònoma de Barcelona, Bellaterra, Spain [3]Consorci Hospitalari Parc Taulí, Servei d'Urologia, Sabadell, Spain [4]Consorci Hospitalari Parc Taulí, U.D.I.A.T., Sabadell, Spain

*Correspondence address. Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Medicina, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Spain. Tel: +34-935811773; Fax: +34-935811025; E-mail: jordi.benet@uab.cat

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BACKGROUND: The analysis of sperm DNA fragmentation has become a new marker to predict male infertility, and many techniques have been developed. The sperm Comet assay offers the possibility of differentiating single- and double-stranded DNA (ssDNA and dsDNA) breaks, which could have different effects on fertility. The objective of this study was to perform a descriptive characterization of different groups of patients, such as those with asthenoteratozoospermic (ATZ) with or without varicocele, oligoasthenoteratozoospermic (OATZ) or balanced chromosome rearrangements, as compared with fertile donors. The Comet assay was used to investigate sperm samples for ssDNA and dsDNA breaks.

METHODS AND RESULTS: The analysis of alkaline and neutral Comet assays in different groups of patients showed different sperm DNA damage profiles. Most fertile donors presented low values for ssDNA and dsDNA fragmentation (low-equivalent Comet profile), which would be the best prognosis for achieving a pregnancy. OATZ, ATZ and ATZ with varicocele presented high percentages of ssDNA and dsDNA fragmentation (high-equivalent Comet assay profile), ATZ with varicocele being associated with the worst prognosis, due to higher levels of DNA fragmentation. Rearranged chromosome carriers display a very high variability and, interestingly, two different profiles were seen: a high-equivalent Comet assay profile, which could be compatible with a bad prognosis, and a non-equivalent Comet assay profile, which has also been found in three fertile donors.

CONCLUSIONS: Comet assay profiles, applied to different clinical groups, may be useful for determining prognosis in cases of male infertility.

Key words: sperm / DNA fragmentation / Comet assay / chromosomal rearrangement / varicocele

Introduction

Infertility is a health problem affecting 15% of all couples of reproductive age. The male factor is present in ~50% of all infertility cases; moreover, an exclusive male factor accounts for ~20% of cases (de Kretser, 1997). Consequently, the study of implicated causes of male factor infertility is a subject of increasing interest. Traditional methods to assess male infertility diagnosis have been mainly based on seminogram parameters. Although this information is necessary, results obtained are not conclusive in accurately determining the fertility status of many patients (Lewis, 2007). More recently, the genomic status of the sperm cell has been investigated in meiotic studies to determine synapsis alterations and recombination (Egozcue *et al.*, 1997; Carrell, 2008; Templado *et al.*, 2011). At a single sperm level, determination of chromosomal aneuploidy using fluorescent *in situ* hybridization methods have also significantly improved the field of male infertility diagnosis (Benet *et al.*, 2005; Martin, 2006). However, prediction of infertility in a reliable manner is still not possible (Collins *et al.*, 2008b). In spite of the progress made, the diagnosis of sperm quality remains controversial (Practice Committee of American Society for Reproductive Medicine, 2008; Zini and Sigman, 2009; Lewis and Simon, 2010; Zini, 2011).

In recent years, the analysis of sperm DNA fragmentation (SDF) has become another marker of genome quality, and for this reason, many tests have been developed for both research and clinical applications (Evenson *et al.* 1980, 2002; Gorczyca *et al.*, 1993; Evenson and Jost 2000; Fernandez *et al.*, 2003; Sharma *et al.*, 2010; Mitchell *et al.*

2011). Characterization of mechanisms and causes of DNA fragmentation are not easy, because there are many intrinsic and extrinsic factors involved. Different factors causing SDF have been proposed (Aitken and De Iuliis 2010; Sakkas and Alvarez 2010). Principally, oxidative stress (Agarwal *et al*., 2008; Makker *et al*., 2009; Aitken and Koppers, 2011), endogenous endonuclease and caspase activation (Maione *et al*., 1997; Sailer *et al*., 1997), alterations to chromatin remodeling during spermiogenesis (Marcon and Boissonneault, 2004; Carrell *et al*., 2007) and apoptosis of germ cells at the beginning of meiosis (Pentikainen *et al*., 1999; Sakkas *et al*., 1999, 2004) have been identified as intrinsic factors. External factors causing DNA damage have also been described, such as radiotherapy, chemotherapy and environmental toxicants (Morris, 2002; Rubes *et al*., 2007; O'Flaherty *et al*., 2008). All of these mechanisms can affect DNA strands in a various manners, producing, in the end, single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) breaks.

Although conventional DNA damage methodologies have established a threshold value based on the percentage of sperm with fragmented DNA (Sergerie *et al*., 2005; Evenson and Wixon, 2008; Sharma *et al*., 2010), the methods previously mentioned are not capable of distinguishing between ssDNA and dsDNA breaks in a separate form. Characterization of the type of DNA break could be interesting from the clinical point of view because it can give guidance regarding which mechanisms may be relevant in producing the DNA damage. Single-cell gel electrophoresis (Comet assay) allows the distinction between ssDNA and dsDNA breaks, depending on whether alkaline denaturing or neutral conditions are performed (Singh *et al*., 1988; Morris *et al*., 2002; Van Kooij *et al*., 2004; Enciso *et al*., 2009). This information from the Comet assay could provide DNA strand break profiles in patient subgroups classified according to their clinical features.

Therefore, this research was conducted to characterize the ssDNA and dsDNA fragmentation profiles, assessed by alkaline and neutral Comet assays, in fertile donors and different groups of patients. The patients were selected according to anomalies in sperm count, motility and morphology, such as oligoasthenoteratozoospermic (OATZ) and asthenoteratozoospermic (ATZ), or due to having pathologies with a high incidence of infertility such as varicocele or balanced chromosomal rearrangements.

Materials and Methods

Semen samples and cryopreservation

Semen samples from 73 men were divided into 5 groups: 15 fertile donors with proven fertility, 15 ATZ with clinical varicocele, 15 ATZ without varicocele, 15 OATZ and 13 patients with structural chromosome rearrangements that include: 9 reciprocal translocations, t(1;13), t(2;13), t(3;8), t(3;19), t(4;8), t(9;17), t(10;14), t(11;17), t(12;16); 1 Robertsonian translocation, t(14;21); 2 double translocations, both t(2;17), t(14;21) and 1 inversion, inv7. Sperm counts (spermatozoa/ml), motility (A + B %) and morphology (normal forms %), respectively, were: 83 ± 48 sperm/ml, 37 ± 23% and 8 ± 3% for fertile donors; 140 ± 122 sperm/ml, 17 ± 10 and 5 ± 2% for ATZ with clinical varicocele; 94 ± 51 sperm/ml, 14 ± 7 and 5 ± 5% for ATZ without varicocele and 11 ± 4 sperm/ml, 16 ± 7 and 5 ± 2% for OATZ. Details of seminograms and meiotic chromosome segregation of 9 reciprocal translocation patients and of the inversion patient have been reported elsewhere (Perrin *et al*., 2009).

Samples were obtained by masturbation after a minimum of 3 days of abstinence. Seminograms were performed according to the World Health Organization 2010 criteria (WHO, 2010), and samples were cryopreserved in the Test-yolk buffer (14% glycerol, 30% egg yolk, 1.98% glucose and 1.72% sodium citrate; Garcia-Peiro *et al*., 2011a,b). Informed consent was obtained from all patients and the study was approved by the appropriate ethics committee.

Neutral and alkaline Comet assay

The Comet assay protocol was performed on all semen samples according to the Enciso *et al*. (2009) method, with slight modifications. Neutral and alkaline Comet assays were carried out simultaneously in two different slides. First, an aliquot of the total semen was thawed and washed three times in phosphate-buffered saline. Then, sperm cells were diluted to a concentration of 10×10^6 spermatozoa/ml, and 25 µl was mixed with 50 µl of 1% low-melting point agarose (Sigma Aldrich, St Louis, MO, USA) in distilled water. Quickly, 15 µl of the mixture was placed on two different pre-treated slides for gel adhesion (1% low-melting point agarose), covered with coverslips and allowed to gel on a cold plate at 4°C for 5 min. Next, coverslips were carefully removed and slides were submerged for 30 min in two lysing solutions (Comet lysis solutions, Halotech, Madrid, Spain) and washed for 10 min in tris borate EDTA (TBE) (0.445 M Tris–HCl, 0.445 M Boric acid and 0.01 M EDTA). For the neutral Comet assay, electrophoresis was performed in the TBE buffer at 20 V (1 V/cm) for 12 min and 30 s, with a subsequent wash in 0.9% NaCl for 2 min. For the alkaline Comet assay, the slide was incubated in a denaturing solution (0.03 M NaOH and 1 M NaCl) for 2 min and 30 s at 4°C, and electrophoresis was then performed in 0.03 M NaOH buffer at 20 V (1 V/cm) for 4 min. Both neutral and alkaline slides were then incubated in the neutralizing solution (0.4 M Tris–HCl, pH 7.5) for 5 min, in TBE for 2 min and finally dehydrated in an ethanol series of 70, 90 and 100% for 2 min each.

Induction of ssDNA breaks: H_2O_2 treatment

In order to induce ssDNA breaks, incubations of 30 min at room temperature with hydrogen peroxide (Sigma Aldrich) at 0, 0.03, 0.15 and 0.30% were performed on five samples from fertile donors with a known low percentage of neutral and alkaline Comet SDF. After hydrogen peroxide treatment, samples were diluted at 10×10^6 spermatozoa/ml and the Comet assay protocol was performed as described above.

Induction of dsDNA breaks: AluI restriction enzyme treatment

Induction of dsDNA breaks was performed on the same five samples from fertile donors with a known low DNA fragmentation rate as mentioned above.

After two lysis treatments, slides were rinsed with 50 µl of reaction buffer and treated with the AluI restriction enzyme (Sigma Aldrich) for different times: 15 IU for 15 min, 15 IU for 25 min and 0 IU as a control. Afterwards, slides were washed in TBE for 5 min and the protocol was continued at the electrophoresis step, depending on neutral or alkaline Comet assays as described above.

Staining and evaluation of samples

All Comet assay samples were stained with DAPI SlowFade® Gold antifade (Invitrogen, Eugene, OR, USA) and were evaluated using a fluorescence microscope (Olympus AX70), counting at least 400 spermatozoa per sample. Sperm cells were classified according to fragmented and non-fragmented sperm. Different levels of DNA damage are shown in Fig. 1.

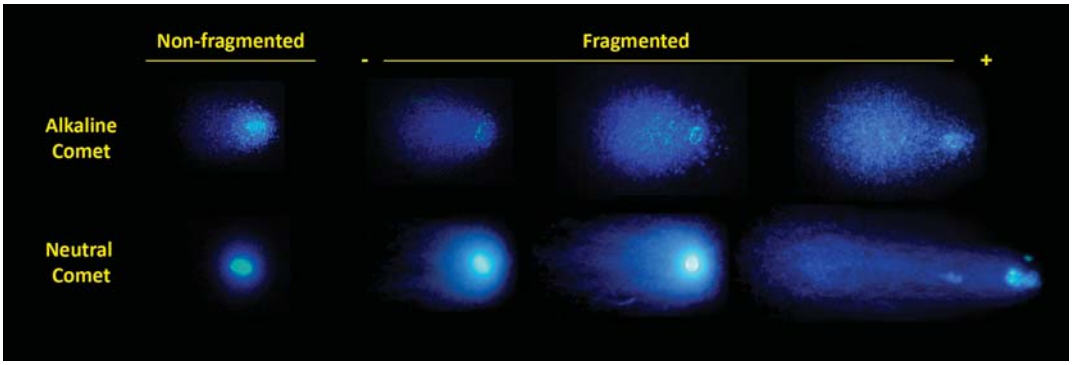

Figure 1 Non-fragmented and fragmented spermatozoa in alkaline and neutral Comet assays. Different levels of sperm DNA fragmentation (SDF) are shown for fragmented spermatozoa (DAPI staining).

Statistical analysis

Statistical analyses of data were performed using the Statistics Package for the Social Sciences software, version 17 (SPSS, Inc., Chicago, IL, USA). Values were compared using a non-parametric, Mann–Whitney *U*-test. A 95% confidence interval was set as being statistically significant.

Results

Oxidative and enzymatic DNA damage induction

Figure 2 shows data pertaining to SDF induction in samples from five donors with proven fertility and a known low alkaline and neutral SDF (<25%; Simon *et al.*, 2011). The effect of incubation of these samples with increasing concentrations of hydrogen peroxide, on both the alkaline and neutral Comet assay SDF, is shown in Fig. 2A. The effect of oxidative stress treatment in controls significantly increased SDF in both alkaline and neutral Comet assays ($P = 0.008$; $P = 0.032$), respectively. This effect was about three times higher in the alkaline Comet assay, with respect to the neutral Comet assay, even at low concentrations of H_2O_2. Contrasting results were obtained when restriction enzyme incubations were performed on samples from the same five fertile donors (Fig. 2B). Alu1 incubation statistically increased SDF ($P = 0.009$; $P = 0.009$) shown by both alkaline and neutral Comet assay, respectively, but produced more than two times more SDF in the neutral Comet assay, with respect to the alkaline Comet assay, after 15 min of incubation.

SDF assessment in different groups of patients

SDF values from both alkaline and neutral Comet assays for different clinical patient groups are shown in Table I and Fig. 3. Statistically significant differences were observed in both alkaline and neutral Comet assays between fertile controls and the entire group of infertile patients ($P < 0.01$). Attending to their clinical classification, statistical differences were also found between fertile controls and each of the infertility subgroups ($P < 0.01$).

Higher values of SDF were observed in the ATZ with varicocele subgroup, being statistically different for both Comet assay methods

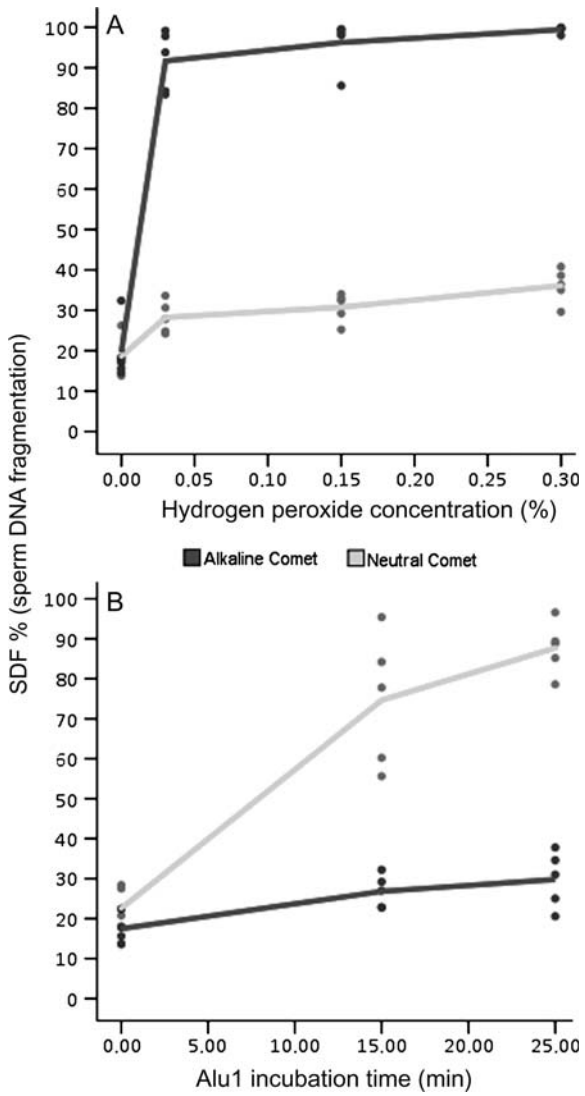

Figure 2 Alkaline and neutral Comet assays evaluating ssDNA and dsDNA breaks, respectively, in incubations with (**A**) increasing concentrations of H_2O_2 and (**B**) Alu1 restriction enzyme for different times.

Table I Sperm DNA fragmentation (SDF) values (mean ± SD) in different groups of patients.

	% SDF	
	Alkaline Comet	Neutral Comet
Fertile controls ($n = 15$)	21.1 ± 5.91	31.59 ± 26.85
Infertile patients		
ATZ without varicocele ($n = 15$)	60.52 ± 11.05[a,b]	65.38 ± 11.18[a,b]
OATZ ($n = 15$)	60.81 ± 11.08[a,b]	61.86 ± 16.48[a,b]
ATZ with varicocele ($n = 15$)	78.98 ± 8.49[a]	78.80 ± 13.66[a]
Rearranged chromosome carriers ($n = 13$)	73.24 ± 27.63[a]	66.61 ± 27.99[a]
Total infertile ($n = 58$)	68.22 ± 17.46[a]	68.22 ± 18.74[a]

[a]Significant differences, with respect to fertile controls ($P < 0.01$).
[b]Significant differences, with respect to ATZ with varicocele ($P < 0.01$).

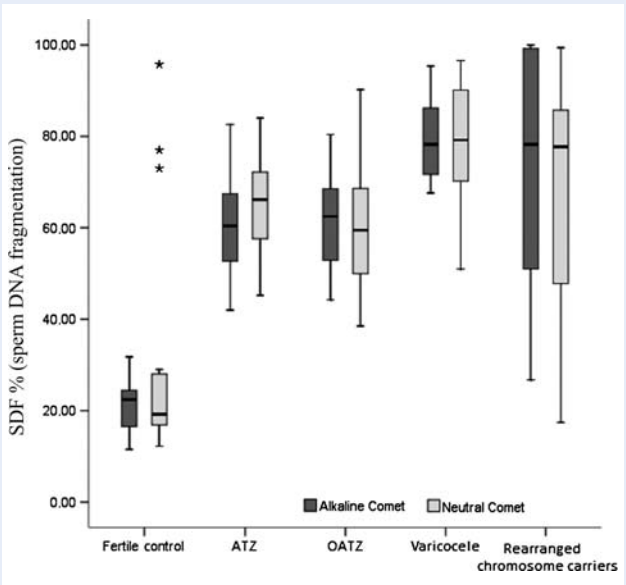

Figure 3 Alkaline and neutral Comet assay SDF in fertile controls and four groups of patients.

when compared with ATZ without varicocele and OATZ subgroups ($P < 0.01$).

No significant differences was observed between the ATZ without varicocele, OATZ and rearranged chromosome carrier subgroups, although a high variability of SDF was observed in both alkaline and neutral Comet assays for the carriers of balanced chromosomal rearrangements (Table I and Fig. 3).

Discussion

Interest in SDF has mainly been focused on predicting male infertility. Although different threshold values for the different methodologies have been proposed (Evenson *et al.*, 1999; Evenson and Wixon, 2008; Velez de la Calle *et al.*, 2008; Sharma *et al.*, 2010; Simon *et al.*, 2011), no differentiation about the relative presence of ssDNA or dsDNA breaks has been reported in infertile or subfertile patients. This distinction may have significant consequences for fertility because sperm DNA damage can occur through different mechanisms (Aitken and De Iuliis, 2010; Sakkas and Alvarez, 2010), and the resulting DNA damage profile could be linked with yet unknown pathophysiological aspects of the patient. Regarding this assumption, to our knowledge, only one report has demonstrated an association between ssDNA breaks and oxidative stress, and dsDNA breaks and enzymatic nuclease activity in human sperm cells using the 2D-Comet assay methodology (Enciso *et al.*, 2009). In the present work, similar results were found using the same experimental conditions but applying alkaline and neutral Comet assays separately. Although there were different levels of fragmentation in each assay, our results did show a statistical increase in both alkaline and neutral DNA strand breaks for both H_2O_2 and AluI treatments, suggesting that the two types of DNA damage may be linked in some way. Recently, it has been proposed that oxidative stress can activate caspases and endonucleases in sperm (Sakkas and Alvarez, 2010). Results reported here are in agreement with this proposal. Therefore, oxidative and enzymatic DNA damage are probably related in infertile patients. Despite this, the alkaline Comet assay was much more sensitive in detecting ssDNA breaks, while the neutral Comet assay was more sensitive in detecting dsDNA breaks (Fig. 2).

Once the sensitivity of alkaline and neutral Comet assay for ssDNA and dsDNA breaks was confirmed, the analysis of different groups of patients was performed in order to characterize their DNA damage profile.

Fertile control group

Low percentages of SDF were observed in the fertile control group for both alkaline and neutral Comet assays (Fig. 3). Similar low values for both alkaline and neutral Comet assay will be referred to as a low-equivalent Comet assay profile. Alkaline Comet assay DNA fragmentation in all controls was lower than the fertility threshold value recently proposed for native semen using ART (52%; Simon *et al.*, 2011) and the majority showed lower DNA fragmentation than the 25% threshold value for natural conception (Simon *et al.*, 2011). Mostly low levels for the neutral Comet assay were shown, although three fertile donors presented high values (Fig. 3). Profiles showing low levels of alkaline SDF (< 52%) and high levels of neutral SDF are referred to as a non-equivalent Comet assay profile. There are no data in the literature about the amount of sperm DNA damage from the neutral Comet assay for fertile males. However, it has been suggested that, in somatic cells, the neutral Comet assay may be more related to the chromatin structure rather than to DNA breaks (Collins *et al.*, 2008a), although our results point out that there is a relationship between neutral Comet assay results and double-stranded breaks caused by nuclease activity. In the three fertile men, there appears to be a DNA damage mechanism that is not related to oxidative stress and has unknown consequences on fertility. In this regard, activation of nucleases has been proposed (Sotolongo *et al.*, 2005). Since the cleavage of dsDNA breaks is one of the origins of chromosomal rearrangements, dsDNA damage may contribute to an increased

risk of having embryos with chromosomal instability (Voet et al., 2011). Consistent with this, sperm DNA damage has been related to an increased risk of recurrent miscarriage (Carrell et al., 2003; Lewis and Simon, 2010).

ATZ without varicocele and OATZ patients

Oligoasthenoteratozoospermic patients are known to have the worst prognosis for becoming fertile, due to their low number of spermatozoa. This low number may lead one to think that a complex etiology could affect them (Burrello et al., 2004). High levels of DNA fragmentation for both alkaline and neutral Comet assays were found in both OATZ and ATZ samples (Fig. 3). Profiles showing high values of alkaline and neutral Comet SDF are referred to as high-equivalent Comet assay profiles. This reinforces the idea that oxidative and enzymatic DNA damage are related, at least in these groups of patients. Moreover, our results suggest that a low sperm number is not related to DNA fragmentation. According to this, in IVF/ICSI treatments, OATZ patients would have the same fertilization potential as would ATZ patients.

ATZ with varicocele

Varicocele patients have an altered spermatogenesis due to different factors (Naughton et al., 2001). High levels of oxidative stress are known to be one of the major contributors to damaging sperm function and DNA (Hendin et al., 1999; Aitken and Krausz, 2001; Hauser et al., 2001). Therefore, the results expected in varicocele patients would be higher in the alkaline Comet than in the neutral Comet assay. However, the high-equivalent Comet assay profile found in varicocele shows higher values of SDF than that in ATZ without varicocele for both alkaline and neutral Comet assays, suggesting that varicocele oxidative stress conditions intensify the two types of DNA damage. These results reinforce the fact that there is a relation between oxidative DNA fragmentation assessed by the alkaline Comet assay and enzymatic DNA damage assessed by the neutral Comet assay. Due to their oxidative damage etiology, varicocele patients could be a group likely to be successfully treated with antioxidants. Nevertheless, there are several antioxidant treatments, and they have a different effect depending on the antioxidant and on the patient (Greco et al., 2005; Agarwal and Sekhon, 2011; Gharagozloo and Aitken, 2011; Zini and Al-Hathal, 2011). Assuming that an antioxidant treatment would work on varicocele patients, we would expect a decrease in DNA fragmentation not only for the alkaline, but also for the neutral Comet assay.

Chromosomal rearrangement carriers

Chromosomal rearrangements have been traditionally associated with an increased risk of miscarriage and infertility (De Braekeleer and Dao, 1991; Benet et al., 2005). Some papers have reported that there are abnormally increased values of SDF in patients carrying Robertsonian translocations (Brugnon et al., 2010), reciprocal translocations and inversions (Perrin et al., 2009, 2011). However, a high variability of SDF has also been observed using TUNEL, SCSA and SCD, suggesting that susceptibility to DNA damage could depend on each specific type of chromosomal reorganization (Garcia-Peiro et al., 2011a,b). In order to gain information about the origin of DNA fragmentation, alkaline and neutral Comet assays were performed, and a high variability was observed for both techniques in these patients (Fig. 3).

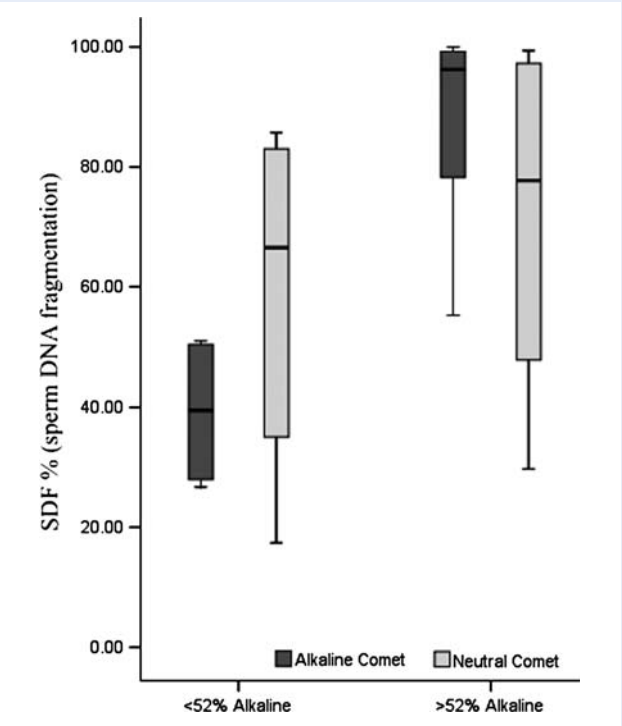

Figure 4 Alkaline and neutral Comet assay SDF in rearranged chromosome carriers, classified according to the 52% alkaline Comet threshold value. Mean ± standard deviations for the <52% alkaline Comet group were 39.18 ± 12.96% for alkaline Comet and 59.03 ± 31.28% for neutral Comet. For the >52% alkaline Comet group, the values were 88.37 ± 15.65% for alkaline Comet and 69.97 ± 27.70% for neutral Comet.

Interestingly, two DNA fragmentation profiles were found when samples were classified according to the 52% alkaline Comet assay fertility threshold proposed (Simon et al., 2011). First, a high-equivalent Comet assay profile was found when the alkaline Comet assay was >52% and, second, a non-equivalent Comet assay profile was found in patients with an alkaline Comet assay <52% (Fig. 4). The high-equivalent Comet assay profile in carriers was similar to that found in ATZ, OATZ and varicocele, and the levels of DNA fragmentation were more similar to varicocele patients than to the other groups of patients, although differences are not significant. This may lead one to think that oxidative stress could be one of the main origins of DNA fragmentation in chromosomal reorganization carriers with a high-equivalent Comet assay profile. This oxidative stress could increase neutral Comet assay DNA fragmentation by activating caspases or endonucleases (Sakkas and Alvarez, 2010). Chromosomal reorganization carriers with a non-equivalent Comet assay profile should have a better prognosis for achieving a pregnancy, considering that they had <52% alkaline Comet assay (Simon et al., 2011), and their profile was similar to the three fertile donors analyzed who also had a non-equivalent Comet assay profile, although there are not enough cases to compare them statistically.

Moreover, in our set of patients carrying chromosomal reorganizations, the analysis of the alkaline–neutral DNA profile in two brothers carrying a double translocation 45,XY,t(2;17)(q14.2;q23); t(14;21)(q10;q10) was performed and the data obtained revealed that they had different Comet assay profiles. In particular, one had a non-equivalent Comet assay profile and a baby born naturally, while the other had a high-equivalent Comet assay profile and a baby born after two cycles of PGD (Rius *et al.*, 2011). This may suggest that a non-equivalent Comet assay profile may have a better prognosis than a high-equivalent Comet assay profile, while low-equivalent Comet assay profile would correspond with the most fertile donors. In this regard, the 52% alkaline Comet assay threshold may predict infertility (Simon *et al.*, 2011), but high values for neutral Comet assay could be indicative of another unknown alteration. In this regard, further studies are needed.

Conclusion

In summary, the combination of alkaline and neutral Comet assays allows researchers to establish relationships between oxidative stress and enzymatic DNA damage, providing a very high sensitivity. DNA fragmentation profiles showed no difference between OATZ and ATZ, while the worst DNA integrity was found in varicocele patients, probably caused by oxidative stress. Different Comet assay profiles can be distinguished in carriers of balanced chromosomal rearrangements, such as the high-equivalent Comet assay profile and the non-equivalent Comet assay profile. Our results suggest that the former would have the worst prognosis, while the latter may have a better chance of achieving a pregnancy.

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Authors' roles

J.R.-M. contributed to the experimental procedure, statistical analysis, graphics and table elaboration and document writing. A.G.-P. contributed to the experimental design, results, discussion and document writing. C.A. and M.J.A. contributed to recruitment of patients, sample collection and storage. J.N. and J.B. contributed to the experimental design and direction and coordination of the work.

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Conflict of interest

None declared.

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