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

Running Title: Alkaline and neutral sperm DNA fragmentation profiles

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ABSTRACT

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 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 to fertile donors. The Comet assay was used to investigate sperm samples for single and double stranded DNA 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 single and double stranded DNA 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 single and double stranded DNA 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.

CONCLUSION: 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 about 50% of all infertility cases; moreover, an exclusive male factor accounts for about 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 JA et al., 2008). In spite of the progress made, the diagnosis of sperm quality remains controversial (Practice Committee of American Society for Reproductive Medicine, 2008; Zini & Sigman, 2009; Lewis & 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 and 2002; Gorczyca et al., 1993; Evenson & Jost 2000; Fernandez et al., 2003; Sharma et al., 2010; Mitchell et al. 2011). Characterization of mechanisms and causes of DNA fragmentation is not easy, because there are many intrinsic and extrinsic factors involved. Different factors causing sperm DNA fragmentation have been proposed (Aitken & De Iuliis 2010; Sakkas & Alvarez 2010). Principally, oxidative stress (Agarwal et al., 2008; Makker et al., 2009; Aitken &

Koppers, 2011), endogenous endonuclease and caspase activation (Maione et al., 1997; Sailer et al., 1997), alterations to chromatin remodelling during spermiogenesis (Marcon & Boissonneault, 2004; Carrell et al., 2007) and apoptosis of germ cells at the beginning of meiosis (Pentikainen et al., 1999; Sakkas et al., 1999; Sakkas et al., 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; Evenxon & 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)

103 and asthenoteratozoospermic (ATZ), or due to having pathologies with a high incidence
104 of infertility such as varicocele or balanced chromosomal rearrangements.
105

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 %) are 83±48 sperm/mL, 37±23 % and 8±3% respectively 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 WHO 2010 criteria (WHO, 2010), and samples were cryopreserved in Test-yolk buffer (14% glycerol, 30% egg yolk, 1.98% glucose, 1.72% sodium citrate) (Garcia-Peiro et al., 2011). 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 PBS. Then, sperm cells were diluted to a concentration of 10 x 10^6 spermatozoa/ml, and 25 µl was mixed with 50 µl of low melting point agarose 1% (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 4ºC for 5 minutes. Next, coverslips were carefully removed and slides were submerged for 30 minutes in two lysing solutions (Comet lysis solutions, Halotech, Madrid, Spain) and washed for 10 minutes in TBE (0.445M Tris-HCl, 0.445M Boric acid, 0.01M EDTA). For the neutral Comet assay, electrophoresis was performed in TBE buffer at 20 V (1V/cm) for 12 minutes and 30 seconds, with a subsequent wash in 0.9% NaCl for 2 minutes. For the alkaline Comet assay, the slide was incubated in denaturing solution (0.03M NaOH, 1M NaCl) for 2 minutes and 30 seconds at 4ºC, and electrophoresis was then performed in 0.03M NaOH buffer at 20V (1V/cm) for 4 minutes. Both neutral and alkaline slides were then incubated in the neutralizing solution (0.4M Tris-HCl, pH 7.5) for 5 minutes, in TBE for 2 minutes and finally dehydrated in an ethanol series of 70%, 90% and 100% for 2 minutes each.

Induction of ssDNA breaks: H_2O_2 treatment

In order to induce ssDNA breaks, incubations of 30 minutes at room temperature with hydrogen peroxide (Sigma Aldrich; St Louis, MO, USA) 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 10x10^6 spermatozoa/ml and the Comet assay protocol was performed as described above.

Induction of dsDNA breaks: Alu1 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 Alu1 restriction enzyme (Sigma Aldrich; St Louis, MO, USA) for different times: 15 IU for 15 minutes, 15 IU for 25 minutes, and 0 IU as a control. Afterwards, slides were washed in TBE for 5 minutes 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 was classified according fragmented and non-fragmented sperm. Different levels of DNA damage are shown in Figure 1.

Statistical Analysis

Statistical analyses of data were performed using the Statistics Package for the Social Sciences software, version 17 (SPSS Inc.; Chicago, IL). Values were compared using a non-parametric test, Mann-Whitney U. 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 alkaline and neutral Comet assay SDF, is shown in Figure 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 (Figure 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 minutes of incubation.

Sperm DNA fragmentation 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 Figure 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 ($p<0.01$).

195 Higher values of SDF were observed in the ATZ with varicocele subgroup, being
196 statistically different for both Comet assay methods when compared with ATZ without
197 varicocele and OATZ subgroups($p < 0.01$).
198 No significant differences was observed between the ATZ without varicocele, OATZ
199 and rearranged chromosome carrier subgroups, although a high variability of SDF was
200 observed in both alkaline and neutral Comet assays for the carriers of balanced
201 chromosomal rearrangements (Table I and Fig. 3).
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DISCUSSION

Interest in sperm DNA fragmentation has mainly been focused on predicting male infertility. Although different threshold values for the different methodologies have been proposed (Evenson et al., 1999; Evenson & 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 have 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 & De Iuliis, 2010; Sakkas & 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 single-stranded DNA breaks and oxidative stress, and double-stranded DNA 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 Alu1 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 & 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 single stranded DNA breaks, while the neutral Comet assay was more sensitive in detecting double-stranded DNA breaks (Figure 2).

Once the sensitivity of alkaline and neutral Comet assay for ss and ds DNA 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 sperm DNA fragmentation were observed in the fertile control group for both alkaline and neutral Comet assays (Figure 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 (Figure 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 AR et al., 2008), 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 double-stranded DNA breaks is one of the origins of chromosomal rearrangements, double-stranded DNA 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 & 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 (Figure 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 & Krausz, 2001; Hauser et al., 2001). Therefore the results expected in varicocele patients would be higher in the alkaline Comet than in neutral Comet assay. However, the high-equivalent Comet assay profile found in varicocele shows higher values of SDF than 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 & Sekhon 2011; Gharagozloo & Aitken, 2011; Zini & 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 & Dao, 1991; Benet et al., 2005). Some papers have reported that there are abnormally increased values of sperm DNA fragmentation in patients carrying Robertsonian translocations (Brugnon et al., 2010), reciprocal translocations and inversions (Perrin et al., 2009; Perrin et al., 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., 2011). 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 (Figure 3).

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 higher than 52% and, second, a non-equivalent Comet assay profile was found in patients with an alkaline Comet assay lower than 52% (Figure 4). The high-equivalent Comet assay profile in carriers was similar to high-equivalent Comet assay profiles 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 & 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 less than a 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.

AUTHORS' CONTRIBUTION TO THE PAPER

Jordi Ribas-Maynou contributed to the experimental procedure, statistical analysis, graphics and table elaboration and document writing.

Agustín García-Peiró contributed to the experimental design, results, discussion and document writing.

Carlos Abad and María José Amengual contributed to recruitment of patients, sample collection and storage.

Joaquima Navarro and Jordi Benet contributed to the experimental design and direction and coordination of the work.

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353 **REFERENCES**

354 Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor
355 infertility: an update. *Am.J.Reprod.Immunol.* 2008; **1:** 2-11.

356 Agarwal A, Sekhon LH. Oxidative stress and antioxidants for idiopathic
357 oligoasthenoteratospermia: Is it justified? *Indian.J.Urol.* 2011; **1:** 74-85.

358 Aitken RJ, De Iuliis GN. On the possible origins of DNA damage in human
359 spermatozoa. *Mol.Hum.Reprod.* 2010; **1:** 3-13.

360 Aitken RJ, Koppers AJ. Apoptosis and DNA damage in human spermatozoa. *Asian*
361 *J.Androl.* 2011; **1:** 36-42.

362 Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome.
363 *Reproduction* 2001; **4:** 497-506.

364 Benet J, Oliver-Bonet M, Cifuentes P, Templado C, Navarro J. Segregation of
365 chromosomes in sperm of reciprocal translocation carriers: a review. *Cytogenet.Genome*
366 *Res.* 2005; **3-4:** 281-290.

367 Brugnon F, Janny L, Communal Y, Darcha C, Szczepaniak C, Pellestor F, Vago P,
368 Pons-Rejraji H, Artonne C, Grizard G. Apoptosis and meiotic segregation in ejaculated
369 sperm from Robertsonian translocation carrier patients. *Hum.Reprod.* 2010; **7:** 1631-
370 1642.

371 Burrello N, Arcidiacono G, Vicari E, Asero P, Di Benedetto D, De Palma A, Romeo R,
372 D'Agata R, Calogero AE. Morphologically normal spermatozoa of patients with

373 secretory oligo-astheno-teratozoospermia have an increased aneuploidy rate.
374 *Hum.Reprod.* 2004; **10:** 2298-2302.

375 Carrell DT. The clinical implementation of sperm chromosome aneuploidy testing:
376 pitfalls and promises. *J.Androl.* 2008; **2:** 124-133.

377 Carrell DT, Emery BR, Hammoud S. Altered protamine expression and diminished
378 spermatogenesis: what is the link? *Hum.Reprod.Update* 2007; **3:** 313-327.

379 Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, Campbell B.
380 Sperm DNA fragmentation is increased in couples with unexplained recurrent
381 pregnancy loss. *Arch.Androl.* 2003; **1:** 49-55.

382 Collins AR, Oscoz AA, Brunborg G, Gaivao I, Giovannelli L, Kruszewski M, Smith
383 CC, Stetina R. The comet assay: topical issues. *Mutagenesis* 2008; **3:** 143-151.

384 Collins JA, Barnhart KT, Schlegel PN. Do sperm DNA integrity tests predict pregnancy
385 with in vitro fertilization? *Fertil.Steril.* 2008; **4:** 823-831.

386 De Braekeleer M, Dao TN. Cytogenetic studies in male infertility: a review.
387 *Hum.Reprod.* 1991; **2:** 245-250.

388 de Kretser DM. Male infertility. *Lancet* 1997; **9054:** 787-790.

389 Egozcue J, Blanco J, Vidal F. Chromosome studies in human sperm nuclei using
390 fluorescence in-situ hybridization (FISH). *Hum.Reprod.Update* 1997; **5:** 441-452.

391 Enciso M, Sarasa J, Agarwal A, Fernandez JL, Gosalvez J. A two-tailed Comet assay
392 for assessing DNA damage in spermatozoa. *Reprod.Biomed.Online* 2009; **5:** 609-616.

393 Evenson D, Jost L. Sperm chromatin structure assay is useful for fertility assessment.
394 *Methods Cell Sci.* 2000; **2-3:** 169-189.

395 Evenson DP, Darzynkiewicz Z, Melamed MR. Comparison of human and mouse sperm
396 chromatin structure by flow cytometry. *Chromosoma* 1980; **2:** 225-238.

397 Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P,
398 Claussen OP. Utility of the sperm chromatin structure assay as a diagnostic and
399 prognostic tool in the human fertility clinic. *Hum.Reprod.* 1999; **4:** 1039-1049.

400 Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for
401 detecting sperm DNA fragmentation in male infertility and comparisons with other
402 techniques. *J.Androl.* 2002; **1:** 25-43.

403 Evenson DP, Wixon R. Data analysis of two in vivo fertility studies using Sperm
404 Chromatin Structure Assay-derived DNA fragmentation index vs. pregnancy outcome.
405 *Fertil.Steril.* 2008; **4:** 1229-1231.

406 Fernandez JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm
407 chromatin dispersion test: a simple method for the determination of sperm DNA
408 fragmentation. *J.Androl.* 2003; **1:** 59-66.

409 Garcia-Peiro A, Oliver-Bonet M, Navarro J, Abad C, Amengual MJ, Lopez-Fernandez
410 C, Gosalvez J, Benet J. Differential Clustering of Sperm Subpopulations in Infertile
411 Males with Clinical Varicocele and Carriers of Rearranged Genomes. *J.Androl.* 2011;

412 Garcia-Peiro A, Oliver-Bonet M, Navarro J, Abad C, Guitart M, Amengual MJ,
413 Gosalvez J, Benet J. Dynamics of sperm DNA fragmentation in patients carrying
414 structurally rearranged chromosomes. *Int.J.Androl.* 2011;

415 Gharagozloo P, Aitken RJ. The role of sperm oxidative stress in male infertility and the
416 significance of oral antioxidant therapy. *Hum.Reprod.* 2011; **7:** 1628-1640.

417 Gorczyca W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual
418 apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation
419 assays. *Cancer Res.* 1993; **8:** 1945-1951.

420 Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J. Reduction of the
421 incidence of sperm DNA fragmentation by oral antioxidant treatment. *J.Androl.* 2005;
422 **3:** 349-353.

423 Hauser R, Paz G, Botchan A, Yogev L, Yavetz H. Varicocele: effect on sperm
424 functions. *Hum.Reprod.Update* 2001; **5:** 482-485.

425 Hendin BN, Kolettis PN, Sharma RK, Thomas AJ,Jr, Agarwal A. Varicocele is
426 associated with elevated spermatozoal reactive oxygen species production and
427 diminished seminal plasma antioxidant capacity. *J.Urol.* 1999; **6:** 1831-1834.

428 Lewis SE. Is sperm evaluation useful in predicting human fertility? *Reproduction* 2007;
429 **1:** 31-40.

430 Lewis SE, Simon L. Clinical implications of sperm DNA damage. *Hum.Fertil.(Camb)*
431 2010; **4:** 201-207.

432 Maione B, Pittoggi C, Achene L, Lorenzini R, Spadafora C. Activation of endogenous
433 nucleases in mature sperm cells upon interaction with exogenous DNA. *DNA Cell Biol.*
434 1997; **9:** 1087-1097.

435 Makker K, Agarwal A, Sharma R. Oxidative stress & male infertility. *Indian J.Med.Res.*
436 2009; **4:** 357-367.

437 Marcon L, Boissonneault G. Transient DNA strand breaks during mouse and human
438 spermiogenesis new insights in stage specificity and link to chromatin remodeling.
439 *Biol.Reprod.* 2004; **4:** 910-918.

440 Martin RH. Meiotic chromosome abnormalities in human spermatogenesis.
441 *Reprod.Toxicol.* 2006; **2:** 142-147.

442 Mitchell LA, De Iuliis GN, Aitken RJ. The TUNEL assay consistently underestimates
443 DNA damage in human spermatozoa and is influenced by DNA compaction and cell
444 vitality: development of an improved methodology. *Int.J.Androl.* 2011; **1:** 2-13.

445 Morris ID. Sperm DNA damage and cancer treatment. *Int.J.Androl.* 2002; **5:** 255-261.

446 Morris ID, Ilott S, Dixon L, Brison DR. The spectrum of DNA damage in human sperm
447 assessed by single cell gel electrophoresis (Comet assay) and its relationship to
448 fertilization and embryo development. *Hum.Reprod.* 2002; **4:** 990-998.

449 Naughton CK, Nangia AK, Agarwal A. Pathophysiology of varicoceles in male
450 infertility. *Hum.Reprod.Update* 2001; **5:** 473-481.

451 O'Flaherty C, Vaisheva F, Hales BF, Chan P, Robaire B. Characterization of sperm
452 chromatin quality in testicular cancer and Hodgkin's lymphoma patients prior to
453 chemotherapy. *Hum.Reprod.* 2008; **5:** 1044-1052.

454 Pentikainen V, Erkkila K, Dunkel L. Fas regulates germ cell apoptosis in the human
455 testis in vitro. *Am.J.Physiol.* 1999; **2 Pt 1:** E310-6.

456 Perrin A, Basinko A, Douet-Guilbert N, Gueganic N, Le Bris MJ, Amice V, De
457 Braekeleer M, Morel F. Aneuploidy and DNA fragmentation in sperm of carriers of a
458 constitutional chromosomal abnormality. *Cytogenet.Genome Res.* 2011; **2-4:** 100-106.

459 Perrin A, Caer E, Oliver-Bonet M, Navarro J, Benet J, Amice V, De Braekeleer M,
460 Morel F. DNA fragmentation and meiotic segregation in sperm of carriers of a
461 chromosomal structural abnormality. *Fertil.Steril.* 2009; **2:** 583-589.

462 Practice Committee of American Society for Reproductive Medicine. The clinical utility
463 of sperm DNA integrity testing. *Fertil.Steril.* 2008; **5 Suppl:** S178-80.

464 Rius M, Obradors A, Daina G, Ramos L, Pujol A, Martinez-Passarell O, Marques L,
465 Oliver-Bonet M, Benet J, Navarro J. Detection of unbalanced chromosome segregations
466 in preimplantation genetic diagnosis of translocations by short comparative genomic
467 hibridization. *Fertil.Steril.* 2011; **1:** 134-142.

468 Rubes J, Selevan SG, Sram RJ, Evenson DP, Perreault SD. GSTM1 genotype influences
469 the susceptibility of men to sperm DNA damage associated with exposure to air
470 pollution. *Mutat.Res.* 2007; **1-2:** 20-28.

471 Sailer BL, Sarkar LJ, Bjordahl JA, Jost LK, Evenson DP. Effects of heat stress on
472 mouse testicular cells and sperm chromatin structure. *J.Androl.* 1997; **3:** 294-301.

473 Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on
474 reproductive outcome, and analysis. *Fertil.Steril.* 2010; **4:** 1027-1036.

475 Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of
476 DNA damage in ejaculated human spermatozoa. *Rev.Reprod.* 1999; **1:** 31-37.

Sakkas D, Seli E, Manicardi GC, Nijs M, Ombelet W, Bizzaro D. The presence of abnormal spermatozoa in the ejaculate: did apoptosis fail? *Hum.Fertil.(Camb)* 2004; **2:** 99-103.

Sergerie M, Laforest G, Bujan L, Bissonnette F, Bleau G. Sperm DNA fragmentation: threshold value in male fertility. *Hum.Reprod.* 2005; **12:** 3446-3451.

Sharma RK, Sabanegh E, Mahfouz R, Gupta S, Thiyagarajan A, Agarwal A. TUNEL as a test for sperm DNA damage in the evaluation of male infertility. *Urology* 2010; **6:** 1380-1386.

Simon L, Lutton D, McManus J, Lewis SE. Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. *Fertil.Steril.* 2011; **2:** 652-657.

Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp.Cell Res.* 1988; **1:** 184-191.

Sotolongo B, Huang TT, Isenberger E, Ward WS. An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. *J.Androl.* 2005; **2:** 272-280.

Templado C, Vidal F, Estop A. Aneuploidy in human spermatozoa. *Cytogenet.Genome Res.* 2011; **2-4:** 91-99.

Van Kooij RJ, de Boer P, De Vreeden-Elbertse JM, Ganga NA, Singh N, Te Velde ER. The neutral comet assay detects double strand DNA damage in selected and unselected human spermatozoa of normospermic donors. *Int.J.Androl.* 2004; **3:** 140-146.

498 Velez de la Calle JF, Muller A, Walschaerts M, Clavere JL, Jimenez C, Wittemer C,
499 Thonneau P. Sperm deoxyribonucleic acid fragmentation as assessed by the sperm
500 chromatin dispersion test in assisted reproductive technology programs: results of a
501 large prospective multicenter study. *Fertil.Steril.* 2008; **5:** 1792-1799.

502 Voet T, Vanneste E, Van der Aa N, Melotte C, Jackmaert S, Vandendael T, Declercq
503 M, Debrock S, Fryns JP, Moreau Y, et al. Breakage-fusion-bridge cycles leading to inv
504 dup del occur in human cleavage stage embryos. *Hum.Mutat.* 2011; **7:** 783-793.

505 WHO. WHO laboratory manual for the examination and processing of human semen.
506 5th edition. *World Health Organization press* 2010.

507 Zini A. Are sperm chromatin and DNA defects relevant in the clinic?
508 *Syst.Biol.Reprod.Med.* 2011; **1-2:** 78-85.

509 Zini A, Al-Hathal N. Antioxidant therapy in male infertility: fact or fiction? *Asian*
510 *J.Androl.* 2011; **3:** 374-381.

511 Zini A, Sigman M. Are tests of sperm DNA damage clinically useful? Pros and cons.
512 *J.Androl.* 2009; **3:** 219-229.
513

TABLES

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

	% SDF (Sperm DNA Fragmentation)	
	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 ($p < 0.01$)

525 **FIGURE LEGENDS**

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

529

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

533

534 Figure 3. Alkaline and neutral Comet assay sperm DNA fragmentation (SDF) in fertile
535 controls and four groups of patients.

536

537 Figure 4. Alkaline and neutral Comet assay sperm DNA fragmentation (SDF) in
538 rearranged chromosome carriers, classified according to the 52% alkaline Comet
539 threshold value. Mean ± Standard deviations for the <52% alkaline Comet group were
540 39.18±12.96% for alkaline Comet and 59.03±31.28% for neutral Comet. For the >52%
541 alkaline Comet group, the values were 88.37±15.65% for alkaline Comet and
542 69.97±27.70% for neutral Comet.