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ORIGINAL ARTICLE

Characterisation of a subpopulation of sperm with massive nuclear damage, as recognised with the sperm chromatin dispersion test

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Summary

Assessment of human sperm DNA fragmentation by the sperm chromatin dispersion (SCD) test is based on the detection of haloes of spreading DNA loops after sequential DNA denaturing and protamine removal. After the SCD test, sperm without DNA fragmentation show chromatin haloes emerging from the central nuclear core, while sperm containing fragmented DNA present small or no haloes. The nuclear degraded sperm are recognised as a differentiated category within the sperm with fragmented DNA, whose cores appear irregularly and/or faintly stained. This subpopulation is more prevalent in patients with varicocele. Protein staining with 2.7-dibrom-4-hydroxy-mercury-fluorescein demonstrated that degraded sperm intensely lose nuclear core proteins after the SCD processing. Moreover, degraded sperm are 65% more faintly labelled for DNA breaks after *in situ* nick translation (ISNT) on average, due to extensive DNA loss. A two-dimensional comet assay under sequential neutral and alkaline conditions demonstrated that degraded sperm contain both massive double- and single-strand DNA breaks. The degraded sperm appear as a subpopulation with stronger nuclear damage, affecting both DNA and protein fractions, possibly due to intense intratesticular oxidative stress, what could explain its higher proportion in patients with varicocele.

Introduction

The frequency of spermatozoa containing fragmented DNA is being recognised as a new potential parameter of semen quality. In fact, the presence of fragmented DNA sperm is higher in infertile patients and in subjects with low sperm quality (Evenson *et al.*, 2002; Evenson & Wixon, 2006). Sperm DNA fragmentation (SDF) may be attributed to abnormal chromatin remodelling during spermiogenesis, and it can also be influenced by oxidative stress or apoptosis (Agarwal & Said, 2003). Increased SDF may adversely affect fertilisation rate, embryo quality, blastocyst rate, implantation rate and pregnancy outcome (Virro *et al.*, 2004; Muriel *et al.*, 2006; Collins *et al.*, 2008; Nuñez-Calonge *et al.*, 2012). Moreover, the SDF assessment may provide relevant information in most andrological pathologies, such as varicocele (Allamaneni *et al.*, 2004; Enciso *et al.*, 2006), genital infections (Gallegos *et al.*, 2008) and cancer (Meseguer *et al.*, 2008).

Several techniques have been applied to analyse SDF. The terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) and the *in situ* nick translation (ISNT) assays are based on the enzymatic incorporation of labelled nucleotides at the free 3′-hydroxyl group of DNA breaks (Gorczyca *et al.*, 1993; Agarwal & Said, 2004). In the single-cell gel electrophoresis (SCGE) or comet assay, protein-depleted nucleoids are exposed to an electric field producing comet images, whose tail lengths and/or amount of DNA are related to the DNA breakage level (Simon *et al.*, 2011). Otherwise, in the sperm chromatin structure assay (SCSA), the sperm are incubated in an acid solution that denatures the DNA when fragmented, producing single-stranded DNA. After staining with acridine orange, sperm with nonfragmented DNA

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containing native double-stranded DNA fluoresce green, whereas sperm with fragmented DNA, single-stranded, fluoresce red when excited with the appropriate light from the flow cytometer (Evenson *et al.*, 2002). These procedures cannot be performed routinely in the conventional semen analysis laboratory, because they are complex, difficult to implement, time-consuming and relatively expensive.

The sperm chromatin dispersion (SCD) test, on the other hand, is a very simple, rapid and accurate procedure to determine SDF (Fernández *et al.*, 2003, 2005). The sperm cells are embedded in an agarose microgel on a slide, incubated in an acid solution that denatures the DNA only in those spermatozoa with fragmented DNA, and then in a lysing solution that removes the protamines, so the DNA loops tightly packed in the nucleus are spread producing DNA haloes emerging from a central core. After staining, the spermatozoa without fragmented DNA show nucleoids with big haloes of spreading of DNA loops, whereas those with fragmented DNA appear with a small or no halo. This perfect correlation was established by sequential labelling of DNA breaks after the SCD test, by DNA breakage detection–fluorescence *in situ* hybridisation (DBD-FISH; Fernández *et al.*, 2003, 2005).

Interestingly, using the SCD test, a group of spermatozoa without halo, that is, with fragmented DNA, appears with an irregularly and/or faintly stained nucleus. This subpopulation of sperm with fragmented DNA is categorised as 'degraded' type (Fernández *et al.*, 2003, 2005). Their proportion within the population of sperm with fragmented DNA was found increased in samples from individuals with varicocele (Enciso *et al.*, 2006; García-Peiró *et al.*, 2012). Although they should correspond to strongly damaged sperm, this assumption has not been confirmed. The present work focuses in a deeper characterisation of the sperm with degraded nucleus, as observed with the SCD test, analysing both the nuclear DNA and protein components.

Materials and methods

Sperm samples were obtained from normozoospermic donor individuals ($n = 3$) and patients with varicocele ($n = 9$) attending an infertility clinic. Patients signed an informed consent allowing the use of the specimen, that otherwise would have been discarded, for the present research purpose.

Sperm DNA fragmentation (SDF) assessment

4 *DNA and protein staining*

SDF was assessed by the SCD test, using the Halosperm G2 kit® (Halotech DNA, SL, Madrid, Spain). The SDF-index (SDFi) was calculated as the percentage of fragmented sperm in the whole analysed sample (500 spermatozoa per analysis).

Minimal changes to the manufacturer recommended methodology were made and were mainly related to the staining procedures. Briefly, the staining steps for bright field microscopy recommended for Halosperm® were removed, and fluorescence microscopy was used. To analyse SDF and/or simultaneous stain DNA and proteins, Gel Red for DNA staining-red fluorescence (Biotium, Hayward, CA, USA) combined with 2.7-dibrom-4-hydroxy-mercury-fluorescein for protein staining-green fluorescence (Sigma-Aldrige, Barcelona, Spain) was used (Santiso *et al.*, 2007). This staining strategy can selectively stain the remaining protein core after DNA denaturation and protein depletion.

Sperm classification according to the images provided by the SCD methodology is shown in Fig. 1a. Sperm containing nonfragmented DNA were scored as the sperm population showing large- or medium-sized haloes of dispersed chromatin surrounding a compact and well-defined core. Sperm with fragmented DNA show small haloes of dispersed chromatin or no haloes, that is, leaving only the chromatin core visible. Degraded sperm, besides not having haloes, were characterised by the presence of a faint and/or nonuniformly stained chromatin core. Degraded sperm were included in the category of fragmented DNA, but the frequency of these particular sperm was also scored separately. Therefore, the SDF takes into account not only those sperm with very small or absent haloes of chromatin dispersion but also the degraded sperm present in the total number of scored spermatozoa.

For sperm visualisation, a Leica DMLA model motorised fluorescence microscope controlled with software for automatic scanning and image digitisation (Leica Microsystems, Barcelona, Spain) was used. The microscope was equipped with a Leica EL6000 metal halide fluorescence light source and Fluotar 40 × objectives for routine scanning with three independent filter blocks (DAPI-5060B; FITC-3540B and TRITC-A; Semrock, Rechestern NY, USA). A charge-coupled device (Leica DFC350 FX, Leica Microsystems) was used for image capture.

In situ nick translation (ISNT) DNA breakage labelling

In situ nick translation was performed after the SCD procedure to test for sperm DNA damage. This technique allows the incorporation of labelled nucleotides in free 3′-hydroxyl termini resulting from single- or double-strand DNA breaks. Special interest was given to the degraded sperm. The relative amount of DNA labelling was compared using semiquantitative image analysis of ISNT

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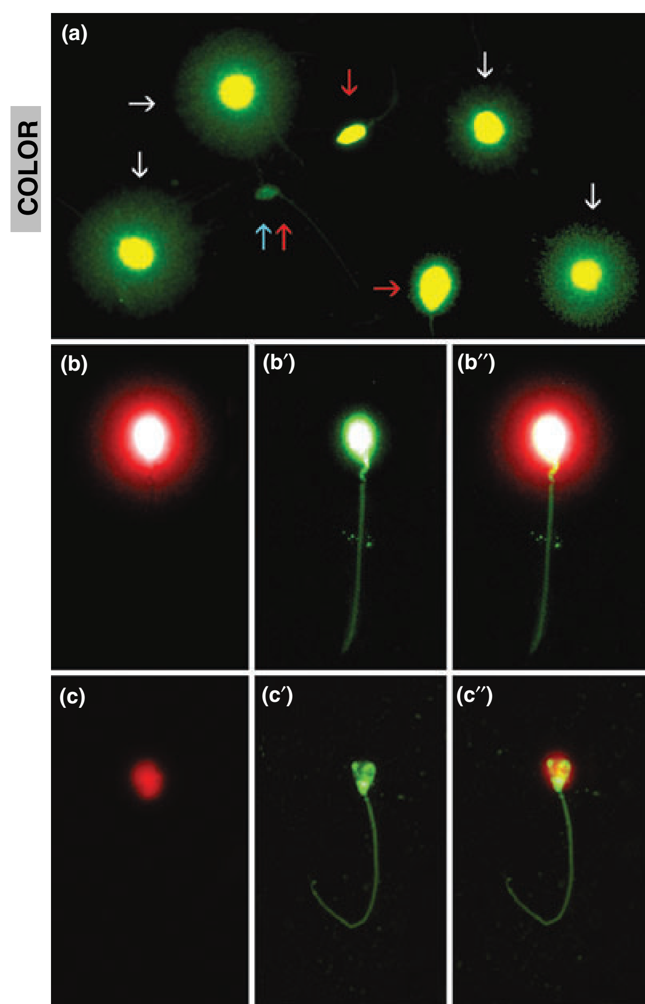

Fig. 1 Sperm DNA damage visualised after the sperm chromatin dispersion (SCD) test. (a) Simultaneous DNA staining with Gel Red (red fluorescence) and protein staining with 2.7-dibrom-4-hydroxy-mercury-fluorescein (green fluorescence). White arrowed sperm corresponds to normal sperm, red arrowed sperm corresponds to fragmented sperm, and double red-blue arrowed sperm corresponds to degraded sperm. (b-b′-b′′) normal sperm after DNA staining (b), protein staining (b′) and merging of both images (b′′). c-c′-c′′: degraded sperm after DNA staining (c), protein staining (c′) and merging of both images (c′′).

spermatozoa. DNA labelling was performed using ISNT rather than the classical terminal transferase deoxyuridine 5-triphosphate (dUTP) nick end labelling (TUNEL) assay, because the level of DNA labelling is typically higher with ISNT, and it was suspected that highly degraded spermatozoa would exhibit low amounts of DNA after DNA denaturation and protein depletion. In the case of Halosperm®-processed slides, after the lysis step, each slide was thoroughly washed in phosphate-buffered saline and incubated at room temperature 4 times in excess reaction buffer for DNA-polymerase I (10 mM Tris-HCl, 5 mM MgCl2, 7.5 mM DTT, pH 7.5) to remove any trace of the lysing solution that might cause enzyme inactivation. For the ISNT procedure, 100 ml of reaction buffer containing 25 units of *E. coli* DNA-polymerase I (Roche Diagnostics GmbH, Mannheim, Germany) and digoxigenin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) in the nucleotide mix were pipetted directly onto the slide, covered with a plastic coverslip and incubated in a humidified chamber for 5, 10, 20 and 30 min at 37 °C. After washing in Tris-Borate-EDTA buffer (pH 8), the slides were dehydrated in sequential 70–90–100% ethanol baths and air dried. The incorporated 16-dUTP was detected after incubation for 30 min with anti-digoxigenin conjugated with fluorescein isothiocyanate (Roche Diagnostics GmbH, Mannheim, Germany). The slides were analysed directly or, alternatively, counterstained with propidium iodide (2 mg ml^{-1}) in Vectashield (Vector, Burlingame, CA, USA). As a control, a designated area of the slide was incubated with the reaction buffer alone, omitting the DNA-polymerase I. The microgel between the areas with and without the polymerase was scratched off to avoid possible diffusion of the enzyme into the control area.

Two-dimensional comet assay

Sperm DNA damage, especially that affecting degraded sperm, was characterised using a two-dimensional, two-tail (2T)-comet assay, as elsewhere described (Enciso *et al.*, 2009). The comet assay was first performed under neutral conditions to assess double-strand DNA breaks (DSBs). Using the same slide, a second 90º electrophoresis was performed, in this case under alkaline conditions, to assess single-strand DNA breaks (SSBs). The comet tail in the *X*-axis corresponded with DSBs. On the *Y*-axis, the comet tail corresponding to the SSBs was clearly differentiated from the previous one.

Statistical analysis

The Kolmogorov–Smirnov test was used to check distribution normality. Variables were compared using the *U*-Mann–Whitney test. Correlation analysis was performed using a Spearman's rank test. $P = 0.05$ was considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences 17 (SPSS Inc., Chicago, IL, USA).

Results

After the SCD procedure, sperm without DNA fragmentation show haloes of chromatin emerging from the

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central nuclear core, whereas these haloes appear small or absent in those sperm containing fragmented DNA (Fig. 1). The nuclear degraded sperm is distinctively recognised because the core appears irregularly and/or faintly stained (Fig. 1a double arrow). This subpopulation is more prevalent in varicocele patients. In this case, the proportion of degraded sperm in donors was 2% (SD 3.3) over a whole SDF of 14.3% (SD 3.5), while for varicocele patients, the values obtained for degraded sperm were significantly higher 7.05% (SD 4.1: *U*-Mann–Whitney 0.00; $P \leq 0.05$) over a whole SDF of 17.8% (SD 20.1).

Evaluation of residual nuclear protein in degraded sperm

Simultaneous staining of SCD processed sperm with Gel Red and 2.7-dibrom-4-hydroxy-mercury-fluorescein revealed a residual protein fluorescence in the core of sperm without fragmented DNA (Fig. 1b′), while the halo fluoresces in red (Fig. 1b). Comparing these sperm with those with fragmented DNA but not degraded, a decrease in the halo size was observed, but the core is maintained or slightly diminished (Fig. 1a compare white and red arrowed sperm). However, protein removal was much stronger in the core of the degraded sperm, remaining 5–31% of the fluorescence intensity from the sperm without DNA fragmentation (*U*-Mann–Whitney; $P < 0.001$; compare Fig. 1b′,c′). The DNA observed in these degraded sperm is residual (Fig. 1c).

Characterisation of the DNA damage in degraded sperm using (ISNT)

In situ nick translation was used to characterise sperm DNA damage after the SCD technique in a varicocele sample, and special attention was given to the morphology of the degraded sperm. Sperm haloes of chromatin, characteristic of those sperm without fragmented DNA, appear unlabelled by ISNT as expected (white arrow in Fig. 2a). The sperm heads without halo, that is, standard fragmented DNA according with the SCD test, are strongly labelled by ISNT (red arrows in Fig. 2a,b). On the other hand, degraded sperm showed lower DNA breakage labelling when compared with the standard fragmented sperm, as well as an irregular morphology and distribution of fluorescence (double red-blue arrows in Fig. 2a,c). Interestingly, after using an intensive ISNT (more than 30 min of polymerase extension), those spermatozoa representing the degraded class exhibited a visible halo of small-extended DNA fragments displaying a huge halo of stellar appearance (Fig. 2a, double red-blue arrow). This stellar halo, formed from *in situ* nick translated small DNA fragments, could not be visualised on equivalent spermatozoa after using a direct fluorochrome for DNA staining (Fig. 1a). This characteristic appearance of degraded spermatozoa on ISNT was not observed in the spermatozoa identified as 'standard fragmented' and probably represented tiny fragments of DNA detached from a remnant core. A semiquantitative analysis of the fluorescence intensity is showed in Fig. 2d. Statistical

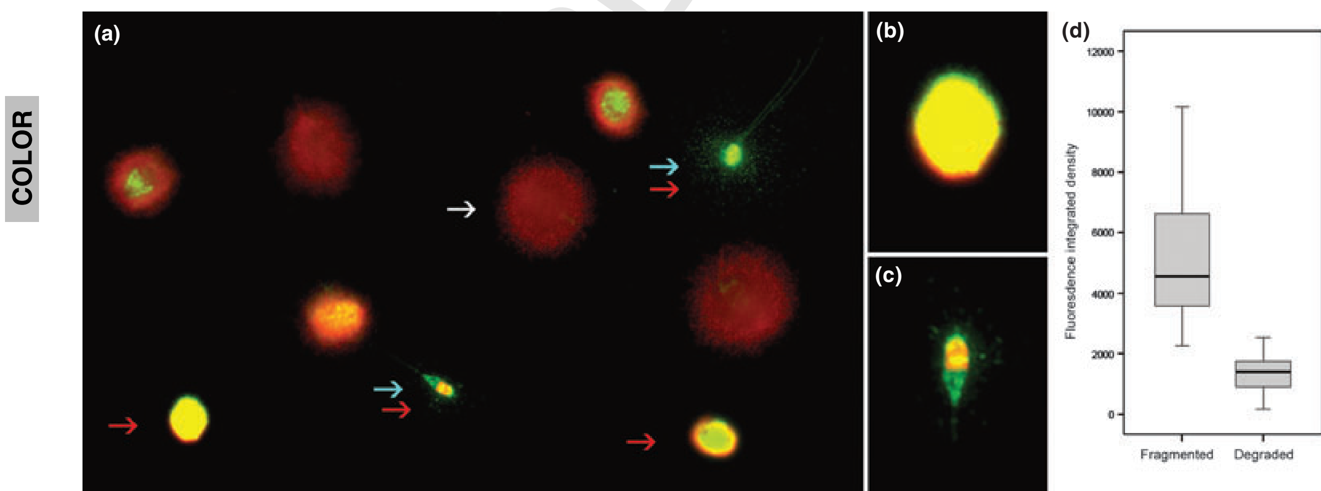

Fig. 2 *In situ* nick translation (ISNT) of a varicocele sperm sample after the sperm chromatin dispersion (SCD) test protocol. (a) Red arrows identify standard fragmented spermatozoa, while double red-blue arrows identify degraded spermatozoa, both with labelled DNA breaks (green fluorescence). An example of unlabelled sperm propidium iodide counterstained (red fluorescence) is indicated by white arrow. Enlarged selected standard fragmented spermatozoon (b) and degraded spermatozoon (c) after ISNT, for comparison. d: Box and whisker plots to illustrate the distribution of the mean fluorescence intensity after ISNT registered in standard fragmented (left) and degraded (right) spermatozoa.

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differences existed in the amount of the integrated fluorescence intensity observed when standard fragmented and degraded subpopulations were compared (Fig. 2d *U*-Mann–Whitney $P < 0.001$). On average, the degraded sperm exhibited 65% less *in situ* nick translatable DNA than the standard fragmented DNA sperm.

Characterisation of the DNA damage in degraded sperm using 2T-comet

Samples were obtained from normozoospermic donor individuals ($n = 3$) and patients with varicocele ($n = 9$). Each sample was independently processed for SCD and a 2T-comet assay to assess and compare the presence of DNA damage considering the degraded sperm class and the presence of single-strand DNA breaks (SSBs) or double-strand DNA breaks (DSBs). This makes approximately a characterisation of about 1.200 comets and a higher number of SCD spermatozoa, close to 3.000. The frequency of whole SDF and the frequency of degraded sperm observed using both SCD test and 2T-comet were compared, and a correlation analysis was performed.

Using the 2T-comet assay, a continuous series of sperm DNA damage was observed with variations among the contribution of DNA to the comet mass. As a representation of this variable DNA damage, a series of different comets is shown in Fig. 3. Basically, sperm without DNA damage had a compact chromatin displacement in the sense of the alkaline migration (*Y*-axis in Fig. 3; see yellow square comet in Fig. 3b). This was due to the presence of alkali-labile sites in all spermatozoa (Singh *et al.*, 1989), and this represented the comet image showing an absence of detectable DNA damage under our experimental conditions. From this general figure, a series of different amounts of DNA damage could be observed in different spermatozoa. SSBs were represented as extra displacement in the *Y*-axis (see green square comet in Fig. 3b, while the *X*-migration corresponded to DSBs (see red square in Fig. 3b). For the purpose of the present study, we paid special attention to those sperm showed in the red panel in Fig. 3b (red square) and to those highlighted in a blue square also in Fig. 3b. Both spermatozoa corresponded to the type we identified as degraded sperm visualised with the 2T-comet assay. They were characterised by the presence of high masses of DNA fragments produced from both SSBs (green-coloured comet in Fig. 3a′) and DSBs (yellow-coloured comet in Fig. 3a′). The chromatin core of the nucleoid (pink colour in Fig. 3a′) was reduced to a minimal expression. When the sperm DNA damage was extremely intense, comets were not visualised or were displaced far from the nucleoid, and only ghost-like remnants were observed (blue square in Fig. 3b).

An initial analysis was performed to test for the differences between the whole level of SDF as assessed using the SCD test and a 2T-comet. The mean values for SDF obtained using the SCD test were 17.8 (SD 20.1; $n = 9$). The results obtained with the 2T-comet assay were 24.6

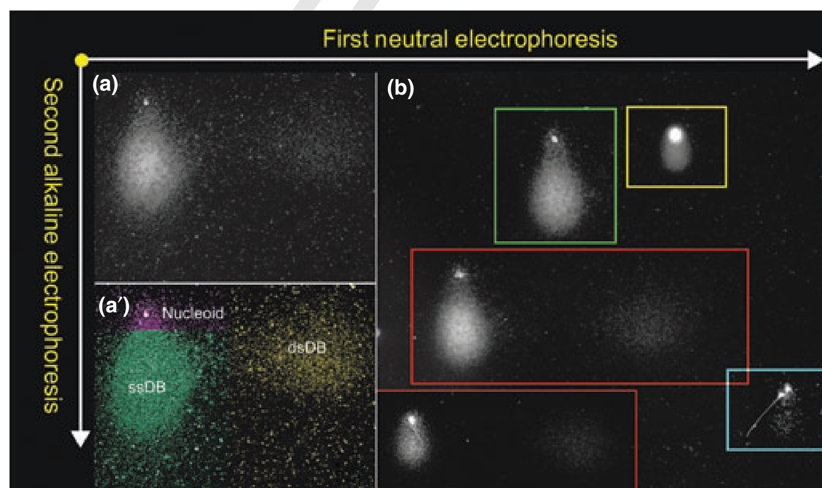

Fig. 3 Two-tail comet assay (2T-comet) panel showing different sperm morphologies and classification criteria. (a-a′) Characteristic 2T-comet emerging from a degraded spermatozoon. Two white arrows show the bidirectional electrophoresis used to identify single-strand DNA breaks (ssDB; vertical DNA displacement and green pseudo-colour (a′) and double-strand DNA breaks (dsDB; horizontal DNA displacement and yellow pseudo-colour (a′); the remnant of the chromatin core is highlighted in pink (a′). (b) Sperm 2T comets showing different levels of DNA damage based on the single- or double-strand nature of the DNA breaks. Normal sperm (yellow square), sperm with single-strand DNA damage (green square), sperm with both single- and double-strand DNA damage (red square) and highly degraded sperm where only remnant of the sperm core is visualised (blue square).

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(SD 20.1; $n = 9$). The *U*-Mann–Whitney test did not show significant differences ($P < 0.05$) although a tendency to score higher levels of DNA damage was observed in the 2T-comet assay. When a correlation analysis using both measures was performed, a good correlation coefficient was obtained ($r = 0.73$; $P < 0.05$).

Additionally, the frequency of degraded sperm, as observed after the SCD test, was compared with the frequency of highly degraded comets using all varicocele patients. The mean values for degraded sperm visualised after the SCD test and 2T-comet assay were 7.2% (SD 7.3; $n = 9$) and 9.3% (SD 9.3; $n = 9$), respectively. Significant differences between both scoring methods were not observed (*U*-Mann–Whitney 0.47; $P < 0.05$). A high correlation coefficient ($r = 0.98$; $P < 0.01$) was obtained between the frequency of degraded sperm identified with the SCD test and with the 2T-comet assay.

Discussion

Human ejaculate is composed of different sperm subpopulations that may be identified by their kinetic, membrane, nuclei or physiological characteristics (Thurston *et al.*, 2001; Buffone *et al.*, 2004). Patients with varicocele have a higher proportion of spermatozoa with abnormal DNA and immature chromatin when compared with fertile men (Talebi *et al.*, 2008). The results presented in this investigation corroborate such evidence but additionally clearly identify two different levels of sperm DNA damage affecting these types of sperm. In the particular scenario of sperm nuclei identification, the degraded subpopulation observed after the SCD test is different from the high DNA stainability sperm subpopulation (HDS) recognised after SCSA due to DNA fragmentation characteristics (García-Peiró *et al.*, 2012). In previous studies, it has been suggested that the HDS sperm seemed to correspond to the amount of immature spermatozoa, which failed to undergo complete histone/protamine replacement or protein assembly during spermiogenesis (Evenson & Wixon, 2006). The results presented in this investigation indicate that within the sperm class that was considered to have fragmented DNA, a particular subclass of sperm (called degraded sperm) can be identified after massive protein and DNA depletion produced after applying the SCD test and the 2T-comet. The strong correlation between the frequencies of degraded sperm identified with the SCD test and with the 2T-comet assay suggested that they were the product of massive SSBs and DSBs and also the protein component resulted affected. Sperm nuclei with heavily damaged DNA and proteins should be especially susceptible to the lysing conditions of the SCD procedure. The low level of staining achieved on these nuclei after SCD treatment is because most of the chromatin was selectively and differentially removed after incubation in the lysing solution of the SCD test. In fact, spermatozoa exhibiting standard DNA fragmentation are not so massively affected by the SCD or the 2T-comet treatments. The neat result is that, on average, degraded sperm exhibited 65% less *in situ* nick translatable DNA, and only retain 5–31% of the integrated fluorescence for remnant proteins when compared with sperm with standard fragmented DNA. This massive DNA chromatin alteration may even lead to ghost-like spermatozoa as demonstrated with the 2T comet assay and is similarly observed after the SCD test.

The degraded class was not exclusive of varicocele patients but was highly present in this group of patients (Enciso *et al.*, 2006; García-Peiró *et al.*, 2012). The dilatation of internal veins, which characterise varicocele, suggests that most of the sperm damage produced by this pathology is produced at the testicular level. The damage that leads to degraded sperm would be produced intratesticularly but not when sperm is circulating within the genital duct system. According to other studies, this type of spermatozoa easily identified after SCD or 2T-comet assay, would emerge as a consequence of the intensive oxidative stress environment, which is produced in the testicle of varicocele patients (Türkyilmaz *et al.*, 2004; Agarwal *et al.*, 2006, 2009, 2012). Such intense oxidative stress in varicocele would be the result of an increase in reactive oxygen species (ROS) production and a decrease in the antioxidant capacity (Barbieri *et al.*, 1999; Hendin *et al.*, 1999). Moreover, the increase in seminal ROS level seems correlated with varicocele grade (Allamaneni *et al.*, 2004). High amounts of nitric oxide (NO) are released by the dilated spermatic veins, which may react with superoxide producing the strong oxidant peroxynitrite, thus contributing to oxidative stress in testis with varicocele (Mitropoulos *et al.*, 1996; Romeo *et al.*, 2001; Türkyilmaz *et al.*, 2004). ROS may also be released by the cytoplasmic droplets from immature spermatozoa (Ollero *et al.*, 2001), which seem to be frequent in the varicocele samples (Zini *et al.*, 2000). ROS attack macromolecules in an unspecific way. Damage to sperm DNA may result in the massive SSBs and DSBs breaks present in the degraded sperm (Sonntag, 1987; Cooke *et al.*, 2003). Additionally, the proteins are also very susceptible to the impact of the oxidative stress, as observed in the degraded subpopulation (Zwart *et al.*, 1999). Finally, the influence of oxidative stress in the generation of the degraded sperm subpopulation is also inferred from their significant decrease after oral antioxidant therapy (Abad *et al.*, 2013).

Overall, degraded sperm, as detected with the SCD test, represent a singular class of sperm characterised by a massive nuclear single- and double-strand DNA damage

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as well as strong nuclear protein alteration. The intensity of the sperm DNA damageseems stronger than that observed in other affected spermatozoa. The singularity of this subpopulation is going to be evaluated as a potential biomarker to characterise or identify patients with varicocele after a simple SDF test.

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