Available online at www.sciencedirect.com
SCIENCE DIRECT
Theriogenology
ELSEVIER
Theriogenology 65 (2006) 308–316
www.journals.elsevierhealth.com/periodicals/the

A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy

María Enciso [a], Carmen López-Fernández [a], José Luis Fernández [b], Pedro García [c], Altea Gosálbez [a], Jaime Gosálvez [a,*]

[a] *Unidad de Genética, Edificio de Biología, Universidad Autónoma de Madrid, C/Darwin n° 2, 28049 Madrid, Spain*
[b] *Sección de Genética y Unidad de Investigación, Complejo Hospitalario Universitario Juan Canalejo, As Xubias, 84, 15006 A Coruña, Spain*
[c] *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Crta. de la Coruña, km. 7,5, 28040 Madrid, Spain*

Received 12 April 2005; received in revised form 10 May 2005; accepted 12 May 2005

Abstract

We present a new, rapid and simple method to study DNA Fragmentation Index (DFI) in sperm samples from boar under bright-field and fluorescence microscopy. Discrimination of sperm cells containing fragmented DNA relies on the extreme peripheral diffusion of their chromatin fragments, whereas those sperm nuclei without DNA fragmentation do not disperse or show very restricted spreading of DNA loops close to the flagellum. The basic methodology provided in the commercial kit Sperm-*Sus*-Halomax® allows, in addition to a direct estimation of DFI in a sperm sample under bright field microscopy, a direct visualization of DNA breaks by incorporation of labelled nucleotides using the DNA polymerase I following the in situ nick translation assay (ISNT methodology not provided in the kit). An external control using DBD-FISH (DNA breakage detection-fluorescence in situ hybridization) on human and boar sperm samples was used in this experiment. The results obtained show (i) low levels of background DNA fragmentation (from 0.7 to 10%), (ii) no significant differences for DFI after the application of Sperm-*Sus*-Halomax and ISNT, with a tendency to be underestimated after using DBD-FISH and (iii) a characteristic chromatin organization in boar sperm

* Corresponding author. Tel.: +34 914978210; fax: +34 914978344.
E-mail address: jaime.gosalvez@uam.es (J. Gosálvez).

0093-691X/$ – see front matter
doi:10.1016/j.theriogenology.2005.05.044

nucleus, with a particular response to chromatin loop relaxation and preferential DNA labelling by ISNT at the proximal nuclear area, close to the flagellum. This methodology allows the routine assessment of boar sperm samples for DFI, as well as basic and clinical research on this relevant topic in any laboratory of semen analysis.

Keywords: Sperm DNA fragmentation; DNA damage; Sperm chromatin; Sperm chromatin dispersion test; Sperm chromatin structure assay

1. Introduction

Studies in humans indicate that the frequency of sperm cells containing fragmented DNA may be a new independent parameter of semen quality and a potential fertility predictor [1,2]. Several techniques exist to analyse sperm DNA fragmentation. Some current methods are as follows: in situ nick translation (ISNT), Terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL), comet assay, and mainly the Sperm Chromatin Structure Assay (SCSA) [1,2]. Nevertheless these techniques are either labour intensive, require the use of enzymes whose activity and accessibility to DNA breaks may be irregular, especially when used on fixed chromatin or as is the case of the SCSA, require expensive instrumentation for optimal and unbiased analysis. As a consequence, some of these procedures are more feasible intended for research purposes, but not for clinical andrology laboratories. So the study of the DNA fragmentation continues but without being incorporated in the majority of clinical semen studies and publications. Recently, a new procedure for the determination of DNA fragmentation, has been developed for human sperm cells [3,4]. Intact spermatozoa are immersed in an agarose matrix on a slide and treated with an acid solution to denature DNA breaks. Membranes and proteins were removed using a lysing solution. This methodology gives rise to nucleoids with a central core and a peripheral halo of dispersed DNA loops. In human spermatozoa, sperm nuclei containing DNA fragmentation produce very small or an absence of halos of DNA dispersion, whereas those sperm without undamaged DNA release their DNA loops forming large halos. These results were confirmed by sequential DNA breakage detection-fluorescence in situ hybridization (DBD-FISH), a procedure in which the restricted single-stranded DNA motifs generated by the acid treatment from DNA breaks can be detected and quantified using in situ DNA hybridization [3–5]. Thus, the presence or absence of DNA fragmentation can be accurately determined merely by examining the halo size; therefore, the SCD becomes a simple, highly reproducible and inexpensive technique with results highly correlated with those obtained with other procedures like the SCSA [4].

The aim of the present investigation was to compare the efficiency of the SCD test when used in boar sperm samples, so DFI could be confidently performed in every basic laboratory of semen analysis using conventional bright-field microscopy. Additionally, another endeavour of the present investigation was to adapt the basic methodology, provided in a commercial kit, for fluorescence microscopy applications, in order to attain a simultaneous and direct visualization of DNA fragmentation in sperm nuclei of boar.

2. Materials and methods

Sperm samples from 10 adult boars aged 18–24 months were used in this study. Human sperm samples were processed as a control for DNA fragmentation using the Halosperm® kit (Indas Biotech, Madrid, Spain) [4]. This methodology was also used on boar sperm samples using the conditions recommended for humans. Both samples were processed with DBD-FISH using correspondent whole genomic DNA probes in accord with the methodology elsewhere described [5]. All sperm samples were studied the same day of sperm collection. The DBD-FISH procedure was performed as follows. Boar sperm nuclei were diluted and stored at 15 °C with Acromax (GVP, Madrid, Spain). Working samples were adjusted to 5–10 million spermatozoa per milliliter using Acromax as extender. Twenty-five microliters of diluted sperm were added to a vial with low melting agarose and mixed at 37 °C. Then, 50 ml of the mixture was pipetted onto a precoated slide provided in the kit. Coverslips were gently removed and the slides immersed in an acid unwinding solution (0.08N HCl) for 7 min, at room temperature to generate the restricted single stranded DNA motifs starting from the ends of the DNA breaks. A lysing solution containing 0.4 M Tris–HCl, 2 M NaCl, 1% SDS, 0.05 M ethylene diamine tetraacetate (EDTA), pH 7.5, for 25 min at 22 °C was used to produce partial protein depletion. These nuclei were sequentially washed, for 5 min each, in distilled water and finally, dehydrated by sequential 70, 90, and 100% ethanol baths, 2 min each, and air dried. A whole DNA genomic probe from boar was used for FISH. Only those sperm nuclei containing fragmented DNA were positively labelled. Compare Fig. 1a with 1b for boars and Fig. 1c for humans.

The technical variant of the SCD test to determine DFI in boar sperm cells was performed using Sperm-*Sus*-Halomax® kit (ChromaCell SL, Madrid, Spain). Working samples were used as above described for DBD-FISH. Twenty-five microliters of diluted sperm were added to a vial with low melting agarose and mixed. Provided pre-treated slides were placed onto a metallic plate which has been previously cooled at 4 °C. A drop of the cell suspension was spread onto the treated face of the cooled slide and covered with a glass coverslip for 5 min. The coverslip was smoothly removed, and the slide was horizontally placed in 10 ml of the lysing solution provided in the kit. Finally, slides were washed for 5 min, dehydrated in sequential 70, 90 and 100% ethanol baths and stained in Wright solution: phosphate buffer (1:1).

For visualization of DNA damage under fluorescence microscopy, ISNT of DNA breaks was performed in sperm samples treated with the lysing agent provided in the kit. It is important to note that after lysis of microgel-embedded cells, the slides were thoroughly washed four times in PBS for 5 min and incubated four times for 5 min in excess reaction buffer for DNA-polymerase I (10 mM Tris–HCl, 5 mM $MgCl_2$, 7.5 mM DTT, pH 7.5). Then, 100 μl of reaction buffer containing 25 units of DNA-polymerase I (New England BioLabs, Beverly, USA) and biotin-16-dUTP in the nucleotide mix, were pipetted onto the slide, covered with a plastic coverslip and incubated in a moist chamber for 5, 10, 20 and 30 min at 37 °C. After washing in TBE buffer, the slides were dehydrated in sequential 70–90–100% ethanol baths, and air-dried. The incorporated biotin-16-dUTP was detected by incubation with the appropriate antibody conjugated with FITC for 30 min. The slides could be directly analyzed or alternatively counterstained with propidium iodide (2 μg/ml)

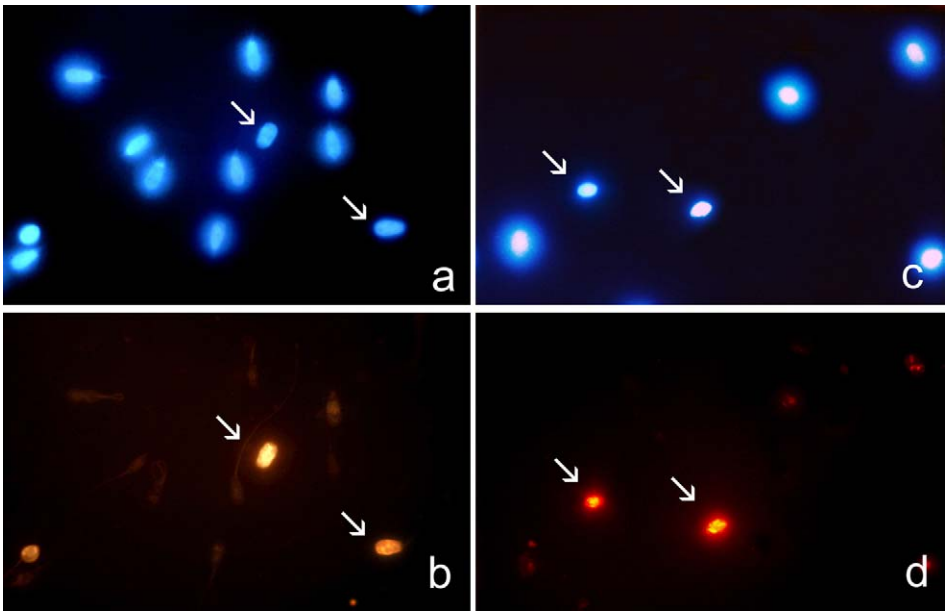

Fig. 1. Boar (left: a, b) and human (right: c, d) sperm cells processed with the Halosperm® kit, treated with an acid solution and then lysed and stained with DAPI (blue: a, c). Sequential DBD-FISH labelling of DNA breaks with the respective whole genome probe Cy3 labelled (red: b, d), demonstrates that those sperm cells without halo or with very small halo (above) contain fragmented DNA (below) in both species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in Vectashield (Vector, Burlingame, CA). As a control, an 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 to avoid possible diffusion of the enzyme into the control area. Using this methodology, and as difference with DBD-FISH, sperm nuclei which showed large halos of chromatin dispersion were those positive labelled. Fluorescence images were captured using a Leica DMRB microscope coupled with a cooled Photometrics CCD camera. Bright field images were acquired using a Motic BA300 microscope equipped with a Motic 2000 CCD camera. Image analysis was performed using Motic Images Advanced 3.2 Software. A minimum of 500 spermatozoa per experimental point were scored under 40× objective. For routine analysis, 20× objectives could be used.

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS v.11). Two-way ANOVA was performed by using DFI as dependent variable and individual and protocol as grouping factors.

3. Results and discussion

Boar sperm cells processed with the SCD protocol developed for humans (Halosperm®) (this method includes an acid treatment prior to lysis), give rise to images of similar

characteristics to those obtained in humans (Fig. 1a and c). Sperm cells with fragmented DNA do not show or show very small halos of dispersion of DNA loops, whereas those without DNA fragmentation release peripheral halos from the central core (boar sperm: Fig. 1a; human sperm: Fig. 1c). DNA fragmentation was confirmed by sequential DBD-FISH labelling of the single-stranded DNA generated by the acid treatment from the end of the DNA breaks, using a boar whole DNA genome probe (Fig. 1b), or with a human whole DNA genome probe (Fig. 1d). Only sperm nuclei containing damaged DNA were labelled, and they correlate with an absence of dispersion halos (Fig. 1). However, discrimination attending the size of the halos is not easy to establish in boar sperm samples because the size of the halos is not as large as those obtained in humans (compare Fig. 1a with 1c). Certain variability in the intensity of hybridization was also observed among different labelled sperm nuclei (compare different labelled nuclei in Fig. 1b).

The technical variant included in Sperm-*Sus*-Halomax only includes a brief incubation in lysing solution. The use of this method in humans gives rise to halos in fragmented DNA sperm nuclei, but they usually are not easily discriminated from those with unfragmented DNA. So the acid-lysis SCD protocol is the most discriminative method for humans, but is seems unsuccessful to estimate DNA fragmentation in boar samples. Boar sperm treated with Sperm-*Sus*-Halomax, gave rise to nucleoids with remarkably large halos of diffusion of chromatin spots in those sperm nuclei containing fragmented DNA. Sperm nuclei without DNA fragmentation did not show halos or showed a minimal relaxation of DNA loops in the proximal area, close to the tail (Fig. 2). According to the morphology of the small halo of dispersion for undamaged DNA nuclei, sperm heads can be clustered in three

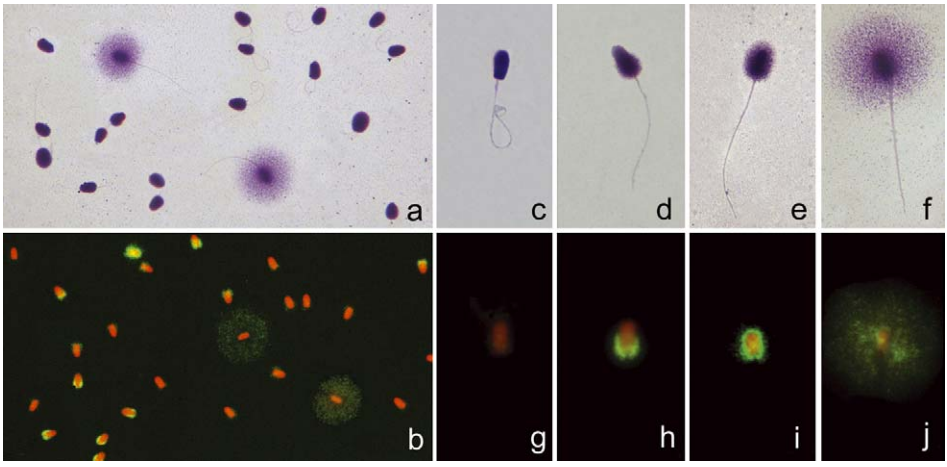

Fig. 2. Nucleoids from boar sperm cells obtained with the adapted SCD procedure developed in the Sperm-*Sus*-Halomax kit. (a) Bright-field microscopy after Wright staining. (b) In situ nick translation using the DNA polymerase I to label DNA breaks (green), and propidium iodide counterstaining (red). Nucleoids with large halo of diffusion of chromatin spots correspond to those containing fragmented DNA. (c)–(f) Different morphology of sperm heads under bright field microscopy. (g)–(j) Correspondent images after INTS under fluorescence microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

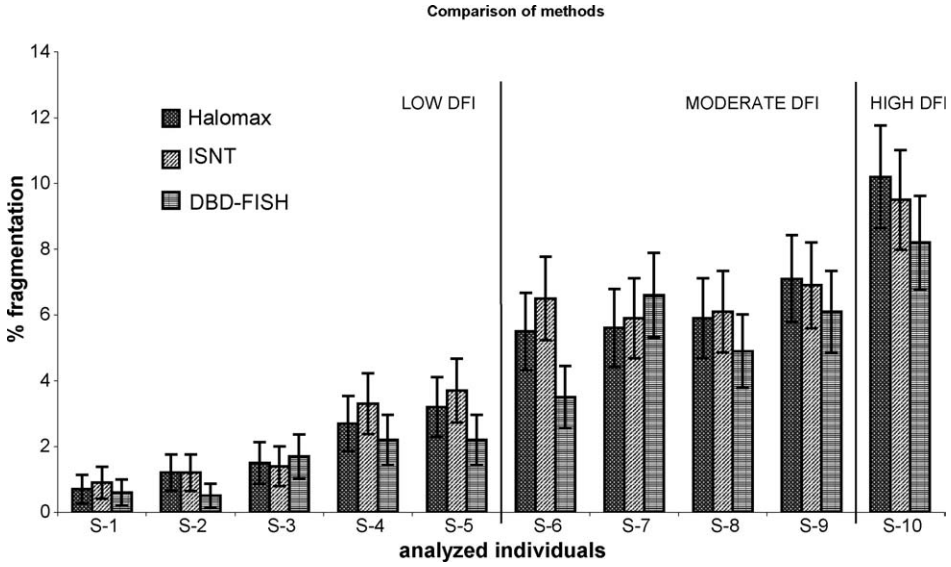

Fig. 3. DFI in 10 individuals (S-1 to S-10) using three different methodologies (Sperm-*Sus*-Halomax, right; ISNT, center; DBD-FISH, right). Each class corresponds to 1500 scores made by three different observers (500 each) where no significant differences were obtained.

types (Fig. 2c–e). Only one type was established for those sperm heads with fragmented DNA and they display a large halo of dispersion (Fig. 2f). According to this criterion, DFI ranged from 0.7 to 10% (Fig. 3). The intraindividual (three different scorings by the same observer) and the interindividual (three different observers) variability showed no significant differences.

Fluorescence microscopy was used in these experiments for two main reasons: (i) as a control technique to detect DNA breakage, and (ii) to compare data from bright-field microscopy (inferred DNA breakage) and fluorescence microscopy (direct visualization of DNA breakage). The ISNT performed on slides processed with Sperm-*Sus*-Halomax revealed that only those sperm nuclei with large halos resulted in highly labelled diffused chromatin spots (Fig. 2b); that being a direct in situ confirmation of the presence of DNA breaks in expanded halos. Interestingly, and according to the level of nucleotide incorporation, three additional and different classes of sperm nuclei, similar to the results described above using bright field microscopy, were observed. Among all the individuals studied we found that between 5 and 10% of the nuclei showed complete absence of DNA labelling (Fig. 2g), 75–90% revealed labelling at the proximal end of the head of the sperm, close to the flagellum insertion (Fig. 2h), 3–8% showed DNA labelling all around the sperm head (Fig. 2i), and finally 0.7–10% showed large halos and intense nucleotide incorporation, thus, corresponding to those containing DNA fragmentation (Fig. 2j).

Two-way ANOVA showed significant differences both among individuals and protocols, though the amount of variance explained by differences among individuals is greater than that explained by differences among protocols. Bonferroni test showed significant differences between DBD-FISH and the other two protocols (DBD-FISH versus

Sperm-*Sus*-Halomax: $p = 0.048$; DBD-FISH versus ISNT: $p = 0{,}011$), but no significant differences were found between Sperm-*Sus*-Halomax and ISNT ($p = 1$). Most differences between protocols seem to be associated to individuals S-5 and S-6 (see Fig. 3). In fact, when these individuals are excluded from the analysis, no differences were shown between protocols ($p = 0.081$). DBD-FISH technique, adapted to boar, shows a tendency to underestimate the ratio of sperm DNA fragmentation. This effect was not detected in humans. The tendency to underestimate DNA fragmentation using the DBD-FISH boar adapted protocol seems to be related to a loss of efficiency for labelling single stranded DNA motifs and sperm nuclei with a reduced DNA labelling were observed. Tukey analysis of the differences between individuals allows for the establishment of three different groups with respect to DFI: low, moderate and high DFI (Fig. 3). Though, as we showed above, differences exist among protocols, the classification of individuals with respect to their DFI is congruent irrespectively of the protocol used for DFI evaluation, i.e. those boars with low DFI after Sperm-*Sus*-Halomax/ISNT also showed low DFI after DBD-FISH, while those boars with high DFI after Sperm-*Sus*-Halomax/ISNT also showed high DFI when DBD-FISH was used for DNA fragmentation evaluation. These results give further validation to DBD-FISH protocol as an experimental control for Sperm-*Sus*-Halomax and ISNT.

Boar is one of the species where the assessment of sperm DNA integrity could be of paramount interest. Recently, it has been partially demonstrated that the intensity of DNA fragmentation in boars is correlated to conception or failure to conceive [6], such as has been established in humans. Thus, the development of a simple technique to determine the DFI in any basic laboratory would be of great interest. It would complement the standard semen analysis, mostly in samples directed for artificial insemination, in the evaluation of DNA quality in frozen samples and/or for research purposes on sperm chromatin and influence of male factor. Within this scenario, the methodology here contrasted could be a logical alternative to the TUNEL assay, Comet-Assay or SCSA test.

The acid-lysis protocol employed in humans seems suitable, as demonstrated by the sequential labelling of ssDNA generated from the ends of the breaks through the DBD-FISH procedure. Nevertheless, the diffusion of the chromatin fragments that results after lysis, without previous acid treatment [7], was clearly found as a much better system for the discrimination of boar sperm cells with fragmented DNA. This is interpreted as a particularly much higher resistance, exhibited by boar sperm nuclei without fragmented DNA, to release the compacted DNA loops by the lysing agent compared to human sperm cells. An extremely restricted relaxation of DNA loops was observed only in boar sperm in the basal nuclear area, close to the flagellum, thus, providing evidence that interspecific (human versus boar) differences occur in the chromatin structure and/or organization in the sperm cells. In this case, and for efficient analytical purposes, the modified SCD test is even simpler and faster than that used for humans and it is now specific for boar sperm cells. This allows its incorporation to any basic reproduction laboratory without the need for new complex or expensive instrumentation. The results obtained with the SCD variant developed in the kit were strongly correlated to those from the ISNT assay. In fact, the ISNT performed on sperm cells processed with the Sperm-*Sus*-Halomax kit directly confirmed that those sperm heads with large halos of diffused spots contain fragmented DNA. Compared with humans, where 30% or higher of sperm count with fragmented DNA is considered low or poor quality to be used in assisted reproduction (2), the DFI found in

boar samples studied (0.7–10%) could be considered acceptable. In fact, Rybar et al. [6] using SCSA and assuming the thresholds for DFI established in humans, proposed that 15% in boar sperm samples could be considered high. Values obtained in the present investigation fit in the range of DFI obtained in the group of lower DFI determined by these authors in a sample of 68 boars. Low levels of DNA fragmentation were also reported, under different experimental conditions of sperm boar conservation, using the comet-assay [8]. In this sense, it could be stated that the sample included in this study would show a moderate DFI. However, with the scarce information now available, additional data is necessary to establish a solid threshold value to this parameter on boar sperm samples.

Although the methodology here presented is effective under bright-field microscopy for routine analysis, it may be adapted for more specific studies with other variants to the visualization of the DNA damage, as could be those based on fluorescence microscopy, like ISNT and DBD-FISH. And probably could be expanded to TUNEL or other FISH strategies. This possibility is of interest because a more direct visualization of the DNA damage could be achieved and probably different subcategories of sperm DNA fragmentation or possible differential chromatin organization patterns could be identified with much more confidence than under bright-field microscopy. In fact, the higher sensitivity of the fluorescence microscopy, coupled with the ISNT analysis, allowed the categorization of different halo patterns in sperm nuclei without extensive DNA fragmentation. Whereas a minority do not show halos and DNA labelling, most of them exhibit small nuclear and basal halos which reveal a regional and ISNT sensitivity. This was not found in human sperm cells [7]. Thus, the sequential use of this test coupled with ISNT reveals differences among cells within the same semen sample. This topic deserves additional investigation.

Overall, the SCD test variant developed in the Sperm-*Sus*-Halomax kit is a simple and rapid procedure to determine DNA fragmentation, specifically in boar sperm cells. The discrimination of spermatozoa with fragmented DNA is extremely sharp, with great reproducibility. This would allow for the routine assessment of boar sperm samples for DNA fragmentation, as well as basic and clinical research on this relevant topic in any laboratory of semen analysis. The kit is versatile enough to be used in more focused research areas like, for example, those based on fluorescence microscopy.

Acknowledgements

The work has been possible through grants from the MCYT (BOS2002-00232 and BOS2003-04263) and Xunta de Galicia (PGIDIT 04BTF916023PR).

References

[1] Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:331–45.

[2] Evenson DP, Larson KJ, 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.

[3] Fernández JL, Muriel L, Rivero MT, Goyanes V, Vázquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl 2003;24:59–66.
[4] Fernández JL, Muriel L, Goyanes VJ, Segrelles E, Gosálvez J, Enciso M, Lafromboise M, De Jonge C. Simple determination of sperm DNA fragmentation with an improved Sperm Chromatin Dispersion (SCD) test. Fertil Steril, in press.
[5] Fernández JL, Gosálvez J. Application of FISH to detect DNA damage: DNA breakage detection-FISH (DBD-FISH). Meth Mol Biol 2002;203:203–16.
[6] Rybar R, Faldikova L, Faldyna M, Machatkova M, Rubes J. Bull and boar sperm DNA integrity evaluated by sperm chromatin structure assay in the Czech Republic. Vet Med 2004;49:1–8.
[7] Muriel L, Segrelles E, Goyanes V, Gosálvez J, Fernández JL. Structure of human sperm DNA and background damage, analyzed by in situ enzymatic treatment and digital image analysis. Mol Hum Reprod 2004;10: 203–9.
[8] Fraser L, Strzeżek J. The use of comet assay to assess DNA integrity of boar spermatozoa following liquid preservation at 5 °C and 16 °C. Folia Histochem Cytobiol 2004;42:49–55.