DNA Status on Thawed Semen from Fighting Bull: A Comparison Between the SCD and the SCSA Tests

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Contents

The assessment of sperm chromatin status is compulsory in a complete spermiogram. Here we applied the sperm chromatin structure assay (SCSA) and the sperm chromatin dispersion (SCD) test to assess the chromatin status of three fighting bulls. Cryopreserved semen (two straws/bull) were analysed by duplicate after thawing and after 6 h at 37°C with and without oxidative stress (1 mM Fe^{24}). Results (SCD: percentage of spermatozoa with halo; SCSA: SD-DFI, %DFI and HDS) were analysed for differences between bulls and treatments, sensitivity and specificity (receiver operating characteristic curves) and repeatability (repeatability coefficients as 2SD of duplicate differences).%DFI for the three bulls was below 2% at 0 h, indicating no risk for fertility according to previous reports. It increased slightly for two of the bulls after Fe^{24} treatment (%DFI < 5%) and more pronouncedly for the other bull (C, %DFI~10%), which merits further investigation. SCD rendered higher percentage of halos for bull C, but could not discriminate between samples with and without oxidizing treatment (AUC: 0.52). SCSA (%DFI) showed a high discriminating ability between treatments (AUC: 0.96). The repeatability coefficient was also higher for SCD (5.9) than for %DFI (1.8), indicating lower repeatability for SCD. Overall, %DFI might be the most useful parameter for assessing sperm chromatin on fighting bull. SCD might yield different information than SCSA, hence further research is warranted.

Introduction

2 Among the huge choice of tests for semen analysis developed in the last years, DNA assessment has gained increasing attention. Indeed, DNA defects are recognized as one factor responsible for uncompensable defects, meaning that the fertility of the given sample cannot be improved by increasing the number of spermatozoa for insemination (Evenson 1999; Saacke et al. 2000). These defects also reduce the success rates **3** of IVF or ICSI. Taking into account the great number of factors that can impair the DNA integrity of the spermatozoa, either during spermatogenesis (male genetics, health, environment) or afterwards (infections, oxidative damage, manipulation), an effective and affordable test to assess the sperm DNA integrity should be considered as a requirement for a complete spermiogram (Agarwal and Allamaneni 2005). The evaluation of sperm DNA has been based on many strategies: direct study of DNA breaks (TUNEL, DBD-FISH, *in situ* nick translation, Comet assay), chromatin affinity to staining (toluidine blue, chromomycin A3), *in situ* acid-induced DNA denaturation [sperm chromatin structure assay (SCSA)], DNA dispersion after using low pH and reducing agents [sperm chromatin dispersion (SCD) test] and many others (Agarwal and Allamaneni 2005; Erenpreiss et al. 2006). There is ample literature on these techniques, with many interpretations of the results and, very often, disagreement on its effectivity and clinical value (Agarwal and Said 2003; Agarwal and Allamaneni 2005). In this article we present a study in which two techniques for assessing sperm DNA integrity on frozen/thawed bull spermatozoa were tested. The golden standard in this study was the SCSA™, currently the only technique that has been extensively tested and whose clinical value seems to be sufficiently contrasted (Erenpreiss et al. 2006). The other technique tested was the SCD test, a recently developed technique for assessing sperm chromatin, which is relatively simple and inexpensive. SCSA™ is based on the *in situ* acid-induced DNA denaturation, followed by staining the sample with the metachromatic stain acridine orange (Evenson et al. 2002).This stain intercalates in the DNA, giving green fluorescence if the intercalating site is double-strand DNA (native), and red fluorescence if the site is single-strand DNA (denatured). The cells are run through a flow cytometer, and the fluorescence values of each cell are used for obtaining a DNA fragmentation index (DFI), which is the relation between red and total fluorescence. DNA regions with breaks are highly susceptible to denature during acid treatment, and thus cells with damaged DNA have a higher DFI. Spermatozoa are then classified according to whether their DFI is low, moderate or high (typically, DFI is considered moderate when it is between 20, 25 and 75, or high if it is above 75). The percentage of spermatozoa with moderate or high DFI (also called DFI) is then used to determine the DNA integrity of the sample. SCSA™ has been extensively used for human semen and is considered as reliable and repeatable (Evenson et al. 2002). Indeed, there are prognostic cut-off values for the DFI of a human semen sample. Thus, when DFI is higher than 30% it is considered that the fertility of the sample will be greatly compromised (Bungum et al. 2007). The technique has been successfully tested in other mammals, such as boar (De Ambrogi et al. 2006; Hernandez et al. 2006), ram (Martinez-Pastor et al. 2004; Garcia-Macias et al. 2006b,c; Kasimanickam et al. 2006b), stallion (Lo et al. 2002; Dias et al. 2006), dog (Nunez-Martinez et al. 2005; Garcia-Macias et al. 2006c) and several wild species (Garcia-Macias et al. 2006b,c; Fernandez-Santos et al. 2007). Domestic

cattle has received much more attention than any other species, apart from humans. In fact, it has been known for 20 years that bull fertility is related to SCSA results (Ballachey et al. 1987). Subsequent studies have confirmed the relationship of SCSA not only with field fertility but also with other physiological and morphological characteristics of bull spermatozoa (Evenson 1999; Bochenek et al. 2001; Ostermeier et al. 2001; Januskauskas et al. 2003; Garcia-Macias et al. 2006a; Kasimanickam et al. 2006a; Waterhouse et al. 2006) and it has been suggested that values of DFI ≥ 20% might announce lower fertility (Evenson 1999). Nevertheless, clear DFI cut-off values for fertility prognosis have not been defined, neither for cattle nor for other non-human species, although there are some efforts in this sense (Love 2005). In contrast, the SCD has been in use for less than 5 years (Fernandez et al. 2003). This technique is based on the inclusion of spermatozoa in a gel matrix, applying a high-salt low-pH treatment (including reducing agents to break disulphydryl bonds in the chromatin). After this treatment, the sample is analysed by brightfield or fluorescence microscopy, obtaining the percentage of sperm heads with a halo. Depending on the commercial kit used (presence or not of a denaturation treatment in order to optimize the test for different species, according to the authors), the halos indicate either good or bad condition of the sperm DNA (Fernandez et al. 2003; Perez-Llano et al. 2006). The SCD is relatively easy to perform and does not require expensive equipment. However, it is recommended to count at least 500 spermatozoa, which can be very time-consuming if analysing many samples. The technique has been used to evaluate semen samples from humans, boars and bulls (Fernandez et al. 2005a; Enciso et al. 2006; Garcia-Macias et al. 2006a; Perez-Llano et al. 2006; Muriel et al. 2007). However, due to the short time this technique has been on use, it is not sure whether it has a relationship to fertility (Muriel et al. 2006b), although some studies indicate that a relationship indeed may exist (Muriel et al. 2006a), and that it may have a performance comparable to SCSA or TUNEL (Chohan et al. 2006). Nevertheless, this test has yet to be fully validated for its clinical use. Its usefulness is still subject to controversy, especially due to its high variability, which may hamper its diagnostic and prognostic use (Evenson and Wixon 2005a; Schlegel and Paduch 2005). In this study we show the results of the first chromatin assessment of fighting bull spermatozoa. This breed is highly valued in Spain and **4** many South American countries, being sensible to establish germplasm banks for the conservation of its genetic resources (Blackburn 2004). However, these animals are reared following traditional and extensive **5** procedures, and males are frequently killed before testing fertility or seminal characteristics. Thus, the use of seminal doses from males killed in bullfighting (epididymal sperm) or kept as breeders (electro ejaculation) would have to rely on the assessment of the cryopreserved semen prior to its use. In this context, the application of sensitive analysis for assessing sperm DNA integrity would be especially interesting, in order to discard samples with bad fertility prognosis. The aim of the present study was to determine the potential of

SCSA and SCD for evaluating cryopreserved fighting bull doses. To achieve this objective, we aimed at detecting between-bull differences. As these differences are usually small and the number of different doses from this breed is scarce, we submitted the samples to oxidative stress, thus inducing alterations on the DNA . **6** Having set up this positive treatment, we tested the repeatability and sensitivity (ability to correctly discriminate samples treated with the oxidant from those untreated) of each test. **7**

Materials and Methods
Experimental design
All chemicals were at least of Reagent grade, and acquired from Sigma (Madrid, Spain). Sperm-Bos-Halomax® (for fluorescence microscopy) was acquired from ChromaCell SL (Madrid, Spain). For the experiment, we collected and froze samples from three fighting bulls (from now on termed bulls A, B and C) from the germplasm bank we maintain in our laboratory for this breed. We thawed two straws from each bull, which were processed separately. Semen was diluted in phosphate-buffered saline (PBS) and split between a Control and an Oxidant treatment. For the Oxidant treatment, we added $FeSO_4$ (100 μM) and sodium ascorbate (1 mM). Fe^{2+} oxidizes to Fe^{3+}, which is recycled by the ascorbate, producing the highly reactive hydroxyl radical (HO). Samples were incubated for 6 h in order to induce oxidative stress. It has been previously demonstrated that this kind of treatment causes sperm DNA damage, including strand breaks (Chen et al. 1997; Lloyd and Phillips 1999). The samples were kept in a water bath at 37°C. We analysed DNA integrity at 0 and 6 h by using SCSA (Evenson and Wixon 2005b) and SCD (Halomax®).

Animals, electroejaculation and semen cryopreservation
Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation, RD223/1988, which conforms to European Union Regulation 86/609. The males were selected fighting bulls used for breeding. They were kept isolated in individual enclosures and were healthy and reproductively mature at the time of the collection of the study. Previous to electroejaculation, bulls were anaesthetized using xylazine (0.1 mg/kg Rompun 2%; Bayer, **8** Germany) and Mepivacaine (0.16 mg/kg Mepivacaine HCl 2%; Braun, Germany) (Álvarez et al. 2006). Semen was collected by electroejaculation using a sine-wave stimulator (P.T. Electronics, Boring, OR, USA). The stimulator was capable of monitoring voltage and amperage and used an AC current of 220 V/60 Hz, with a transformer producing a maximum of 55 V and 1.5 A. The stimulating voltage was delivered using rectal probes with three longitudinal surface-mounted electrodes. Probe diameter, probe length and electrode length were 4.5, 37.5 and 8.5 cm respectively. The probe was lubricated and gently inserted into the rectum, and orientated so that the electrodes were positioned ventrally. The penis was

prolapsed beyond the prepuce and semen collected using a 30-ml sterile plastic container, which was kept warm by covering it with the hand. The electroejaculation regime used was based on that employed previously for ungulates (Howard et al. 1981; Roth et al. 1998) with various modifications. It consisted of consecutive series of 5-s pulses of similar voltage, each separated by a 5-s break. Each series consisted of a total of four pulses (Garde et al. 2003). Semen was placed in a water bath at 30°C until processed. For freezing, we supplemented Biladyl (Fraction A and B; IMV, L'Aigle, France) with 20% egg yolk.

Semen was diluted to 100×10^6 cells/ml in glass tubes with Biladyl Fraction A at 30°C. The tubes were placed in beakers with 100 ml of water at the same temperature, and the beakers were placed in a walk-in fridge at 5°C (slow cooling). When water temperature reached 5°C, Biladyl Fraction B (12% glycerol) was added 1 : 1 (final concentration of glycerol: 6%). The samples were left to equilibrate for 4 h and then packed in 0.5 ml straws (50×10^6 cells/straw). Freezing was carried out in liquid nitrogen vapours (4.5 cm above liquid nitrogen level; −20°C). Straws were stored in liquid nitrogen for a year.

Thawing and sample processing

Thawing was carried out by dropping the straws into a water bath at 37°C for 30 s. Semen was diluted in PBS (10^7 spz./ml), from which we took 1 ml as a Control and 1 ml as the Oxidant treatment. To the Oxidant tube, we added 10 μl of 10 mM $FeSO_4$ and 100 mM sodium ascorbate in water (final: 100 μM Fe^{2+} and 1 mM ascorbate). Both tubes were placed in a water bath at 37°C.

Sperm chromatin dispersion test

For carrying out the SCD test, samples were treated with the commercial kit Sperm-Bos-Halomax® following the protocol included with the kit. Twenty-five microlitres of sperm sample was added to a vial containing 50 μl of liquid low-melting agarose at 37°C. Then, 25 μl of the solution (∼3×10^6 spz./ml) was placed on an agarose pre-treated slide provided with the kit and cooled at 4°C. The drop was covered with a coverslip (22×22 mm) and the slide was placed at 4°C for 5 min. The coverslip was then carefully removed and the slide was immediately put into lysing solution at ambient temperature. After 4 min, the slide was washed for 5 min in distilled water and sequentially dehydrated in ethanol (70%, 90%, 100%). After dehydration, slides were examined for a few hours by fluorescence microscopy, staining for 10 min with 5 μl of staining solution (provided with the kit) mixed with 10 μl of 1 mM of 1,4-diazabicyclo[2,2,2]octane (DABCO) in 30% glycerol in water (antifade solution). Fluorescence was observed using a 510–560 nm excitation filter and a 590 nm emission filter, at ×400 magnification. Spermatozoa were classified as having a halo (damaged DNA) or not, recording the percentage of spermatozoa with halo. The same observer, trained on the technique, performed all analysis.

SCSA

The SCSA technique (Evenson et al. 2002) is based in the metachromatic stain acridine orange, which fluoresces green when combined with double-stranded DNA, and red when combined with single-stranded DNA (denatured). Spermatozoa were diluted in TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM EDTA; pH 7.4) to 2×10^6 ml^{-1}. Samples were flash frozen in LN2 and stored at −80°C until analysis. For the analysis, the samples were thawed on crushed ice and 200 μl was put on a cytometry tube. We then added 400 μl of an acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2). Exactly 30 s after adding the acid-detergent solution, we added 1.2 ml of staining solution (6 μg/ml of acridine orange in a buffer containing 37 mM citric acid, 126 mM Na_2HPO_4, 1.1 mM disodium EDTA and 150 mM NaCl; pH 6). We left the sample staining for 3 min, and then we ran it through a Becton Dickinson LSR-1 flow cytometer. We excited the acridine orange using an argon laser providing 488 nm light. The red fluorescence was detected using a long-pass (670LP) filter (FL-3) and the green one using a band-pass (530/28BP) filter (FL-1). Both photodetectors were adjusted using linear scales. Sample acquisition was carried out with the CellQuestv.3 software. Flow cytometry data (FCS files) were processed and saved as tabbed text using WinMDI v.2.8 (The Scripps Research Institute, La Jolla, CA, USA). We calculated the DFI for each spermatozoa as the ratio of red fluorescence with respect to total fluorescence (red + green), expressed as a percentage. The processing of DFI data was performed using the R statistical environment (R Development Core Team, 2007). From the DFI values, we obtained the standard deviation of DFI (SD-DFI). The percentage of spermatozoa with high fragmentation index (%DFI) was calculated as the percentage events with DFI > 25% (Fig. 1). We also obtained the high DNA stainability index (HDS) as the percentage of spermatozoa with green fluorescence intensity above channel 600 (0–1023 channels).
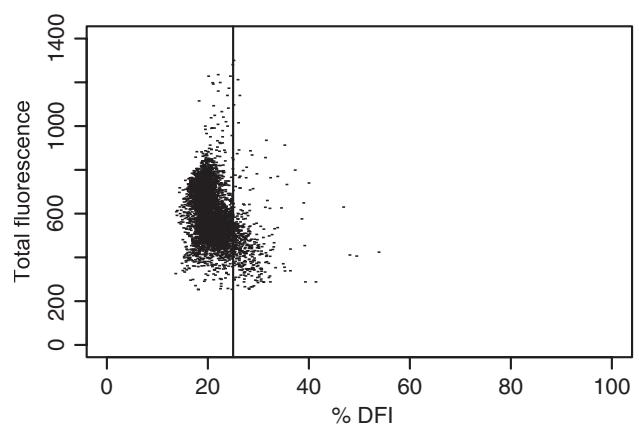

Fig. 1. Example of dot plot for %DFI vs total fluorescence. This kind of graph allows to interpreting %DFI data (each dot represents data from one spermatozoon), defining a main population with low %DFI and a cut-off value for medium and high %DFI values. In this study, this cut-off value was set at 25% (vertical line)

Statistical analysis

Statistical analyses were carried out using the R statistical environment (R Development Core Team, 2007). First, we carried out a general analysis on the effect of treatment on the DNA data. The effect of the treatments was analysed by linear mixed-effects models (male as a random factor). As males seemed to respond differently to some parameters, we also carried out an ANOVA and Tukey test for assessing differences between males within each of the three treatments (0, 6 h Control and 6 h Oxidant). The repeatability of each parameter was assessed by using the test described by Bland and Altman (1986), calculating the repeatability coefficient as twice the standard deviation of the differences between the replicates. We used the Wilcoxon signed rank test for checking whether the differences differed significantly from 0. The discriminating ability of each parameter to distinguish between the Control and Oxidant treatment at 6 h was estimated by using receiver operating characteristic (ROC) curves (Nunez-Martinez et al. 2007). The sensitivity (true positive rate) and specificity (true negative rate) of each parameter for **12** different threshold values were calculated by plotting them as an ROC curve. We then calculated the area **13** under curve (AUC) using the integrate method. AUC varies from 0.5 (test with no discriminatory ability) to 1 (perfect discriminatory ability).

Results

The analysis of data in males showed that bull C differed significantly from the other two bulls because of lower DNA integrity, both for SCD and SCSA (Fig. 2). Nevertheless, the %DFI for that bull was still below the values considered problematic for fertility (Evenson 1999). Considering the response to the Oxidant treatment, SCD failed in detecting any difference with the Control (both at 0 and 6 h). However, only in bull C did the SCD show an increase in the percentage of spermatozoa with halo after incubation at 37°C ($p < 0.001$), both for Control and Oxidant treatments. In this case, the Oxidant treatment yielded a slightly higher percentage of halos than the Control ($p < 0.05$). Contrarily, %DFI did not show differences between Control at 0 and Control at 6 h, but increased significantly in the Oxidant treatment for the three bulls (Fig. 2), indicating a decrease in DNA integrity (higher %DFI). Although this increase was observed in all the three bulls, it was more marked for bull C. SD-DFI increased in the Oxidant treatment too ($p < 0.05$), but a more detailed analysis indicated that this increase was only significant for bull C. HDS showed a very high dispersion in the 0 h analysis, being much more homogeneous within and between bulls at 6 h. At that time and only for the Oxidant treatment, the three bulls differed significantly **14** following the pattern B < A < C. The results for repeatability and discriminating ability (ROC test) of SCD and SCSA are shown in Table 1. Differences between replicates were small, and not different from 0 ($p > 0.05$). Repeatability was relatively high for all techniques (low repeatability coefficients). However, **15** SCD, being based in subjective microscopy assessment,
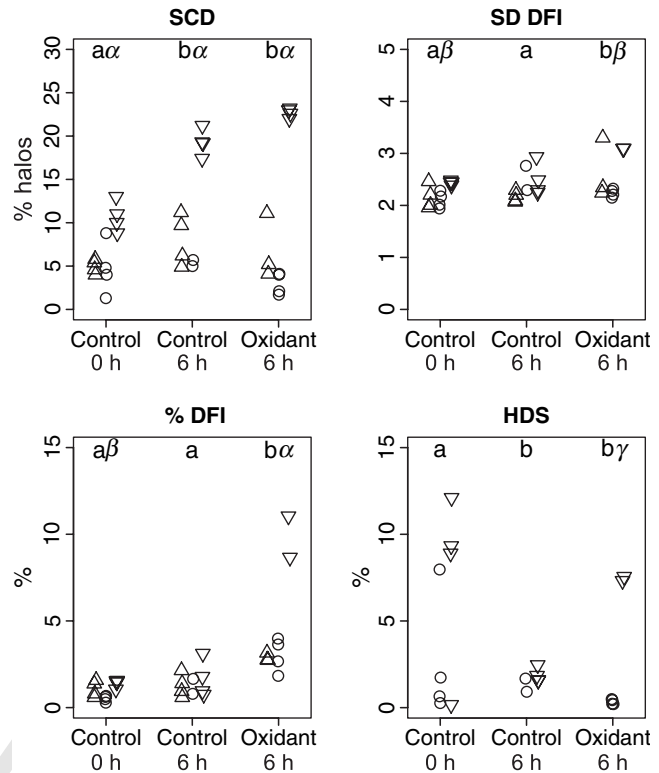

24 Fig. 2. Scatter plot showing the distribution of results for each bull and treatment for SCD and SCSA. Bull A: △ bull B: ○; bull C: ▼. **23** Lower-case Latin letters indicate significant differences between treatments (Control at 0 h, Control at 6 h and Oxidant at 6 h); lowercase Greek letters indicate significant differences between bulls within each treatment: α: A ≠ C, B ≠ C; β: B ≠ C; γ: A ≠ B, A ≠ C, B ≠ C. Notice the different vertical scales used for each parameter

had a lower repeatability. Figure 3 displays a graphical depiction of the test, showing the differences between the repeatability coefficient intervals of SCD and %DFI for **16** SCSA, showing the higher dispersion of the differences for the former. The analysis of the discriminating ability of each parameter, according to the treatment (Control or Oxidant), showed important differences between techniques (Table 1 and Fig. 3). The discriminating ability of SCD according to this criteria was almost null (AUC: 0.517). In the case of SCSA, %DFI had an AUC of 0.956, meaning a very good discriminating ability for this parameter. SD-DFI and HDS showed a low discriminating ability, as expected considering that these parameters only showed differences for bull C between Control and Oxidant.

Discussion

The assessment of semen doses from fighting bulls showed that %DFI results were below 2% overall. Taking the information published about the SCSA test on bull semen, we would identify all these doses as not having its fertility compromised because of chromatin defects. Indeed, it has been suggested that bull samples with %DFI ≥ 20% might have lower fertility (Evenson 1999). Nevertheless, submitting the samples to oxidative stress increased the %DFI of bull C to around 10%,

Table 1. Summary of the results obtained from the repeatability (RPT) and ROC tests. The data shown for RPT are the mean of the differences between replicates, and the repeatability coefficients (2SD of the differences). For the ROC test, data are the area under the curve (AUC) and the optimal cut-off point (OCP) for each curve (the value providing the higher number of true positives and the lower number of false negatives). A higher repeatability coefficient indicates lower repeatability, whereas a higher AUC indicates higher discriminating ability between samples submitted and not submitted to the Oxidant treatment

	SCD	SCSA		
		SD-DFI	%DFI	HDS
RPT				
Mean	0.306	0.004	0.138	1.384
2SD	5.9	0.648	1.764	6.389
ROC				
AUC	0.517	0.706	0.956	0.665
OCP	11.05	2.32	1.83	1.55

while the other two samples still remained below 5%. The reasons behind that difference merit further study, and highlight the importance of supplementary tests to assess the resistance of semen samples to stressing conditions. At this point, our study must be considered preliminary. Because of how fighting cattle are reared, no fertility data were available. Moreover, the number of cryopreserved doses from fighting bull is still very limited —our germplasm bank being one of the few containing such samples – and only a reduced number of males are represented there. The SCD test did not show any discriminating power for detecting oxidative damage caused by Fe^{2+} on bull sperm, contrary to the SCSA results. This contrasts with previous studies on this technique on human sperm, which suggested that SCD might be related to DNA damage as measured with DBD-FISH (Fernandez et al. 2003), SCSA (Fernandez et al. 2005b) and other tests (Chohan et al.2006). **17** We have to consider, though, that the SCD test for human sperm (Halosperm®) has a different lysis solution and a different interpretation than the Halomax® produces a halo when the spermatozoa kit for bovine sperm. Halosperm® has intact DNA, whereas Halomax® (and the variant for boar sperm) produces a halo when the spermatozoa has damaged DNA. This may **18** difficult the comparison between the human and the bull kits. There is only one previous reference to the Halomax® kit for bull sperm (Garcia-Macias et al. 2006a). In that study, the authors found negative correlations between 90-day non-return rates and both the percentage of halos (SCD) and several SCSA parameters. They also combined the percentage of halos with other sperm parameters and SD-DFI from SCSA
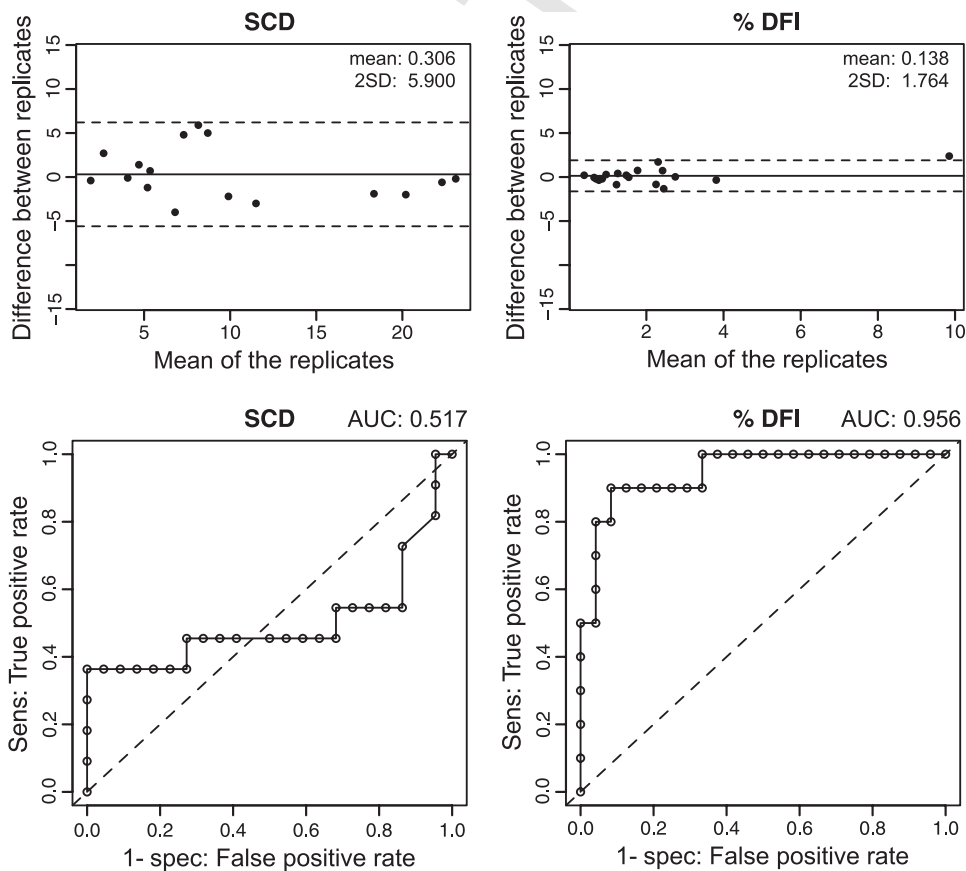
Fig. 3. Bland–Altman plots for repeatability (top) and ROC curves (bottom) for SCD and %DFI (SCSA). Repeatability plots show the mean of the replicates against their difference (overplotted lines represent the mean of the differences and the coefficient of repeatability, $2 \times SD$ of the differences). ROC curves (1 − specificity vs sensitivity) show the discriminating ability of each test between Control and Oxidant treatments. SCD had low repeatability (2SD = 5.9) and no predictive ability (AUC ~ 0.5), whereas %DFI had better repeatability (2SD = 1.764) and a very good predictive ability (AUC ~ 1)

into a multiple regression formula for predicting the non-return rate, although the predictive value was not high ($r^2 = 0.34$). They concluded that SCSA and SCD explained different aspects of sperm DNA damage, both techniques being useful for studying it and even complementary. Unfortunately, these authors did not use other statistical techniques, such as ROC curves or odd ratio calculation, which possibly had improved the comparison between SCSA and SCD for bull sperm. Considering both the results of Garcia-Macias et al. and ours, it seems that at least for the commercial kit Sperm-Bos-Halomax®, the SCD render different information that the SCSA. This may be a difference with the Halosperm®, for human sperm, which was criticized for not providing additional information than previous tests (Evenson and Wixon 2005a; Schlegel and Paduch 2005). The SCD test might detect some kind of sperm chromatin alterations, which may eventually affect sperm fertility, thus the relations found by Garcia-Macias et al. (2006a) and other authors. In our opinion, it is necessary to study the SCD kits for different species, and verify if the information delivered is the same in all cases. In our experiment, the SCD test discriminated bull C at 0 h and indicated changes in the sperm of bull C after the incubation at 37°C, but apparently not related to the oxidative stress. Thus, it seems that SCD detected a kind of chromatin alteration different to those detected by the SCSA. delaTorre et al.(2007), using SCD on boar sperm, could find increased DNA damage after applying an oxidative stress. However, that stress was much higher (15 mM H_2O_2 for 48 h) than the one used in our study, which might have had introduced artefacts in the analysis. Further research is necessary on this relatively new test to assess its validity [20] on detecting DNA damage and to determine whether the interpretation of the data is the same in different species. Considering the SCSA, %DFI was the most informative parameter. HDS had a totally different behaviour than the rest of the parameters, as it showed a broad dispersion at 0 h, which disappeared after the incubation period. Evenson and Wixon (2006) proposed that HDS would indicate the proportion of immature sperm in a sample, thus rendering a different kind of information than SD-DFI or %DFI. In our experiment, the reduction in HDS after 6 h of incubation may indicate changes during the incubation affecting the organization of sperm chromatin. In fact, other authors have reported changes in the degree of chromatin condensation after incubating spermatozoa in different media (Corcuera et al. 2007).

In conclusion, the SCD test for bull sperm did not discriminate among untreated and oxidized samples and it had a lower repeatability than the SCSA. Nevertheless, it discriminated between bull C and the others. The SCSA allowed to discriminating between the oxidized and untreated samples and also between bulls. We propose that both tests may be identifying different features of sperm chromatin. In the study of delaTorre [19] et al.(2007), the SCD was performed simultaneously to an analysis of protein loss from the sperm nucleus. Although limited, the results showed a relationship between nuclear protein alteration and DNA damage (formation of halos). In fact, the authors indicated that the lysis solution used in the SCD test alters the chromatin in a different manner than the acid-detergent used in SCSA, causing a disintegration of the protein scaffold, which may explain the differences between both techniques. The SCD is a potentially useful technique but it must be further studied. The higher subjectivity of this technique is a major drawback, which might be overcome with the development of automated systems for detecting halo sizes. Nevertheless, the lack of responsiveness discriminating among control or oxidized samples raises questions on the interpretation of this test, which must be solved before approaching the extensive use of this test.

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