Title:**DNA fragmentation assessment by flow cytometry (SCSA®) and Sperm-Bos-Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm.**

Short title: SCSA® and Sperm Bos Halomax to analyze bull sperm DNA fragmentation

Key Words: chromatin, SCSA,

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ABSTRACT

The aim of this study was to find the relationship between fertility (as 90-day non-return rates) with DNA fragmentation assessed by two techniques (SCSA and Sperm-Bos-Halomax: SBH). Furthermore, other quality parameters were acheived (motility, morphological abnormalities, cytoplasmic droplets, viability, capacitation and acrosomal and mitochondrial status) and their correlations with fertility were analyzed. Bulls were divided into three fertility groups: high (NRR≥ 80), medium (80 < NRR ≥ 70) and low (70 < NRR > 40). The results of this study indicate that there is a good correlation between fertility and different parameters of sperm quality (SBH and SCSA parameters, % of spermatozoa with head, neck and total abnormalities and % of spermatozoa with proximal cytoplasmic droplets) and differences between fertility groups were observed in some of them (SBH and SCSA parameters and % of spermatozoa with head, neck and total abnormalities). In this sense, SBH parameters rendered good correlations with fertility (r=-0.42 using bright light microscope and r=-0.47 with fluorescence). Also, SD-DFI and DFIh showed good correlations with fertility (r=-0.41 and r=-0.29). No correlations were observed between SCSA and SBH parameters. A multiple regression shows that four parameters (% of proximal cytoplasmic droplets, % of intact astrodomes in total population, SD-DFI and percentage of fragmented DNA detected by bright light microscope) present a good predictive value of the fertility of sperm samples (r^2=0.34, P<0.001).

INTRODUCTION

Conventional tests routinely used for semen evaluation are considered to be inconsistent predictors of reproductive efficiency because they only help by eliminating samples with very poor quality (Rodriguez-Martinez, 2003) [1]. In order to increase production potential, the cattle artificial insemination industry is focusing on the development of accurate methods that can predict field fertility when frozen semen is used. Indeed, in the high selected bull population more sensitive methods are needed to enable elimination of males with potentially low fertility (Hallap et al, 2005) [2]. Thus, characterization of sperm quality must consider motility and sperm viability but also sperm functional competence, such as the ability to undergo capacitation, acrosome reaction and the ability of sperm chromatin to form the male pronucleus. A spermatozoon must be capable of undergoing decondensation at an appropriate time in the fertilization process (Amann, 1989) [3]. Currently, the evaluation of chromatin status is one of the main objectives because it has demonstrated a high correlation with fertility [4]. The sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins.

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 (Evenson et al, 2002; Agarwal and Said, 2003) [5;6]. There are several techniques to assess sperm DNA fragmentation such as In Situ Nick Translation (ISNT), the Terminal deoxyribonucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL) and comet assay and mainly Sperm Chromatin Structure Assay (SCSA) (Evenson et al, 1980; Evenson et al, 2002; Evenson and Tritle, 2004) [6-8]. Recently, a new procedure for the DNA fragmentation assessment has been developed

for human sperm cells (Fernandez et al, 2003) [9] and for boar sperm cells using a commercial kit (Enciso et al, 2005) [10].

In this work, we used the SCSA technique and an adaptation of the technique used in boar sperm. Briefly, intact spermatozoa are immersed in an agarose matrix and slides are treated with a lysis buffer to remove membranes and proteins. Removal of nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loops. DNA fragmentation produces large halos whereas those sperm with low levels of fragmentation show small halos.

The main objective of this work was to determine whether the assessment of in vitro basic sperm characteristics and chromatin status could be used to predict fertility. For the assessment of DNA fragmentation we used SCSA and a new commercial kit adapted to bull sperm for bright field or fluorescence microscopy applications for the determination of DNA fragmentation in bull sperm.

MATERIAL AND METHODS

Reagents and media

Unless otherwise stated, all media components were purchased from Sigma (The Netherlands).

Source of cryopreserved semen

Semen cryopreserved from 60 Holstein Friesian bulls (12-52 months old) from ABEREKIN S.A. AI bull center (Derio, Bizkaia, Spain) was used. It was collected via artificial vagina, diluted in extender (Triladyl® Minitüb, Tiefenbach, Germany), and frozen and packed in 0.25 ml plastic straws frozen using a biofreezer (freezing chamber IMV Digitcool 5300 3T with a ZB350 controller and connected to Cryo Diffusion 200l-XA120 tank). Freezing was carried out at a cooling rate of -5ºC/min to -10ºC, -40ºC/min to -100ºC and finally -20ºC/min to -140ºC. The frozen straws were stored in liquid nitrogen until tested.

Inseminations were performed routinely within 1 year of freezing on heifers and cows of different parity during all seasons of the year. The number of inseminations per bull ranged from 45 to 150. Non return rates (NRRs) 90 days after AI were recorded for each bull.

The males were classified according to their fertility calculated on the basis of the NRRs in three groups of 20 males: high fertility bulls (NRR $\geq$ 80), medium fertility bulls (80 < NRR $\geq$ 70) and low fertility bulls (70 < NRR > 40).

Assessment of Sperm Motility

Post-thawing sperm motility was determined with a computer-assisted motility analyzer (CASA system) using an optical phase contrast microscope (Nikon Labophot-2, equipped with negative phase contrast objectives (10x) and a warming stage at 37 ºC), a Sony XC-75CE camera, and a PC with the Sperm Class Analyzer software (SCA2002,

Microptic, Barcelona, Spain). Samples were diluted (10-20 x 10^6 cells/mL) in a 1% egg yolk buffered (HEPES 20 mM/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7; 320 mOsm/Kg), and warmed on a 37 ºC plate for 5 min. Then, a pre-warmed Makler counting chamber (10 µm depth) was loaded with 5 µl of sample. The proportions of total motile (MT, %), progressive motile (MP, %), as well as kinematic characteristics such as velocity according to the actual path (VCL, µm/s), velocity according to the straight path (VSL, µm/s), velocity according to the average-smoothed-path (VAP, µm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of the lateral displacement of the sperm head (ALH, µm) and frequency of the flagellar beat (BCF, Hz) were recorded. At least five sequences were saved and analyzed afterwards.

Assessment of Sperm Viability

The double stain SYBR-14 with propidium iodide (Molecular Probes, L-7011 LIVE/DEAD® Sperm Viability Kit) using flow cytometer was applied. Sperm samples were diluted with PBS down to 5 x 10^6 spz/ml, and 300 µl were transferred to a polypropylene tube to which we added 3 µl PI and 1.5 µl SYBR-14. The tubes were kept at 37ºC for 20 min in the dark.

We detected three populations corresponding to percentage of live spermatozoa (green), moribund spermatozoa (red+green) and dead spermatozoa (red), but only live spermatozoa (named VIAB) were recorded.

Sperm morphology

Sperm morphology was evaluated in wet preparations of 10 µl of sperm samples fixed with 100 µl of glutaraldehyde 8% in BL-1 medium (glucose 2.9 g, sodium citrate dihydrate 1 g, sodium bicarbonate 0.2 g to 100 ml deionizated water). In these preparations, 200 cells were assessed in randomly selected fields (x 600) under an

optical phase contrast microscope (Nikon Labophot-2). The spermatozoa were classified into 4 groups: spermatozoa with head abnormalities (loss head, piriforms, macrocephal and microcephal spermatozoa, etc...), spermatozoa with neck abnormalities (double piece, bad flagel inserted), spermatozoa with tail abnormalities and finally normal spermatozoa. Moreover, we assessed the presence of cytoplasmic droplets according to their location: proximal, intermediate and distal.

Viability and Acrosomal Status

For acrosomal status we used the double stain PNA-FITC (PNA-FITC; Sigma) and propidium iodide (PI; Sigma) in a stock solution in PBS at 0.1 mM and 7.4 mM respectively. Once more, sperm samples were diluted in PBS (5 x 10^6 spz/ml), and 300 µl were transferred to a polypropylene tube in which we added 2.5 µl PI and 2.5 µl PNA-FITC. Flow cytometer results rendered two populations: the percentage of intact acrosome of the viable population (ACR) and the percentage of total intact acrosome (ACRT).

Capacitation Status

The capacitation status of spermatozoa was assessed by staining with the hydrophobic dye Merocyanine 540 (Sigma). M540 detect a decreased packing order of phospholipids in the outer leaflet of the plasma membrane lipid bilayer (Williamnson et al, 1983; Langer et al, 1993) **[11;12]** which is believed to occur in capacitated spermatozoa. Again, 300 µl of PBS and sperm (5 x 10^6 spz/ml) were put in a polypropylene tube and we added 1.56 µl of M540 stock solution (1 mM in DMSO) and 2.5 µl of PI (stock solution in PBS at 0.1 mM). The tubes were kept at 37ºC for 30 min in the dark.

We recorded the percentage of population of viable (non PI fluorescence) and capacitated spermatozoa (high merocyanine fluorescence) named CAP.

Mitochondrial status

Mitochondrial status was analyzed diluting the sample 300 µl of PBS (5 x 10^6 spz/ml), adding JC-1 (6.8 µM final; Molecular Probes, The Netherlands). After 30 min at 37 ºC, flow cytometer rendered the percentage of sperm with orange stained (high membrane mitochondrial potential) named MIT.

Flow cytometer for chromatin assessment (SCSA®)

We used the SCSA technique [7] (Evenson et al, 1980) to assess chromatin stability using metachromatic staining Acridine Orange (AO). This dyes fluorescent green when combined with double DNA helix, and red when combined with denatured DNA (Darzynkiewicz et al., 1975; Evenson and Jost, 2000) [13;14]. Samples were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM EDTA; pH 7.4) into polypropylene tubes at a final sperm concentration of approximately 1-2 x 10^6 cells/ml. Samples were dropped immediately into LN2 and stored in an ultra-cold freezer (-80ºC) until needed. For analysis, samples were thawed on crushed ice and mixed with 400 µl of an acid-detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% Triton X 100; pH 1.4). Exactly 30 seconds later, 1.20 ml of acridine orange staining solution (0.037 M citric acid, 0.126 M Na2HPO4, 0.0011 M disodium EDTA, 0.15 M NaCl; pH 6.0, 4ºC) containing 6 µg/ml electrophoretically purified AO (Polysciences, Inc, Warrington, PA) was added. The stained samples were analyzed just 3 min after AO staining.

Detection of DNA fragmentation using Sperm-Bos-Halomax

The two variants of the Sperm-Bos-Halomax ® kit (ChromaCell SL, Madrid, Spain) were used: for light microscopy and for fluorescence microscopy.

The first step is placing (?) the sperm sample in agarose microgel. Twenty-five microliters of diluted sperm were added to a vial with low melting agarose and mixed. Provided pretreated slides were placed on a metallic plate which had previously been cooled to 4 ºC. A drop of the cell suspension was spread on the treated face of the

cooled slide and covered with a coverslip for 5 min. The coverslip was then carefully (?) removed and immediately placed in 10 ml of the lysing solution (the recipient must be in contact with ice). Finally, the slides were washed for 5 min in distilled water and dehydrated in sequential ethanol solutions (70, 90 and 100%). The last step is different depending on the kit used. If light microscopy was used, we stained in Wright solution (Merck 1.01383.0500) 1:1 in Phosphate Buffer pH 6.88 (Merck 1.07294.1000). When fluorescence microscopy was used, samples were stained with 5 µl of propidium iodide 0.01mM (Sigma).

The samples were observed using a microscope (Nikon Eclipse E800 coupled with a Nikon digital camera DXM1200F) and 500 spermatozoa per sample were counted and classified into two groups: spermatozoa which exhibited halo and those that did not (Fig.1). Spermatozoa with fragmented DNA (%FragM with bright light microscopy and %FragF with fluorescence microscopy) corresponded with those where the thickness of the halo was equal to or greater than (???) the length of the core minor diameter. Unfragmented spermatozoa were those without halo or with a halo thickness of less than a third of the core minor diameter. These results were confirmed by sequential DNA breakage detection-fluorescence in situ hybridization (DBD-FISH. Personal communication Jaime Gosálvez. Data are not shown).

Cytometer assessment

For flow cytometer evaluation (viability, acrosomal status, capacitation and SCSA) we used a FACScalibur flow cytometer (Becton Dickinson Immunochemistry Systems; San Jose, CA, USA), equipped with standard optics and an argon ion laser, tuned at 488 µm, and running at 200 mW. Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). 10,000 events with a flow rate of ~200 cells/s were analyzed per sample. Data corresponding to the red (FL3 photodetector), orange

fluorescence (FL2 photodetector) and green fluorescence (FL1 photodetector) of acquired particles were recorded. In all the cases we assessed 10,000 events per sample with a flow rate of ~200 cells/s. The flow cytometry dot plots used were: FL3/FL1 for the analysis of viability and acrosomal status and SCSA assay and FL2/FL3 for capacitation assessment (figure 1).

Statistical analysis

Statistical analyses were performed with the SAS^{TM} V. 8 package (SAS Institute Inc., Cary, NC, USA). Comparisons between fertility groups were carried out using general linear model (Turkey). Pearson rank correlations were used to calculate the relationships between sperm quality parameters and for the correlation between fertility and sperm quality parameters. The Bland-Altman test was applied to determine the level of agreement between SCSA assay and Sperm-Bos-Halomax (and also between the two Sperm-Bos-Halomax techniques). Multiple regression analyses were used to calculate regression equations and to predict the NRRs on the basis of the analyses made in vitro. Values are presented as mean ± standard error, and were considered statistically significant when $P<0.05$.

RESULTS

The data of morphology, viability, acrosomal integrity and mitochondrial status depending on the fertility group are shown in Table 1. Only percentage of head, neck and total abnormalities had significant differences ($p<0.05$) between fertility groups, being the highest in the low fertility group. Nevertheless, motility parameters (Table 2) did not show differences between fertility groups.

All the analyzed parameters for (?) chromatin status and DNA assessment (Table 3) showed differences between fertility groups ($p<0.05$). In SCSA, mean DFI and HDS were higher in high and low fertility groups, but SD-DFI was higher in low fertility groups. Instead all of the DFI parameters had higher values in the low fertility group, only DFIh had differences between the low and the rest of the groups.

Bull sperm cells processed with the Sperm-Bos-Halomax gave rise to images of similar characteristics to those obtained in boars (Enciso et al, 2005) [10] (figure 2). Thus, sperm cells with fragmented DNA showed peripheral halos from the central core whereas those without DNA fragmentation showed very small halos of dispersion. In Halomax detection of DNA fragmentation, both bright light and fluorescence microscopy showed significant differences between the high fertility group and the other two with the highest DNA fragmentation figures being recorded in the low fertility group (Table 3).

Some of the semen parameters tested were significantly correlated with field fertility estimates. Correlations between single in vitro tests and fertility values are summarized in Table 4. Pearson´s correlation coefficients were higher in SD-DFI and the two parameters obtained with halomax. The detection of percentage of total abnormalities also had a good correlation with fertility ($r=-0.36$).

We also assessed correlations between all different studied parameters considering significant differences when $p<0.01$ (Table 5). Thus, we observed correlation between the percentage of sperm with head and total abnormalities and the fragmentation DNA indexes calculated by Sperm-Bos-Halomax (with both bright light microscopy and fluorescence microscopy). This relation was not observed between these indexes and percentage of sperm with neck abnormalities. We did not observe correlations between SCSA and Sperm-Bos-Halomax parameters.

The Bland-Altman test was applied to evaluate the level of agreement between the SCSA technique and Halomax and also between the two Halomax techniques (bright light and fluorescence microscopy). The results showed that there was a good correlation between bright light and fluorescence microscopy (0.35 ± 1.67). Nevertheless, this good correlation did not exist between SCSA and both Halomax techniques.

To calculate expected fertility, all tested parameters were included in a predictive equation. Stepwise multiple regression analysis was used to select independent variables that best predicted fertility values. Thus, we obtained a predictive equation ($r^2=0.49$) that included 4 parameters: % of fragmented DNA evaluated by Halomax with fluorescence microscopy (% FragF), SD-DFI, % proximal cytoplasmic droplets (PCD) and % intact acrosomes in total population (ACRT). The best single predictor of fertility ($r^2=0.22$) was the % FragF and the next one was SD-DFI ($r^2=0.12$). Real fertility (NRR) and predictive fertility are shown in figure 3. These two parameters had a good correlation ($r^2=0.34$; $p<0.001$).

DISCUSSION

In the present study, we assessed the relationship between different sperm quality parameters and chromatin and DNA status parameters (by SCSA and Sperm-Bos-Halomax) and (?) fertility.

The absence of differences observed in a great number of parameters among fertility groups could be explained by the narrow range of fertility we are working with.

Head and neck abnormalities, detected by bright light microscopy, showed a correlation with fertility. This observation is in agreement with other authors (Soderquist et al, 1991) [15] that reported statistically significant correlations between the incidence of some sperm abnormalities (abnormal heads, nuclear pouches, proximal cytoplasmic droplets) and fertility (56 NRR). Moreover, we observed that head and total abnormities were correlated with DNA fragmentation detected by Sperm-Bos-halomax. The possible relationship between chromatin organization and abnormalities has been described by Ostermeier et al [16] but it was not observed between SCSA parameters and morphology results. Maybe this is because potential problems with chromatin or damaged DNA will result in only minor changes of sperm head shape which are difficult to assess by subjective evaluation.

The SYBR-14/PI assay is a fast, reliable and sensitive method for assessing the viability of bull sperm (Grundler et al., 2004) [17]. Our results show no differences between the three fertility groups. In this sense, the relationship between fertility and post thawing viability parameters has not always been statistically significant (Garner et al, 1997; Zhang et al, 1998) [18;19] and tends to increase when damage is extensive or when

fertility values are spread widely (Wood et al., 1986) [20]. Neither have other authors (Brito et al, 2003) [21] observed great differences in the results for viability ??? among semen samples of bulls (8 males) producing different in vitro fertilization rates. But our results contrast with Januskauskas et al (2000) [22] and with Christensen et al (2005) [23] who observed a good correlation between viability and fertility (56 NRR). This lack of correlation observed in our work could be also influenced by the fact that ? when a high number of sperm is packed in each insemination dose the effect of selecting the best ejaculate according to sperm viability has a relatively limited effect on NRR (Christensen et al., 2005) [23].

With regard to the capacitation results, only a low percentage of viable spermatozoa were capacitated after cryopreservation. This is in agreement with Guthrie et al (2005) [24] who observed a slight increase in plasma membrane phospholipid disorder in cryopreserved boar semen when compared with fresh sperm (from 1.1% to 3-8%). Furthermore, there was no relation between capacitation and fertility results in spite of the fact that cryopreservation procedures can induce capacitation-like changes in spermatozoa (Bailey et al, 2000) and this cryocapacitation is thought to be partly responsible for the reduced fertility of frozen semen (Cormier et al, 2003) [25].

Motility is the most frequently assessed characteristic routinely used in the AI industry but its correlation with fertility remains controversial. Our results showed no correlation with fertility (neither total nor progressive motility nor velocity parameters). These results are in disagreement with Januskauskas et al [26] who observed a good correlation between CASA-assessed sperm motility and field fertility ($r = 0.61$; $P<0.05$) and Zhang et al [19] who found good correlation between field fertility and post-thaw

linear motility (*r*= 0.59). Nevertheless, Januskauskas et al [27] obtained no correlation between sperm kinematic characteristics and field fertility results.

Assessment of chromatin status and DNA fragmentation showed the most important differences in the three fertility groups and also some parameters showed a good negative correlation with fertility. This suggests that even small changes in fertility could be predicted by SCSA and by Sperm-Bos-Halomax. A close relationship between fertility and DNA status has been established and several papers show that SCSA is a predictor of sub/infertility in bulls [4;28;29]. Our SCSA results showed differences in all the studied parameters between fertility groups but it should be pointed out that SD-DFI and DFIh parameters had better correlation with fertility (r= -0.41 and r=-0.29). This is in agreement with previous data from bull fertility trials and mouse toxicology studies that show the SD-DFI was 20-30% more sensitive than % DFI [4].

Some of the authors that have found a relationship between fertility and SCSA parameters include Ballachey et al, 1987 (SD-DFI r=-0.58 and COMP αt r=-0.40, currently DFI) in mature bulls and Januskauskas et al, 2000 (COMP αt, r=-0.53) and 2003 [26] (COMP αt r= -0.33 in batches and -0.51 in bulls). Bochenek et al, 2001, showed that fertility correlation was with COMP αt (-0.50) and SD αt (-0.55). Rybar et al.[29] worked with two fertility groups (60.6% and 43.3% mean pregnancy rate) and observed differences between these groups in DFIh (0.4% vs 0.5%) and HDS (1.2% vs 1.9%).

The significant lower results of mean DFI in the medium fertility group is not important because this parameter demonstrated that is not the best fertility predictor. The small

percentage of cells with high DNA stainability (HDS) in all the fertility groups could explain the same behaviour for this parameter.

Thresholds of subfertility in humans (DFI > 30%) are very different from those obtained from Rybar et al [29] for bulls (10-20%). Taking into account this threshold, all the fertility groups had good DFI results (highest media of DFIt 8.90 in the low fertility results).

In the case of Sperm-Bos-Halomax, we also observed good correlations with fertility but in this case we observed differences between the high fertility groups and the other two. Our results showed that this technique could be used to detect fertility variations in bulls. This kit is a simple procedure to determine DNA fragmentation, specifically in bull sperm cells and the discrimination of spermatozoa with fragmented DNA is extremely sharp, as occurred in boar semen (Enciso et al, 2005) [10]. This would allow for the routine assessment of bull sperm samples.

The different results between the two DNA assessment (SCSA and Sperm-Bos-Halomax) could be because chromatin condensation depends on the different nature of interactions that occur between DNA and protamines, and among protamines (Madrid-Bury et al, 2005) [30]. SCSA defines abnormal chromatin structure as increased susceptibility of sperm DNA to acid-induced denaturation in situ due to the occurrence of DNA breaks or derailments in protamine quantity and composition or an insufficient level of disulfide groups (Giwercman et al, 2003) [31]. However, Sperm-Bos-Halomax quantifies sperm chromatin dispersion and only assesses DNA breaks after the lysis of chromatin proteins. With these differences, we conclude that SD-DFI and DFIh could

discriminate between low and medium fertility groups but parameters obtained using Sperm-Bos-Halomax only discriminate high fertility from the rest of the groups.

The SCSA had two advantages over Sperm-Bos-Halomax: a high number of cells are analyzed and evaluated objectively. Nevertheless this technique requires expensive instruments (flow cytometry).

Multiple regression analysis showed that only a few single-sperm parameters appear to be correlated to in vivo fertility (Januskauskas et al, 2000) [22]. In this work, we obtained a regression model that combines 4 sperm parameters: % of proximal cytoplasmic droplets, % of intact acrosomes in total population, SD-DFI and percentage of fragmented DNA detected by bright light microscopy to explain differences in fertility (r^2=0.49). This model showed us that SCSA and Sperm-Bos-Halomax test could measure different sperm traits for (?) DNA and chromatin status, so they could be used together to obtain accurate results. As sperm chromatin structure is complex, more than one test may be needed to measure sperm chromatin integrity [32].

To sum up, the results of this study indicate that there is a good correlation between fertility and different parameters of sperm quality (DNA fragmentation, percentage of spermatozoa with head neck and total abnormalities and percentage of spermatozoa with proximal cytoplasmic droplets). A multiple analysis of these parameters shows a good predictive value of the fertility of sperm sample.

Furthermore, SCSA and Sperm-Bos-Halomax test could measure different traits of sperm chromatin and DNA so they could be included at the same time in the analysis of bull sperm.

Table 1. Morphology, viability, acrosomal and mitochondrial status results depending on fertility group (Mean ± standard error).

	Fertility		
Parameter[1]	High	Medium	Low
PCD	1.47±0.31	3.10±0.65	3.40±0.64
ICD	0.26±0.15	0.25±0.12	0.25±0.12
DCD	2.05±0.45	2.75±0.67	3.80±0.90
TCD	3.79±0.63	6.10±0.98	7.45±1.31
HAbn	8.21±1.53[a]	15.25±1.41[ab]	16.75±3.36[b]
NAbn	1.79±1.40[a]	2.15±0.40[ab]	3.25±0.50[b]
TAbn	1.11±0.46	1.65±0.65	1.60±0.26
ToAbn	11.11±1.85[a]	19.05±1.74[ab]	21.60±3.25[a]
VIAB	47.50±2.11	48.66±2.49	47.92±2.65
ACR	99.66±0.06	99.40±0.12	98.86±0.55
ACRT	87.95±2.02	85.45±1.42	81.12±2.36
CAP	4.58±1.15	5.34±0.89	4.71±0.98
MIT	44.28±2.58	47.05±2.62	45.55±3.22

[a,b]: Values with different letters in a row are significantly different ($p<0.05$).

[1]PCD: % spermatozoa with proximal cytoplasmic droplets; ICD: % spermatozoa with intermediate cytoplasmic droplets; DCD: % spermatozoa with distal cytoplasmic droplets; TCD: % total spermatozoa with cytoplasmic droplets. HAbn: % spermatozoa with head abnormalities. NAbn: % spermatozoa with neck abnormalities. TAbn: % spermatozoa with tail abnormalities. ToAbn: % total sperm abnormalities (head, intermediate tract and tail).

VIAB: % viability. ACR: % intact acrosomes in viable population. ACRT: % intact acrosomes in total population. CAP: % live capacitated spermatozoa. MIT: % high membrane mitochondrial potential.

Table 2. Motility descriptors depending on fertility group (mean ± standard error).

	Fertility		
Parameter[1]	High	Medium	Low
MT	55.5±1.88	59.42±2.74	58.07±2.52
MP	36.72±1.87	34.17±2.49	36.01±2.19
VCL	66.97±2.71	61.54±2.41	61.26±2.23
VSL	102.33±2.96	98.66±2.85	95.93±3.69
VAP	57.98±2.77	52.08±2.79	52.64±2.28
LIN	54.42±1.94	50.44±1.81	52.89±2.25
STR	81.00±0.97	78.34±1.60	80.13±1.52
WOB	65.2±1.72	62.19±1.20	63.72±1.67
ALH	3.87±0.12	3.90±0.09	3.69±0.16

BCF	9.39±0.24	9.15±0.31	9.48±0.29

[1]MT: % total individual motility; MP: % progressive motility; VCL (µm/s): velocity according to the actual path; VSL (µm/s): velocity according to the straight path; VAP (µm/s): velocity according to the average-smoothed-path; LIN (%): linearity; STR (%): straightness; WOB (%): wobble; ALH (µm): amplitude of the lateral displacement of the sperm head) and BCF (Hz) frequency of the flagellar beat.

Table 3. DNA parameters (SCSA and Sperm-Bos-Halomax) depending on fertility group (mean ± standard error).

	Fertility		
Parameter[1]	High	Medium	Low
Mean DFI	19.07±0.41[a]	17.51±0.25[b]	19.50±0.33[a]
SD-DFI	8.71±0.84[a]	7.15±0.71[a]	12.93±0.94[b]
HDS	0.98±0.08[a]	0.80±0.03[b]	0.92±0.07[ab]
DFIm	4.14±0.94[ab]	2.15±0.26[a]	5.23±0.53[b]
DFIh	2.50±0.40[a]	1.37±0.23[a]	3.67±0.43[b]
DFIt	6.65±1.20[ab]	3.52±0.49[a]	8.90±0.92[b]
%FragMO	3.60±0.64[a]	5.65±0.55[b]	6.35±0.49[b]
%FragF	3.25±0.53[a]	5.06±0.42[b]	6.15±0.53[b]

[a,b] Values with different letters in a row are significantly different ($p<0.05$).

Table 4. Pearson's correlation coefficient between various quality parameters and fertility.

	Correlation coefficient and significance	
Parameter[1]	r	P-value
PCD	-0.29	0.026
TCD	-0.34	0.009
HAbn	-0.30	0.022
NAbn	-0.35	0.007
ToAbn	-0.36	0.005
SDαt	-0.41	0.001
DFIh	-0.29	0.026
%FragMO	-0.42	0.001
%FragF	-0.47	0.001

[1]See tables 1 and 2.

Table 5. Correlation coefficients and levels of significance of the Pearson's rank correlation among various quality parameters.

	HAbn	NAbn	TAbn	Mean DFI	SD-DFI	HDS	DFIm	DFIh	DFIt
NAbn	0.18		**	-	*	-	-	*	*
TAbn	0.97**	1**		-	-	**	**	-	-
Mean DFI	-0.08	0.33	0.01		-	**	**	**	**
SD-DFI	-0.05	0.34*	0.03	0.80		**	**	**	**
HDS	-0.01	-0.05	0.95**	0.53**	0.39**		**	**	**
DFIm	-0.11	0.31	0.88**	0.87**	0.69**	0.57**		**	**
DFIh	-0.13	0.32*	-0.04	0.81**	0.95**	0.41**	0.71**		**
DFIt	-0.12	0.33*	-0.01	0.91**	0.85**	0.55**	0.96**	0.88**	
%FragMO	0.49**	0.26	0.52**	-0.02	0.10	-0.15	-0.04	0.04	-0.01
%FragF	0.54**	0.27	0.56**	-0.01	0.10	-0.11	-0.05	0.01	-0.03

- P>0.01
* P<0.01
** P<0.001

Figure 1. General flow cytometric plots of complexity/particle size, double dye PI/PNA-FITC , SYBR-14/PI, JC-1, merocyanine/PI and SCSA.

Figure 2. Sperm processed with the Sperm-Bos-Halomax® kit 1. Stained with Wright solution and 2. Stained with propidium iodide. Those with a small halo (a) have normal status of DNA fragmentation and those spermatozoa with a big halo (b) contain a high proportion of fragmented DNA.

Figure 3. Relationship between fertility rates predicted by the equation based on the combination of 4 sperm parameters assessed in vitro and the observed field fertility (X d non return rate) of the bulls tested, (r^2=0.34, P<0.001; n=60). The line shows the trend in the data.