

Sperm DNA fragmentation in boars is delayed or abolished by using sperm extenders

Begoña Pérez-Llano^a, María Enciso^b, Pedro García-Casado^c,
Rubén Sala^a, Jaime Gosálvez^{b,*}

^a *Gestión Veterinaria Porcina S.L. C/Calibre 121 Pol.Ind.P-29, C.Villalba, 28400 Madrid, Spain*

^b *Unidad de Genética, Edificio de Biología, Universidad Autónoma de Madrid, C/Darwin no. 2, 28049 Madrid, Spain*

^c *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Crta. de La Coruña, km. 5.9, 28040 Madrid, Spain*

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Abstract

The semen quality of seven young adult boars was assessed for percentages of sperm motility, normal acrosomes, abnormal sperm, cells positive to sHOST (short Hipoosmotic Swelling Test), HPNA cells (sHOST Positive with Normal Acrosome cells) and the percentage of sperm heads, which exhibited DNA fragmentation using the Sperm Chromatin Dispersion test (SCD). These parameters were analysed in sperm samples both undiluted and diluted using a commercial extender and stored at 15 °C for 21 days. Results showed that semen quality decreases faster in the undiluted semen samples from day 0 to day 7 compared to diluted semen samples that remained with a high quality up to day 11. The undiluted semen exhibited a low DNA fragmentation index (DFI) during the first days and then a significant increase from day 7 up to day 21. This increase in the DFI coincided with the lowest levels of the other semen quality parameters. On the contrary, the samples diluted in the commercial extender showed very low levels of DNA fragmentation in all boars during the preservation period. When the evolution of DNA fragmentation was analysed in the undiluted samples, differences were found among boars. These differences were not shown in the samples diluted in the extender where the basal DFI remained stable during the 21 days. The main conclusion of this study was that some sperm extenders delay or partially prevent sperm DNA fragmentation.

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1. Introduction

Damaged DNA in the single sperm cell that fertilizes a female oocyte can have a dramatic negative impact on foetal development and health of the offspring. Thus, sperm DNA fragmentation tests provide a reliable analysis of the DNA fragmentation index (DFI) that may help to identify individuals who are at risk of

failing to initiate a healthy pregnancy [1]. Information about sperm DNA integrity may help in the clinical diagnosis, management and treatment of male infertility and may be of prognostic value in assessing the outcome of an assisted conception treatment [1,2]. Although most information about DNA fragmentation has been obtained from humans, in animals, especially in mammals, it may be assumed that similar factors are the effectors of sperm DNA fragmentation. However, they have been less studied because of the absence of adapted technologies to visualize such effects. In the particular case of boar sperm samples DFI has been

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

analysed using four different strategies, SCSA (sperm chromatin structure assay; [3], comet-assay [4,5], TUNEL assay [6] and recently a modified SCD test (Sperm Chromatic Dispersion test; [7,8]). These studies point to the fact that DNA fragmentation is positively correlated with a decrease in fertilization success.

In humans, the causes of high DNA fragmentation are usually related with chemical or radiation exposure, heat exposure, several types of infection, testicular cancer, age, and of course, oxidative stress through an increase of the free radical levels in the semen [9]. In addition, it is very important to be aware that DFI can change with time during the life of a male. Therefore, a good tracking of the sperm quality during different periods of life could assure the selection of the best samples, which could be preserved until its utilization. In boars, especially those used for reproductive purposes, a high incidence of those causes, which may produce sperm DNA fragmentation in humans are not expected, although they can not be disregarded. This is mostly because donor males are young and have been selected for reproductive strategies. However, no strict biological policies, such as the use of extenders and storage at 15 °C to fertilize receptive females, are a common practice. Even within this scenario, it is completely unknown how the quality of the DNA, in terms of DNA fragmentation, is affected in sperm samples, which have been stored in diluents. The aim of the present investigation was to analyse the stability of DNA in boar sperm samples under different conditions of preservation, in an attempt to throw some light on the dynamics of DFI.

2. Materials and methods

Seven adult boars aged 18–24 months were used to perform this experiment. These animals were selected from a series of 52 individuals, which were studied for DFI and standard semen parameters. The criterion to select the seven individuals to be included in the analysis was animals with DFI lower than 5% and high quality in standard semen parameters. Thus, semen quality was assessed for percentage of sperm motility, percentage of normal acrosomes, percentage of abnormal sperm, percentage of cells positive to sHOST (short Hypoosmotic Swelling Test), percentage of HPNA cells (sperm cells sHOST positive with a normal acrosome) and percentage of DNA fragmentation.

Boars were fed with a commercial porcine ration. Water was available *ad libitum*. Semen collection was performed with the gloved hand method, discarding gel fraction and seminal plasma.

DFI was independently assessed in two different laboratories and the mean was included in the analysis. From every ejaculate two samples were taken: one sample of 20 ml was kept in a plastic tube without extender and the other sample of 2 ml was extended in 18 ml of ACROMAX[®] (GVP, SL, Madrid, Spain). After an equilibration period of 3 h at room temperature, both samples of each ejaculate were preserved at 15 °C for 21 days. During the preservation period, samples were taken on days 1, 3, 4, 5, 7, 8, 10, 15 and 21 to check for the same parameters of semen quality as stated above.

Sperm motility was checked manually using negative phase contrast microscopy with 20× objectives. Semen samples were placed on a warm slide at 37 °C. Acrosomal status was analysed in semen samples fixed in a 2% glutaraldehyde solution [10]. Observations were performed under phase contrast using 100× objectives. One hundred sperm cells were evaluated as intact or damaged acrosome according to the criteria previously established [10]. Sperm cells with an intact acrosome were considered those with a normal apical ridge. Sperm cells with damaged acrosome were those that showed damaged or missing apical ridges or loose acrosomal caps. In the same slide, the percentage of abnormal sperm was evaluated by counting 100 sperm cells and calculating the percentage of proximal and distal droplets, coiled tails and other sperm abnormalities.

The percentage of sHOST positive cells was calculated by placing a 0.1 ml (undiluted) or 0.35 ml (diluted) semen sample into 1 ml of hypoosmotic solution (75 mOsm/kg) in a water bath at 37 °C for 5 min. After incubation a sample of 0.35 ml was removed and fixed in 0.5 ml of 2% glutaraldehyde solution and evaluated in a phase contrast microscope using 100× objectives and counting in 100 sperm cells those with any degree of a coiled tail (sHOST positive cells) and those with a straight tail (negative sHOST cells) [11]. In the same preparation, the HPNA value was calculated by counting in 100 sperm cells positive sHOST cells with a normal acrosome [12]. This fraction represents the population of sperm cells with the most resistant sperm membrane.

To determine DFI in boar sperm cells, the Sperm-Sus-Halomax[®] kit (ChromaCell SL, Madrid, Spain) was used. 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 had been previously cooled at 4 °C. A drop of the cell suspension was spread onto the treated face of the slide and covered with a glass coverslip for 5 min

at 4 °C. 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).

To assure that, under all experimental circumstances, the production of a large halo of chromatin dispersion was correlated with the presence of fragmented DNA, *in situ* nick translation (ISNT) was performed at days 3, 10 and 21. ISNT of DNA breaks was carried out in sperm samples treated with the lysing agent provided in the kit. After lysis of microgel-embedded cells, and to achieve the best results in polymerase activity, the slides were thoroughly washed four times in PBS for 5 min each and then incubated four times for 5 min in excess reaction buffer for DNA-polymerase I (10 mM Tris-HCl, 5 mM Mg Cl₂, 7.5 mM DTT, pH 7.5) performance. One hundred microliters of reaction buffer containing 25 U 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 30 min at 37 °C. After washing in a 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 were either directly analysed or alternatively counterstained with propidium iodide (2 µg/ml) 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. 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.

A Spearman rank order correlation analysis was performed to study the relationship between the percentage of DNA fragmentation and the other sperm quality parameters. To study the influence of the preservation time and the differences between undiluted and diluted sperm on semen quality and level of DNA fragmentation, a Kruskal-Wallis one-way ANOVA and multiple mean comparison by Dunn's method was used. Variations between boars were determined by Kruskal-Wallis one-way ANOVA and multiple mean comparison by Dunn's method (undiluted samples data) or Tukey test (diluted samples data).

3. Results

The initial semen quality of the seven ejaculates ranged from: 70 to 80% sperm motility, 78 to 88% normal acrosomes, 2 to 12% abnormal sperm, 56 to 88% sHOST, 48 to 86% HPNA, and 0 to 4.25% DFI.

DNA fragmentation in boar sperm treated with Sperm-Sus-Halomax[®] was visualized and classified according to the morphology of the nucleoids produced after protein depletion. Nucleoids with remarkably large halos of diffusion of chromatin spots were those sperm nuclei containing fragmented DNA (Fig. 1a). Sperm nuclei without DNA fragmentation did not show halos or showed an extremely minimal relaxation of DNA loops in the proximal area, close to the flagellum insertion (Fig. 1b). *In situ* nick translation of DNA breaks gave rise to positive DNA labelling in those sperm heads, which exhibited large halos of chromatin dispersion. In the rest of the nuclei, absence or minimal DNA labelling in the proximal end of the sperm head was observed (compare Fig. 1b and c).

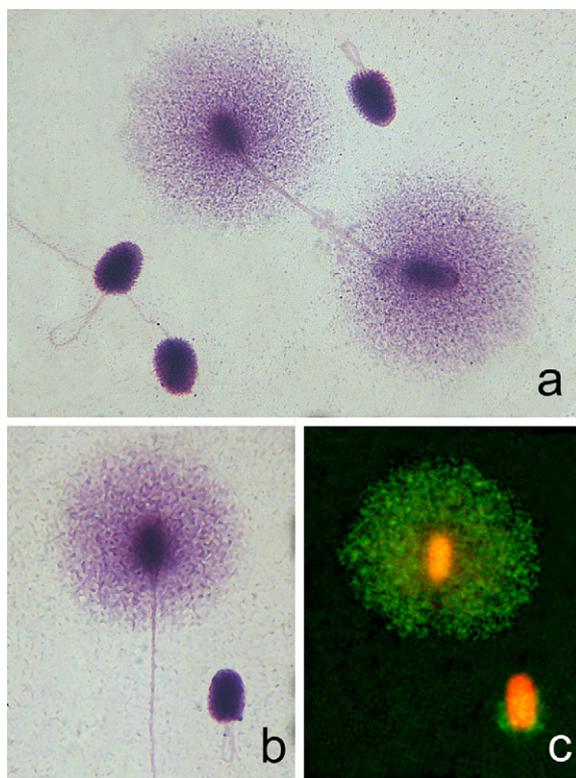


Fig. 1. Boar sperm cells processed with the Sperm-Sus-Halomax[®] kit and stained for bright field microscopy (a and b) or *in situ*-nick translated (c). In both cases, sperm cells with large halos of chromatin dispersion corresponds with sperm heads containing fragmented DNA while those showing absence of halos or minimal chromatin dispersion contains unfragmented DNA.

When undiluted and diluted semen samples were compared, sperm quality parameters analysed during 21 days of preservation at 15 °C were completely different (compare Fig. 2a and b).

Semen quality decreased faster in undiluted semen samples. This event was especially dramatic from day 0 to day 7 (Fig. 2a). On the contrary, diluted semen samples revealed a high quality of semen parameters up to day 11 of preservation (Fig. 2b). Undiluted semen showed low DFI during the first days of preservation, similar to those observed at the beginning of the experiment. From day 7 up to day 21 of the preservation period, a significant ($p < 0.037$) increase in the DFI was observed. Interestingly, and despite the fact that all boars presented a very low DFI at the onset of the experiment, an increase in DFI did not occur at the same time in all boars. However, the onset of DFI in each animal coincided with the loss of motility and the lowest levels of the other semen quality parameters (Fig. 2a). On the other hand, those samples diluted in ACROMAX[®] showed very low levels of DNA fragmentation during the entire preservation period in all boars (Fig. 2b).

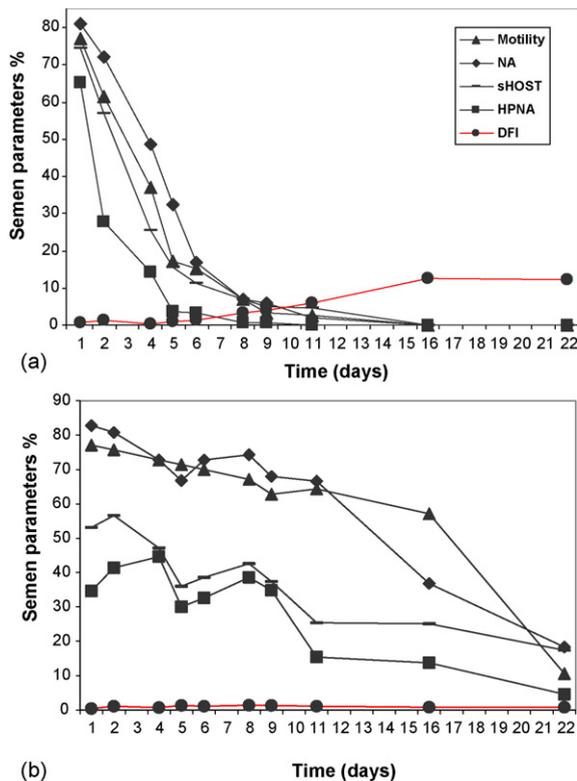


Fig. 2. Evolution of semen quality (NA: normal acrosomes, sHOST: short HOST, HPNA: sHOST positive-normal acrosome sperm cells) and sperm DNA fragmentation (DFI) in boar semen during 21 days at 15 °C in two different preservation conditions: (a) undiluted and (b) diluted (black dots: DNA fragmentation).

When the progression of DNA fragmentation during the preservation period is analysed in the undiluted samples, significant differences ($p < 0.001$) were found among boars (Fig. 3a). These differences were not shown in the samples diluted in ACROMAX[®] (Fig. 3b). The basal DFI was low and constant during the entire preservation period. When undiluted semen samples were assessed for DNA fragmentation, it was observed that boars with the highest levels of DFI at the beginning of the study, also showed the highest DFI at the end of the experiment (Fig. 3a).

The analysis of the correlation between all the semen parameters studied was made separately and compared to the data of undiluted and diluted semen. The results are summarized in Table 1.

Essentially, these results indicated that in undiluted semen, correlation coefficients were high and showed significant correlations among all the semen quality parameters compared. However, they decreased, although the differences remain significant, in diluted semen. Correlation coefficients between the DNA fragmentation level and the other semen quality parameters in undiluted semen fluctuated around $r = -0.40$ ($p < 0.001$). This correlation was not present when the parameters of diluted semen were included in the analysis.

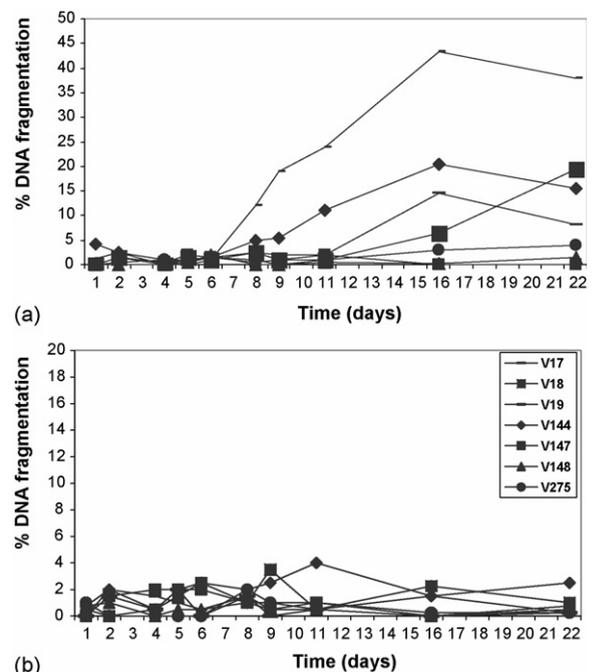


Fig. 3. Evolution of the sperm DNA fragmentation in semen from seven boars during 21 days at 15 °C in two different preservation conditions: (a) undiluted and (b) diluted.

Table 1

Correlation coefficients and *p*-value (parenthesis) between sperm quality parameters and DNA fragmentation in undiluted and diluted boar semen (Spearman rank order test, *n* = 70)

	NA	sHOST	HPNA	DFI
Undiluted semen				
Motility	0.92 (0.001)	0.94 (0.001)	0.92 (0.001)	−0.396 (0.001)
NA		0.90 (0.001)	0.89 (0.001)	−0.43 (0.001)
sHOST			0.93 (0.001)	−0.38 (0.001)
HPNA				−0.389 (0.001)
Diluted semen				
Motility	0.65 (0.001)	0.42 (0.001)	0.55 (0.001)	0.04 (0.7)
NA		0.44 (0.001)	0.53 (0.001)	−0.05 (0.6)
sHOST			0.78 (0.001)	0.03 (0.08)
HPNA				0.11 (0.3)

NA: normal acrosomes; DFI: DNA fragmentation.

4. Discussion

The results presented in this study shows two main important evidences: (i) DNA fragmentation is negatively correlated with standard parameters assessed for sperm quality evaluation and (ii) DNA fragmentation is a dynamic and modulable variable which depends on the conservation strategy and on the biological characteristics of each animal. Because a high incidence of sperm DNA fragmentation has been related with fertility and embryo viability in a series of species such as human [13], bull [13,14], boar [14–16] and stallion [17], it seems evident that a negligible evaluation of this variable is not prudent, especially in protocols where assisted reproduction is performed. However, the tangible reality is that routine evaluation of boar semen offers modest information about sperm chromatin integrity. Boar, and of course all animals included in assisted reproduction programs such as endangered species, are open research areas where the assessment of sperm DNA integrity is of paramount interest and deserves more basic research in the future. The fact, as demonstrated in the present experiment that sperm DNA fragmentation is dependent on the conservation strategy used and that it is not constant, opens interesting questions about the best way to preserve the sperm samples and the optimal time to use it. DFI of each individual has been prevented in diluted sperm samples during the entire experimental preservation period. In addition, diluted semen samples maintain a fine quality in other parameters during a longer period of storage at 15 °C. However, DFI in undiluted boar semen increases during the same preservation period while the rest of the parameters show a clear tendency to be of poor quality. The extended semen has been protected both against the effects of the *in vitro* aging

and against the mechanisms that lead to sperm DNA fragmentation.

The fact that the Halomax test is able to detect an increase in DFI when the sperm is not extended discards the possibility of it being a technical artefact and links the effect to inherent characteristics of the used extender. Effectively, it has been reported that extended boar semen shows an increase in DNA instability from day 0 to day 4 in some extenders [5], and recently Boe-Hansen et al. [18] have reported that increasing storage time of extended boar semen reduces sperm DNA integrity and this could be detected within the first 24 h of the experimental conditions. The presence of endogenous reactive oxygen species (ROS) could play an important role with the induction of membrane damage, which in turn, may affect the DNA integrity [7,19]. Additionally, the release of active proteolytic enzymes such as those contained in the acrosome, as well as the presence of active nucleases as those involved in the apoptotic process [20] may result in DNA damage. In fact, under our experimental conditions, we observe that, in undiluted semen samples, DNA fragmentation starts to increase when the sperm membranes are altered; a critical moment when enzymes could be released, cells turn permeable and the nuclei becomes more accessible to new attacks. These results are now being reanalysed in the light provided by the results of new ongoing experiments where we are considering the role that active quelants may play to block enzyme action.

Another noticeable tendency that derives from this experiment is the differential dynamics of the sperm DNA fragmentation among boars. Although all the selected animals have low DFI at the beginning of the experiment, boars with higher DFI produce the highest increase in DFI during the preservation period in absence

of the extender. On the contrary, those boars with lower DFI, do not undergo an increase of the same magnitude during the entire preservation period. This tendency is congruent with the results presented by Boe-Hansen et al. [18] who found some animals that do not give rise to damaged DNA as other animals do. This is a clear indication that different factors, inherent to the biology of each animal, are probably involved in the dynamics of sperm degradation, even under *para*-biological conditions of sperm conservation. Evidently, individual differences in sperm DNA fragmentation have been previously reported in boar [8,14,15] and, of course, they can be expected when different individuals are analysed. However, the dynamics of DFI observed in this study have different biological connotations. It seems that the DFI observed may hide a higher DNA damage, which is evidenced after the *in vitro* aging in undiluted semen. This effect, in some sense, paragon the “tip of the iceberg” effect such as was presented by Evenson et al. [15]. More than likely, we are visualising only a part of the DNA damage within our sample and as such as Boe-Hansen et al. [18] states “The presence of moribund sperm in an ejaculate increases the production of ROS – reactive oxygen species – which may affect the DNA integrity”. If ROS are involved or not in such effect still must be demonstrated. But the idea that affected DNA may accelerate the degradation process of unaffected cells is a good hypothesis to be tested with new experimental strategies. In the context of our experimental approach, a similar situation could explain why the DNA damage detected within those animals which show the highest DFI, shows an increasing tendency to, in time, be close to exponential and remain being lineal where the DFI is the lowest (see Fig. 3a). Ongoing experiments would throw some light on this point.

We have observed a significant negative and moderate correlation between the percentage of sperm DNA fragmentation and other sperm quality parameters studied in undiluted semen. With regard to sperm motility, this result is in agreement with Giwercman et al. [21], who reported that in humans a significant correlation exists between sperm motility and SCSA parameters. This kind of relationship between semen quality and DNA status implies that there is a subpopulation with a good semen motility, acrosomal morphology and membrane functionality that also has a low DFI. At the same time there are also other sperm subpopulations with high standard parameters that exhibit a high DFI. The goal we must pursue is to detect those ejaculates with a predominantly low DNA fragmentation subpopulation and the best quality for standard parameters. This fact reinforces the idea of

including sperm DNA fragmentation analysis in every ejaculate evaluation. In addition, the next step in boars should be to establish a threshold value to decide if the DFI is suitable to produce abundant and healthy offspring. While in humans, Evenson suggests that the threshold values are around 30%, therefore in boars we may assume, for research purposes and according to the results presented by Rybar et al. [14], that values of DFI lower than 15–20% could be considered as a reasonable DFI with no dramatic incidence in the female pregnancy. Since we have now available technology to further the study of DNA fragmentation in boar sperm samples in standard laboratories, we suggest assuming these values as reasonable and testable to advance in this field of research.

In summary, the knowledge of the level of DNA fragmentation in boar semen represents an essential input in a routine semen analysis in order to gain information about the fertility potential of the ejaculate. Additionally, and as demonstrated in this experiment, given that DFI could differentially fluctuate under different conservation strategies, this parameter seems to be a good candidate for study under other conservation strategies such as frozen sperm samples.

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