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DNA fragmentation in frozen sperm of *Equus asinus*: Zamorano-Leonés, a breed at risk of extinction

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Abstract

The dynamics of sperm DNA fragmentation (sDF) and sperm viability were analyzed in frozen–thawed sperm samples of *Equus asinus* (Zamorano-Leonés), a breed at risk of extinction. Sperm DNA fragmentation was assessed using an adaptation of the sperm chromatin dispersion test developed for stallions in five different frozen samples. Sperm were thawed and incubated at different temperatures (37 °C, 25 °C, and 4 °C) and sDF was assessed at different times and compared. The mean sDF after thawing at the beginning of the experiment was 18.20 ± 14.77% and did not differ significantly from the results of a neutral comet assay (22.0 ± 19.34%). The tendency in the sDF of all donkeys indicated that sperm DNA is more sensitive to breakage when incubated at 37 °C than when incubated at 25 °C or 4 °C. Interestingly, the tendency was not the same when different animals were compared, and differences in sDF dynamics were established among individuals. sDF correlated negatively with sperm viability in some individuals but not in others. From a conservation perspective, sDF analysis may offer a new way to assess sperm quality in endangered breeds in order to identify and select the best semen samples for artificial reproduction purposes. In particular, we recommend for artificial insemination the use of semen samples with a slow increase in sDF with time after thawing.

Keywords: Male fertility; DNA fragmentation; Reproduction; Sperm chromatin dispersion; *Equus asinus*; Endangered species

1. Introduction

The history and geographic distribution of the donkey are particularly interesting because, since its domestication about 5000 years ago in either Egypt or Mesopotamia [1] it has always had economic importance linked to the activities of human populations. However, in the last century, these animals have had no relevance in industrially developed countries and its ancestral role is only retained in some poorly developed cultures. These populations act as genetic reservoirs for the gene pool of these animals. On the Iberian Peninsula, as in other northwestern regions of the Mediterranean Basin, populations of donkeys are at serious risk of extinction. On the Iberian Peninsula, this situation might be even more critical because the genetic variation of the donkey is linked to five different breeds (Andaluza, Catalana, Mallorquina, Encartaciones, and Zamorano-Leonés), all of which have suffered a rapid and substantial decrease in population size and consequently in their genetic variability during the last 100 years [2]. This reduction is so dramatic that

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in certain breeds, no more than 50 animals are considered of high genetic value. These donkeys are included in the United Nations Food and Agricultural Organization (FAO) list of domestic animals to be preserved (FAO, DAD-IS http://fao.org/dad-is). The population of the Zamorano-Leonés breed has been reduced to only 1200 animals, of which only 200 females and 35 males are considered of genetic value. In this context, efforts to preserve the heritable gene pool must be made to reduce, as much as possible, the risk of reaching a no-return point in the reproduction of these animals.

Sperm quality is considered a parameter of crucial importance in assisted reproduction within conservation programs for species at risk of extinction. Disturbances in the organization of the genomic material through inadequate sperm handling (when using cooled or frozen–thawed sperm samples) are detrimental for fertilization and successful embryonic development. For example, severe changes in temperature are a common feature of the semen preservation protocols used in assisted reproduction, which are not a biological characteristic to which the spermatozoan has become adapted. In fact, temperature excursion episodes, or changes in the constant temperature required by the sperm in its passage from the male to the female, have deleterious effects on spermatozoan survival and lower conception rates after artificial insemination (AI) [3–5]. This reduction in fertilizing ability after thawing is also associated with a reduced rate of sperm motility and the production of morphological abnormalities [5,6]. Cryopreservation may also cause damage to the sperm plasma membrane [4], with a subsequent increase in oxidative stress. The *de novo* formation of free radical oxygen species results in a raised basal level of DNA damage [7–9]. Although preservation of sperm membrane functions and motility are important measures of sperm quality, they may not reveal the inherent pathology associated with changes in the orthodox sperm DNA configuration. Recently, the analysis of sperm DNA fragmentation (sDF) and chromatin stability has helped to explain the infertility of individuals with apparent normal seminograms [10,11]. This approach is particularly interesting because DNA fragmentation correlates poorly with classical parameters of semen quality [12]. Thus, the omission of DNA damage assessment from a seminal analysis would result in a significant proportion of male-factor infertility remaining undetected.

The aims of this study were to analyze the prevalence and dynamics of sDF and sperm viability in frozen–thawed sperm samples of *Equus asinus* (Zamorano-Leonés breed), using an experimental model in which different temperature ramps were applied after thawing. The general idea was to understand the sensitivity of sperm DNA to degradation. Although this is the first report of sDF in donkeys, the broad intention of this and other ongoing investigations is to improve the techniques and methodologies for sperm handling after thawing in these animals.

2. Material and methods

2.1. Semen

Frozen spermatozoa from five different donkeys located at the Depósito de Sementales de Ávila Fondo de Explotacion de los Sevicios de Cria Caballar y Remonta (FESCCR) in Spain were analyzed. The animals ranged in age from two to four years, and were currently kept under controlled conditions of feeding, stabling, and physical exercise. All these animals were considered of high genetic value and were healthy at the time of sperm collection.

Semen samples were collected with an artificial vagina and cryopreserved according to conventional methods [13]. All the animals included in this analysis exhibited seminal characteristics within the normal ranges established for these animals.

The extender used for storage was INRA-96 (IMV Technologies, Paris, France). Immediately after collection, the semen was diluted to a final concentration of 50×10^6 mL^{-1} and cooled from 34 °C to 22 °C by allowing samples to reach room temperature over a period of approximately 60 min. The semen sample was then cooled to 5 °C by placing it in a water jacket in a standard refrigerator. This represented a cooling rate of approximately −0.6 °C/min. For the sperm cryopreservation procedure, the ejaculate was centrifuged at $300 \times g$ for 5 min, the supernatant was removed, and the pellet was resuspended in a skim milk–egg yolk (2%) Tris–glycerol-based extender [13]. The diluted mixtures were cooled from 34 °C to 4 °C at a rate of −0.5 °C/min. Once at 4 °C, the diluted sample was packaged into precooled (4 °C) 0.5 mL polyvinylchloride straws (IMV International, St Paul, MN, USA) and frozen in liquid nitrogen vapor (4 cm above the surface) for 15 min, after which they were plunged directly into liquid nitrogen (−196 °C) for storage.

2.2. Experimental design

All samples were thawed by immersion in a 37 °C water bath for 30 s and diluted at 10–15 × 10^6 spermatozoa/mL in a skim milk–egg yolk sperm

extender (INRA-96). Different aliquots, including replicates, were thawed and incubated at 4 °C, 25 °C, or 37 °C for 48 h taking samples for sDF assessing at 1 h, 4 h, 6 h, 24 h, and 48 h. For individual sDF assessments, replicates were used as the internal controls. The degree of sperm DNA damage in each sample was calculated using the sperm DNA fragmentation index (sDFI: ratio of fragmented versus total spermatozoa in the analyzed sample, expressed as a percentage). The samples were assessed for sDF just after they were thawed, when the sDFI was considered as T0 (basal sDFI), and after 1 h (T1), T4, T6, T24, and T48.

2.3. Sperm viability assessment

Sperm viability was assessed using a supravital stain based on the red/green emission of two fluorescent dyes, acridine orange (AO) and propidium iodide (PI), respectively (Fig. 1; Duo-Vital Kit, ChromaCell SL, Madrid, Spain). Color emission differences were automatically scored using a computer-assisted semen analysis system fitted with a specific module for this purpose (SCA-Vital Module, Microptic SL, Barcelona, Spain).

2.4. sDF assessment

sDF was analyzed using the Sperm-Halomax® kit (Halotech-DNA; Madrid, Spain). The kit series that we used was specifically designed for sDF evaluation in stallions [14], but produced consistent results in donkeys [15]. This methodology is based on the sperm chromatin dispersion (SCD) test and has been used to assess sDF in a range of mammalian species, including humans [16,17], pig [18,19], bull [20,21], and other species [22]. For each experiment, 25 μL of diluted spermatozoa (10–15 × 10^6 spermatozoa/mL) were added to a vial containing low-melting-point agarose and mixed. A small aliquot of the agarose–sperm mixture (10 μL) was then spread onto pretreated slides (provided in the Halomax® kit), covered with a glass coverslip, and placed in a refrigerator on a cold metallic plate for 5 min. Following solidification, the cover slip was carefully removed and the 'sperm–gel' slide placed horizontally for 5 min in 10 mL of the lysing solution provided in the Halomax® kit. The sperm–gel preparation was then washed in distilled water (dH_20), before dehydration in a sequential series of 70%, 90%, and 100% ethanol.

DNA damage after the SCD test was visualized with fluorescence microscopy. In this case, the dual emission DNA–red/protein–green fluorochrome combination provided in the Halomax® kit gives sufficient information for manual scoring by allowing the ready discrimination of sperm heads containing either fragmented DNA (large halos of chromatin dispersion) or unfragmented DNA (small halos or no halos of chromatin dispersion; Fig. 3a). The sperm flagellum and those proteins that resist lysis in the SCD protocol showed strong green fluorescence, which contrasts with the red emission associated with the DNA. For automatic scoring, the dehydrated slides were stained only with the DNA-directed fluorochrome. Fluorescence microscopy was used for automatic sperm counting using the Sperm Class Analyzer (SCA) DNA module (Microptic SL). The SCA software discriminates spermatozoa that show a small and peripheral halo of chromatin dispersion from those that exhibit a large and "spotty" halo of chromatin dispersion. This software was coupled to a Leica DMLA motorized fluorescence microscope (Leica SA, Germany) controlled with Leica-based software for automatic scanning and image digitalization of each slide. For automatic evaluation procedures, a 20× magnification lens was used. A minimum of 300 spermatozoa per sample were counted. The proportion of spermatozoa with fragmented DNA was then calculated as a percentage of the total spermatozoa scored.

Because the SCD kits were used here for the first time with donkey sperm, sDF was assessed in parallel using the direct incorporation of labeled nucleotides in putative DNA breaks using in situ DNA nick translation (ISNT) and the comet assay. To confirm that the SCD test used in this study appropriately identified DNA damage, the direct incorporation of labeled nucleotides using Klenow polymerase was measured as previously described by Enciso et al. [18] for boar spermatozoa. This method directly labels DNA breaks, which can be observed in spermatozoa as halos of chromatin dispersion (Fig. 3b). DNA damage was also assessed using both neutral comet assays for double-stranded DNA analysis and alkaline comet assays to assess single-stranded DNA breaks. The alkaline comet assay was performed according to Singh et al. [23]. Sperm–gel slides were prepared as described previously for the SCD test. The slides were immersed in two lysis solutions for 30 min each, at room temperature. The first solution containing 0.4 M Tris-HCl (pH 7.5), 0.8 M DTT and 1% SDS) and the second one containing 0.4 M Tris-HCl (pH 7.5), 0.8 M DTT, 1% SDS, and 0.05 M EDTA. The slides were washed in 1× TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) buffer

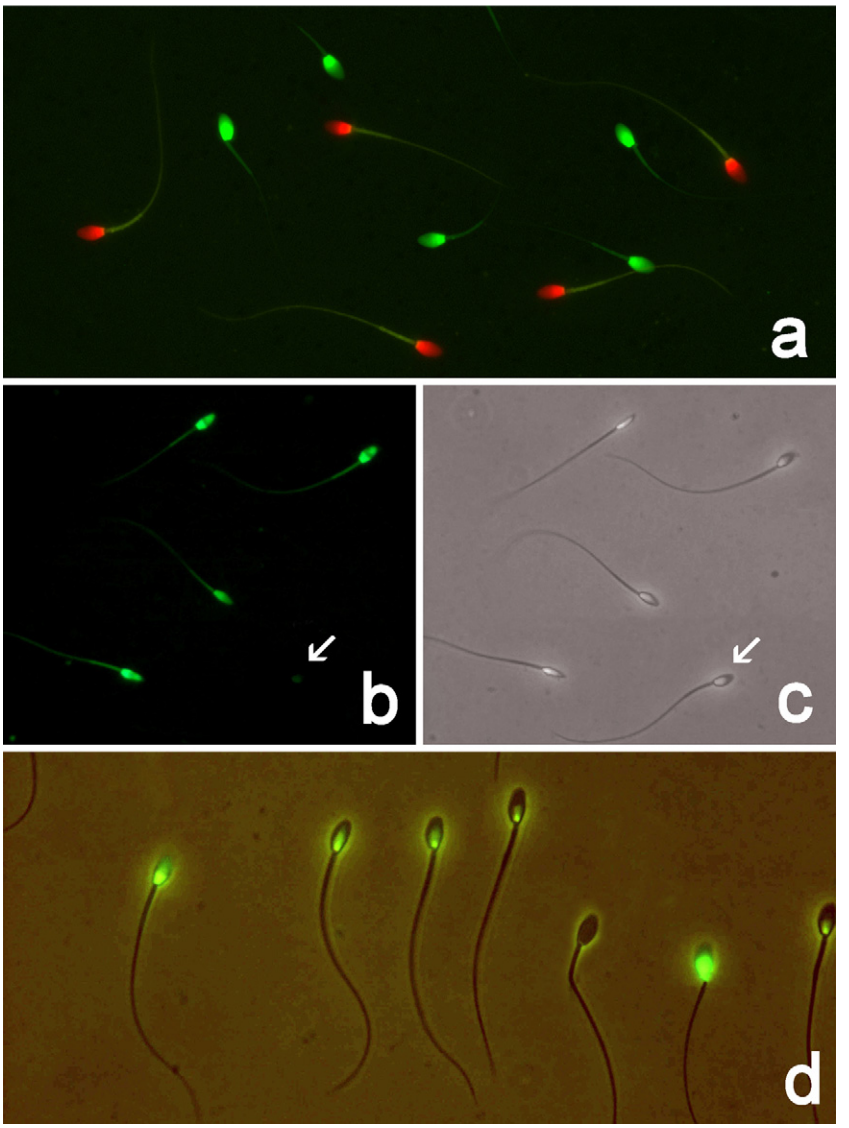

Fig. 1. Sperm cells stained with acridine orange (AO) and propidium iodide (PI) ((a) nonviable sperm in red; viable sperm in green). Sperm stained with AO were analyzed independently with fluorescence microscopy (b) and phase contrast microscopy (c), and then analyzed simultaneously (d). The arrow indicates a viable sperm cell not detected with AO. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

for 10 min and then treated with fresh alkaline solution (0.03 M NaOH, 1 M NaCl) for 2.5 min to cleave the alkali-labile sites (ALS). The slides were placed horizontally in an electrophoresis tray, which was filled with fresh alkaline electrophoresis solution (0.03 M NaOH, pH 13). Electrophoresis was conducted at 20 V for 20 min at room temperature. After electrophoresis, the slides were gently removed from the tray and washed with neutralizing buffer (0.4 M Tris–HCl, pH 7.5) for 5 min. The slides were washed in dH_2O for 5 min and then dehydrated in a sequential series of 70%, 90%, and 100% ethanol.

For the neutral comet assay, the slides were treated with the lysis solutions described above and electrophoresis was performed in neutral buffer (1× TBE) at 20 V for 20 min. Finally, the slides were stained with PI (20 μg/mL) and visualized under a fluorescence microscope. At least 100 cells per experiment were analyzed at 600× magnification.

2.5. Image analysis and statistics

Image analysis was performed to compare the fluorescence intensities obtained after ISNT. For this

purpose, integrated density (a correlation between area and fluorescence intensity after background subtraction) was calculated using the Leica Q-Win image analysis software.

All data were analyzed with Microsoft Excel, and statistical comparisons were made with StatGraphics Plus 5.1 (Manugistics Group Inc., Rockville, MO, USA). The association between the sDFI and the viability index was analyzed with the Spearman correlation. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Standard seminal characteristics

The standard sperm characteristics of volume, concentration, total motility, progressive motility, hyposmotic test, and abnormal morphology were assessed for all five donkeys before the sperm samples were frozen (D1 to D5; Table 1).

3.2. Dynamics of sperm viability

The sperm viability test used in this study was based on AO, a fluorochrome that stains DNA irrespective of the membrane status, and PI, which only stains DNA if the membrane is damaged (Fig. 1a; nonviable sperm are red).

It is interesting that when this test was used in donkeys, in some case the level of sperm viability observed at T0 was lower than that observed after sperm incubation at T1 (see Fig. 2; continued line for vitality). To understand this discrepancy, a parallel experiment was conducted in which only AO was used as the primary DNA dye. The slides stained with AO were analyzed independently with fluorescence microscopy and phase contrast (Fig. 1b, and c respectively), or simultaneously under phase contrast and fluorescence microscopy (Fig. 1d). When the first analysis was performed, some nuclei did not fluoresce, but were visible under phase contrast microscopy (compare Fig. 1b with c). When the analysis was performed using simultaneous epifluorescence excitation and phase contrast microscopy, the results were the same, but the images could be observed and captured in one frame (Fig. 1d). In a similar analysis at T1, the vitality results were those expected based on the rationale of the test, and all nuclei fluoresced either red or green. Our conclusion was that at T0, we underestimated the frequency of viable nuclei because AO was unable to reach the DNA. A comparison of the increasing values for sperm viability from T0 to T1 allowed us to calculate that around 10% of viable nuclei at T0 were not fluorescent. We thus distinguished three main subpopulations of sperm at T0 in donkeys (viable, green; nonviable, red; and supraviable, no staining). In terms of orthodox viability assessment, only this system of nuclei classification (when the viable and supraviable groups were included in one class) produces valid values at T0 or alternatively after long incubations.

The levels of sperm viability obtained after sperm thawing were generally low (even when corrected) compared with those reported for chilled samples. In these thawed samples, no values higher than 40% viability were observed. Sperm viability decreased dramatically after 4 h incubation at 37 °C, and viability was completely lost after 24 h (Fig. 2; continued line for viability).

Table 1
Standard seminal characteristics of five different donkeys before the semen samples were frozen

Seminal characteristics	Donkeys				
	D1	D2	D3	D4	D5
Volume (mL)	75	65	88	56	54
Concentration 10^6/mL	345	456	453	91	127
Total motility (%)	70	73	70	85	60
Progressive motility (%)	60	61	55	75	52
Hiposmotic test (+) (%)	75	58	64	71	62
Normal morphology (%)	85	90	90	91	84
Abnormal morphology (%)	15	10	10	9	16
Viability (%)	33	19	21	17	12
sDFI (%)	6	22	14	7	42
Seminal quality	0.69	0.61	0.63	0.69	0.52

Sperm quality: $\Sigma Vi/100n$, where "Vi" is an independent variable and "n" is the number of variables. The independent variables included in this formula were progressive motility, hyposmotic test (+), normal morphology, viability, and sDFI. sDFI: sperm DNA fragmentation index. D1 to D5: donkey 1 to donkey 5.

3.3. Sperm morphology after the SCD test

Semen samples processed with the SCD test for DNA fragmentation assessment could be separated into two different sperm categories according with the morphology obtained. Some nuclei remained compacted or displayed small halos of chromatin dispersion, which should correspond to spermatozoa containing unfragmented DNA, whereas other sperm nuclei exhibited large halos of chromatin diffusion with a stellar appearance (Fig. 3a), which should contain fragmented DNA. However, after selective DNA labeling using nucleotide incorporation in DNA breaks, both types of nuclei were positively labeled (Fig. 3b).

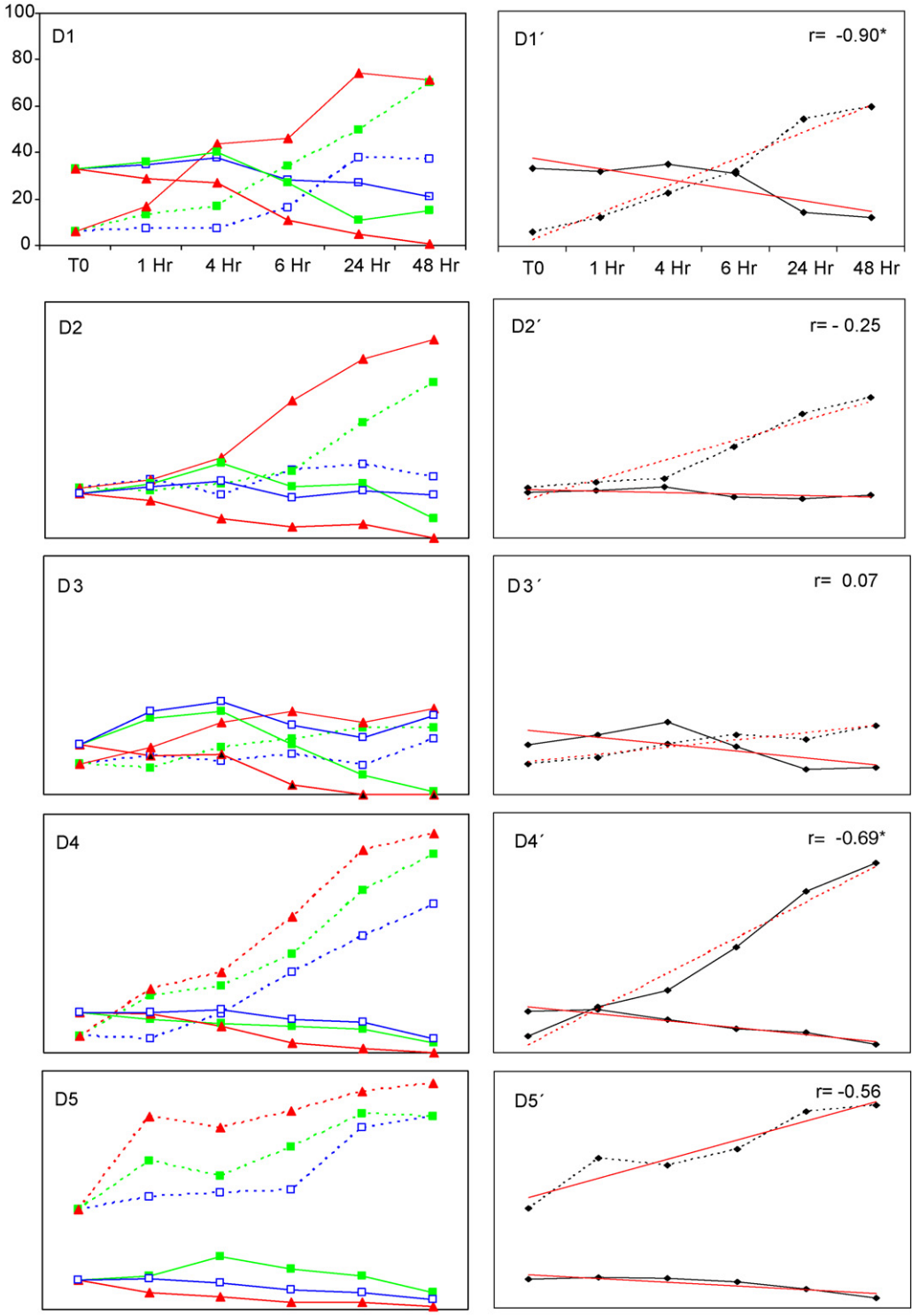

Fig. 2. Dynamics of sDF (dotted line) and viability index (continued line) of the five donkeys studied (D1–D5). Red line = incubation at 37 °C, green line = incubation at 25 °C, blue line = incubation at 4 °C. Regression analysis between sDF, and viability index (D1′–D5′) (white line) and, regression line (red line). (*) Significant correlation ($P \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Nevertheless, the fluorescence intensity of the stellular nuclei was 7.4 times greater (44,181 ± 9566) than that of the nuclei considered to have normal DNA status (5,926 ± 606). This high level of background was unexpected and is also high compared with the results obtained for other species. Halos larger than those observed after direct staining with a DNA-specific fluorochrome were always recovered after ISNT (compare Fig. 3a with b). This occurred because DNA polymerases incorporate labeled nucleotides with high efficiency, even in small DNA fragments that are not large enough to be visualized with direct fluorescence. When double fluorescence staining was used (red emission for DNA and green emission for proteins), the flagellum was clearly separate from the chromatin. Halos of stellular dispersed chromatin consisted mainly of DNA and no traces of proteins were detected (Fig. 3a). Sperm heads containing fragmented DNA showed smaller protein cores than those in sperm heads with unfragmented DNA.

Because of the high level of background in cells with a small halo or cells in which the halo was absent, the use of alternative experimental strategies to analyze sDF in detail is recommended. These results call into question any direct association between the presence of large halos of chromatin dispersion and sperm containing fragmented DNA. The comet assay was used to resolve this ambiguity.

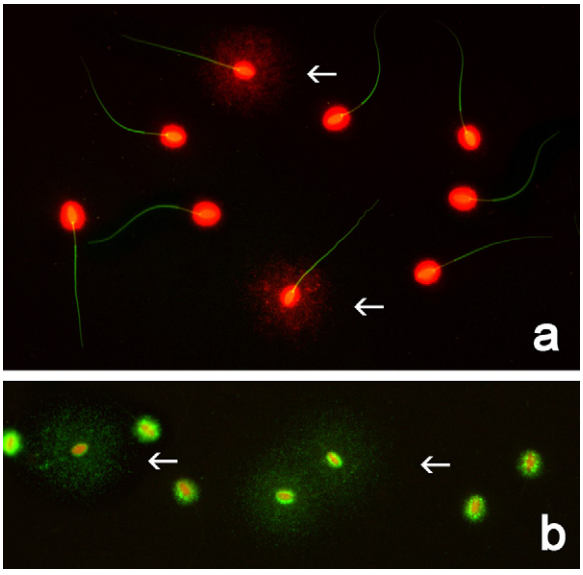

Fig. 3. Fluorescence microscopic analysis of DNA fragmentation in donkey sperm following the SCD test. (a) SCD-processed sample with dual fluorescence; some nuclei remained compacted or displayed small halos of chromatin dispersion, other showed large halos of chromatin dispersion contained fragmented DNA (arrows) and (b) sperm after SCD and *in situ* nick translation; both types of nuclei were receptive to modified nucleotides after treatment with polymerase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.4. Comet assay

When a neutral comet assay (Fig. 4a) was performed to analyze sDF, the results did not differ significantly ($P > 0.05$) from the sDFI obtained with the SCD test, according to the criterion "large halo = fragmented nuclear DNA" (Table 2). Different individuals exhibited similar values for sDFI, although the neutral comet assay tended to produce slightly higher values in general estimates of sDFI. Interestingly, when a denaturing comet assay was used, 100% of the sperm cells produced a visible comet (Fig. 4b and Table 2). The amount of DNA in the tail varied from one sperm head to another, but traces of DNA displacement were always observed.

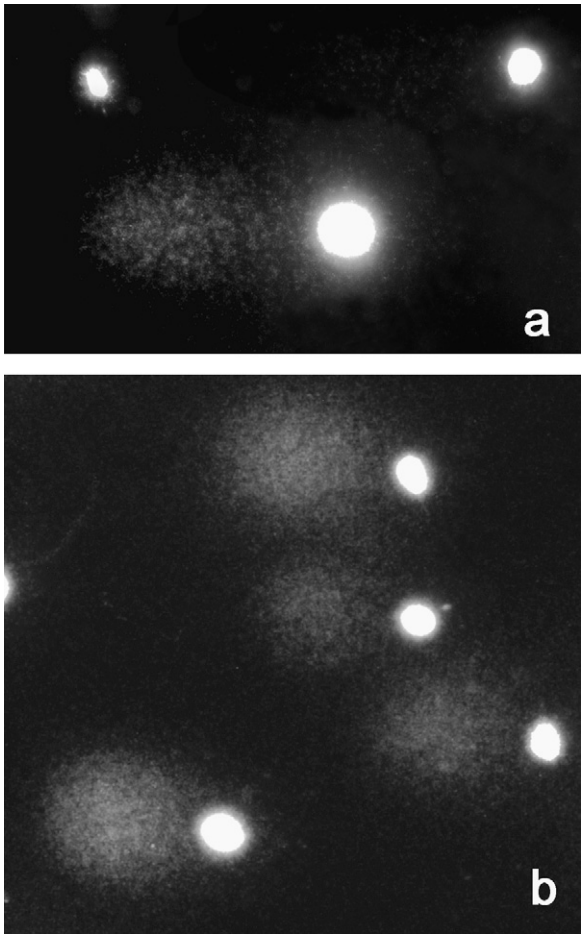

Fig. 4. Donkey sperm cells after neutral (a) and alkaline (b) comet assays.

Table 2
Basal values for sDF, the neutral and alkaline comet assays, ISNT, and the viability of the frozen sperm of the five donkeys studied

Donkey	SCD (%)	N-Comet (%)	D-Comet (%)	ISNT (%) with halo/without halo	Viability (%)
D1	6	8	100	5/95	33
D2	22	26	100	22/78	19
D3	14	14	100	15/85	21
D4	7	8	100	7/93	17
D5	42	54	100	45/55	12
X ± SD	18.20 ± 14.77	22.0 ± 19.34	100 ± 0	18.80 ± 16.13/81.20 ± 16.13	20.40 ± 7.80
Range	6–42	8–54	100–100	5–45/78–95	12–33

SCD, sperm chromatin dispersion; N-Comet, neutral comet; D-Comet, denaturing comet; ISNT, *in situ* DNA nick translation.

Because the results obtained for sDF using the SCD test and the neutral comet assay were not significantly different, an analysis of the dynamics of sDF under different experimental conditions was performed using the SCD test, because technically it is much less expensive and less time consuming.

3.5. Dynamics of sDF

The basal (T0) sDFI values for the five animals studied are presented in Table 2. When the SCD test was used, the mean sDFI was 18.20 ± 14.77 and as previously stated, did not differ significantly from the results obtained with the neutral comet assay (22.0 ± 19.34). The range of distribution of sDFI was wide among the five animals included in the analysis (Table 2), including one animal (D1) that exhibited a relatively low level of sDF (around 6%), whereas in another animal (D5), this value was close to 45%.

The dynamics of sDF after thawing and incubation at different temperatures are presented in Fig. 2 (dotted lines in Fig. 2; D1–D5). The common tendency of sDF in all donkeys indicated that the sperm DNA was more sensitive to DNA breakage when incubated at 37 °C than when incubated at 25 °C or 4 °C (compare dotted lines in Fig. 2). However, it is interesting to note that the tendency of sDF to increase with temperature and time was not the same in different animals. When sDF was compared with sperm viability (Fig. 2; D1′–D5′), D1′ and D4′ showed significant negative correlations ($r = -0.90$, and $r = -0.69$, respectively), whereas D-2′, D-3′, and D-5′ did not show any significant correlation ($r = -0.25$, $r = 0.07$, and $r = -0.56$, respectively).

Regression analysis of the sDFI of frozen semen samples did not yield significant differences between the slopes of individual donkeys when different incubation temperatures were compared (Fig. 5). Only D4 showed a different slope for sDFI when the incubation was performed at 4 °C (Fig. 5). The average regression coefficient (b) was 1.43, indicating that sDFI increased with time at the same rate in all individuals (Fig. 5). However, the intercepts of the regression equations showed significant variation, signifying that each individual semen sample had different sDF dynamics; the average regression coefficient (b) was 1.49. When all the results for sperm incubated at 37 °C were analyzed, three clear tendencies in the dynamics of sDF were identified among individuals. D5 showed a high basal sDFI, which reached high values rapidly, whereas the other four animals had almost identical basal sDFIs, thus establishing two groups. One of these groups includes donkeys D2, D3, and D4, which had similar slopes for sDF dynamics. D1 was clearly separate from the other two groups in having a lower rate of sDF increase.

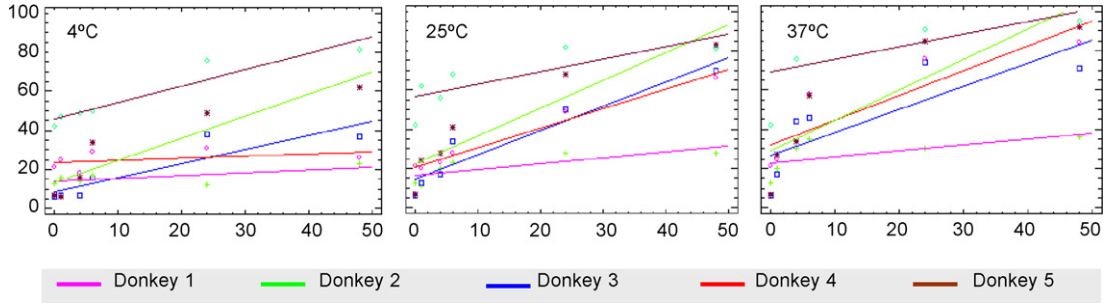

Fig. 5. Multiple regression analysis of sDFI at temperatures of 4 °C, 25 °C, and 37 °C in the five donkeys studied. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

The scant information available regarding the assessment of sperm quality in Spanish breeds of donkeys is not consistent with the risk of extinction of these populations. Whereas the semen characteristics of some males have been analyzed in the Catalonian [24] and Zamorano–Leonés breeds [25], information about their genetic variability is much more abundant [26,27]. We wish to call attention to future programs to preserve frozen sperm samples from these animals. According to the results of this study, sperm DNA quality may be seriously compromised after thawing. Furthermore, the time required for the sperm to reach the oocyte in AI might be reduced by correct management of the temperature excursion episodes experienced by the sperm during handling or storage. The level of sperm DNA damage is also much more intense at a biological temperature of 37 °C.

Our results in this study indicate that sDF in donkeys is a new parameter with which to assess sperm quality, and may reveal interesting differences when various animals are compared. These differences are more noticeable than those apparent with standard sperm parameter evaluation. If we assume that sDF has a similar relevance to the fertility of donkeys as has been demonstrated in other mammalian species [28], sDFI analysis of these animals should be considered in the final decision to use a specific semen sample for AI. This option may be critical in reproduction programs for endangered species, where maximum control of the male factor is necessary to ensure a fully successful pregnancy. It can be concluded from the results of this study that the semen samples of D1 and D4 are the best candidates for use in AI, especially because D1 had both the lowest level of sDF at T0 and the smallest increase in this parameter with time relative to those of the other animals.

By considering the parameters of sperm quality (viability and fragmentation) for each animal before the samples were frozen and after thawing, we estimated the sperm quality (sQ) by integrating the different values in a single value ranging from 0–1 according to the formula $sQ = \Sigma Vi/100n$, where Vi is an independent variable and n is the number of variables included. The independent variables included in this case were: progressive motility, hyposmotic test (+), normal morphology, viability, and sDFI (Table 1). Interestingly, after the integration of all these data, D1 and D4 showed the highest values (0.69) when compared with those of the other animals; they also exhibited the lowest levels of sDF. These characteristics and the level of sperm viability in these two animals were within the ranges expected for the rest of the animals, but their degree of sDF with time was significantly lower. Under these circumstances, greater survival in the female tract would be expected of the sperm from these animals. The sperm of D5 should be discarded for AI and those of the other two animals probably represent the expected average sDF dynamics for donkeys. In stallions comparable clusters of individuals were established after a similar experimental approach [14].

As has been demonstrated in the spermatozoa of other animals, such as the ram [29], boar [30], bull [21], and humans [31], we can assume that DNA damage is not a direct effect of freezing. In fact, D1 and D4 showed a very low level of sDF, probably very similar to that exhibited by this sample before thawing. This DNA behavior as has been observed when fresh and frozen samples of other species. However, the stability of the DNA molecule after freeze–thawing, during the first minutes of thawing, is not consistent with the decrease observed in other parameters, such as sperm viability or sperm motility. These suggest that DNA damage following chilling or cryopreservation is likely to be an indirect consequence of the stressors associated with changes in temperature, osmolarity, and plasma membrane instability. This could be why a good or partial negative correlation was observed in all the samples analyzed in this experiment when sDF was compared with the dynamics of sperm viability. The loss of membrane integrity can be considered a pathological process because it can result in the uncontrolled release of active enzymes (proteases, among others, from the acrosome). This can lead to autodestructive necrotic processes or alternatively to the activation of apoptotic pathways, including the oxidative stress that result in fragmented DNA [32–34]. Furthermore, the shortened longevity of sperm may play an important role in fertilization when semen is placed in the cervix of the female selected for insemination. If this reduced longevity is an effect of sperm handling, intrauterine insemination or conscientious estrus synchronization could help to circumvent this problem in these cases. These alternatives must be seriously considered in species at risk of extinction, especially in species where males of high genetic value are scarce. The selection of semen samples with delayed sDF, high estrus synchronization, and laparoscopy-assisted insemination could improve pregnancy rates.

In addition to establishing the importance of assessing sDF in these particular animals, we serendipitously found two particular characteristics of donkey sperm that recommend the reanalysis of this material in

more detail. One involves the behavior of the sperm membrane in relation to the permeability of AO, a fluorochrome that has been used in combination with PI to assess viability in a large variety of cells [35–37]. The slow penetration of AO into the donkey sperm is characteristic of these animals. Similar trials using human, goat, dog, ram, or stallion sperm have not produced this effect; all these sperm samples are rapidly stained after exposure to the AO/PI fluorochrome combination. This is clear evidence that the composition/structure of the sperm membranes of donkeys is substantially different from those of their relatives, including stallions.

Secondly, the assumption that the level of DNA labeling in sperm after ISNT with DNA polymerase reflects the degree of unfragmented DNA must be analyzed in more detail. The unexpected behavior of donkey chromatin could be indicative of the presence of constitutive DNA nicks in the sperm. The observed phenomenon is wholly consistent with vast numbers of single-stranded DNA motifs, producing 100% DNA migration in the comet assay under DNA-denaturing alkaline conditions. Although the presence of alkali labile sites (ALS) are not easily explained, it seems that there is a good correlation between the presence of ALS in the sperm DNA and the strong labeling of sperm by techniques that demonstrate the presence of DNA breaks. ALS could be interpreted as DNA modifications that can be transformed into DNA breaks or single-stranded DNA motifs by relatively weak alkaline solutions. Our group has reported a high frequency of constitutive ALS in major mouse satellite DNA located in the pericentromeric chromatin regions in somatic cell lines [38], and in human leukocytes, specifically within the pericentromeric 5 bp classical satellite DNA sequence areas, as well as in the constitutive heterochromatic Yqh region [39]. In human sperm cells, the whole genome is enriched in constitutive ALS [40,41] compared with somatic cells. Recently, the differential presence of ALS has been reported in sperm and somatic cells of boars and rams [42]. Ongoing investigation of this phenomenon should throw some light on this motivating observation. However, the behavior of donkey sperm chromatin in terms of ISNT reported in this study has no parallel in the literature and may provide a good model with which to analyze these chromatin conformations in sperm cells. It is possible that this effect is produced by a defective method of freezing the sperm samples. Ongoing experiments with fresh sperm samples should clarify this particular issue.

In conclusion, from a conservation point of view, as we try to preserve the gene pool still available in the different Iberian breeds of donkey, we encourage the development of novel strategies for assessing improvements in sperm longevity and DNA quality after thawing. The particular structure of the sperm chromatin of the donkey exhibits a large proportion of unorthodox DNA configurations in frozen sperm samples. Therefore, it could constitute a good model with which to understand sperm chromatin structure or at least to develop new models with which to analyze it. By corollary, this could be another reason to fight, in research terms, for the genetic patrimony of these species.

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References

[1] Littauer MA, Crouwel JH. Wheeled vehicles and ridden animals in the ancient near east. Leiden and Köln: Brill; 1979.
[2] Jordana J, Folch P. La Raza Asnal Catalana: Programa de Conservación y Mejora de una Población en Peligro de Extinción. Arch Zootec 1998;47:403–9.
[3] Ritar AJ, Salamon S. Fertility and frozen–thawed semen of Angora goat. Aust J Biol Sci 1983;36:49–56.
[4] Parks J, Graham J. Effects of cryopreservation procedures on sperm membranes. Theriogenology 1992;38:209–22.
[5] Holt WV. Basic aspects of frozen storage of semen. Anim Reprod Sci 2000;62:3–22.
[6] Hammerstedt R, Graham J, Nolan J. Cryopreservation of mammalian sperm: what we ask them to survive. J Androl 1990;11:73–88.
[7] Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. Hum Reprod 1998;13:1429–36.
[8] Chatterjee S, Gagnon C. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol Reprod Dev 2001;59:451–8.
[9] Baumber J, Ball BA, Linfor JJ, Meyers SA. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J Androl 2003;24:621–8.
[10] Sergerie M, Laforest G, Bujan L, Bissonnette F, Bleau G. Sperm DNA fragmentation: threshold value in male fertility. Hum Reprod 2005;20:3446–51.
[11] Shafik A, Shafik AA, Shafik I, El Sibai O. Sperm DNA fragmentation. Arch Androl 2006;52:197–208.
[12] Giwercman A, Richthoff J, Hjøllund H, Bonde JP, Jepson K, Frohm B, Spano M. Correlation between sperm motility and sperm chromatin structure assay parameters. Fertil Steril 2003;80:1404–12.

[13] Heitland AV, Jasko DJ, Squires EL, Graham JK, Pickett BW, Hamilton C. Factors affecting motion characteristics of frozen–thawed stallion spermatozoa. Equine Vet J 1996;28:47–53.
[14] López-Fernández C, Crespo F, Arroyo F, Fernández JL, Arana P, Jonhston SD, Gosálvez J. Dynamics of sperm DNA fragmentation in domestic animals. II. The stallion. Theriogenology 2007;68:1240–50.
[15] Cortés-Gutiérrez EI, Crespo F, Serres-Dalmau C, Gutiérrez de las Rozas AL, Dávila-Rodríguez MI, López-Fernández C, Gósalvez J. Assessment of sperm DNA fragmentation in stallion (*Equus caballus*) and donkey (*Equus asinus*) using the sperm chromatin dispersion test. Reprod Domest Anim; 2008, doi:10.1111/j.1365-2605.2007.00781.
[16] Fernández JL, Muriel L, Goyanes V, Segrelles E, Gosálvez J, Enciso M, LaFromboise M, De Jonge C. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil Steril 2005;84:833–42.
[17] Enciso M, Muriel L, Fernandez JL, Goyanes V, Segrelles E, Marcos M, Montejo JM, Ardoy M, Pacheco A, Gosalvez J. Infertile men with varicocele show a high relative proportion of sperm cells with intense nuclear damage level, evidenced by the sperm chromatin dispersion test. J Androl 2006;27:106–11.
[18] Enciso M, López-Fernández C, Fernández JL, García P, Gosálbez A, Gosálvez J. A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy. Theriogenology 2006;65:308–16.
[19] De Ambrogi M, Spinaci M, Galeati G, Tamanini C. Viability and DNA fragmentation in differently sorted boar spermatozoa. Theriogenology 2006;66:1994–2000.
[20] García-Macías V, de Paz P, Martinez-Pastor F, Alvarez M, Gomes-Alves S, Bernardo J, Anel E, Anel L. DNA fragmentation assessment by flow cytometry and Sperm-Bos-Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. Int J Androl 2007;30:88–98.
[21] Fernandez JL, Gosálvez J, Santiso R, Goyanes V, López-Fernández C. Adaptation of the sperm chromatin dispersion (SCD) test to determine DNA fragmentation from bull sperm. In: Valentino RG, editor. New cell apoptosis research. New York: Nova Science Publishers Inc; 2008. pp. 69–84.
[22] Gosálvez J, Fernández JL, Goyanes V, López Fernández C. Análisis de la fragmentacion del ADN en espermatozoides mediante el test de dispersión de cromatina (SCD). Biotechnology 2006;1:38–51.
[23] Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantification of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–91.
[24] Miró J, Lobo V, Quintero-Moreno A, Medrano A, Peña A, Rigau T. Sperm motility patterns and metabolism in Catalonian donkey semen. Theriogenology 2005;63:1706–16.
[25] Crespo Castejón F. Estudio de la congelación de semen e inseminación artificial en el asno Zamorano Leonés. Tesis doctoral, 2005. Facultad de Veterinaria, Universidad Complutense de Madrid.
[26] Aranguren-Mendez J, Gomez M, Jordana J. Hierarchical analysis of genetic structure in Spanish donkey breeds using microsatellite markers. Heredity 2002;89:207–11.
[27] Aranguren-Mendez J, Jordana J, Gomez M. Genetic diversity in Spanish donkey breeds using microsatellite DNA markers. Genet Sel Evol 2001;33:433–42.
[28] Fraser L. Structural damage to nuclear DNA in mammalian spermatozoa: its evaluation techniques and relationship with male infertility. Pol J Vet Sci 2004;7:311–21.
[29] Peris SI, Morrier A, Dufour M, Bailey JL. Cryopreservation of ram semen facilitates sperm DNA damage: relationship between sperm andrological parameters and the sperm chromatin structure assay. J Androl 2004;25:224–33.
[30] Boe-Hansen GB, Ersboll AK, Greve T, Christensen P. Increasing storage time of extended boar semen reduces sperm DNA integrity. Theriogenology 2005;63:2006–19.
[31] Donnelli ET, Steele EK, McClure N, Lewis SE. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod 2001;16:1191–9.
[32] Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation. DNA fragmentation and effectiveness of antioxidants. Hum Reprod 1998;13:1864–71.
[33] Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, et al. Study of apoptotic DNA fragmentation in human spermatozoa. Hum Reprod 2000;15:830–9.
[34] Saleh RA, Agarwal A, Nada ES, EL-Tonsy MH, Sherma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril 2003;79:1597–605.
[35] Foglieni C, Meoni C, Davalli AM. Fluorescent dyes for cell viability: an application on prefixed conditions. Histochem Cell Biol 2001;115:223–9.
[36] Bank HL. Rapid assessment of islet viability with acridine orange and propidium iodide. In Vitro Cell Dev Biol 1988;24:266–73.
[37] Landex NL, Kayser L. Optimization of an acridine orange–bisbenzimide procedure for the detection of apoptosis-associated fluorescence colour changes in etoposide-treated cell cultures. J Mol Histol 2004;35:133–9.
[38] Rivero MT, Vázquez-Gundín F, Goyanes V, Campos A, Blasco M, Gosálvez J, Fernández JL. High frequency of constitutive alkali-labile sites in mouse major satellite DNA, detected by DNA breakage detection-fluorescence in situ hybridization. Mutat Res 2001;483:43–50.
[39] Fernández JL, Vázquez-Gundín F, Rivero MT, Goyanes V, Gosálvez J. Evidence of abundant constitutive alkali-labile sites in human 5 bp classical satellite DNA loci by DBD-FISH. Mutat Res 2001;473:163–8.
[40] Singh NP, Danner DB, Tice RR, McCoy MT, Collins GD, Schneider EL. Abundant alkali-sensitive sites in DNA of human and mouse sperm. Exp Cell Res 1989;184:461–70.
[41] Fernández JL, Vázquez-Gundín F, Delgado A, Goyanes VJ, Ramiro-Díaz J, de la Torre J, Gosálvez J. DNA breakage detection-FISH (DBD-FISH) in human spermatozoa: technical variants evidence different structural features. Mutat Res 2000;453:77–82.
[42] Cortés-Gutiérrez EI, Dávila Rodríguez MI, López-Fernández C, Fernández JL, Gosálvez J. Alcali-labile sites in sperm cells from Sus and Ovis species. Int J Androl 2007. doi: 10.1111/j.1365-2605.2007.00781.