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Refrigerated Storage of Red Deer Epididymal Spermatozoa in the Epididymis, Diluted and with Vitamin C Supplementation

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Abstract

We have approached the problem of refrigerated storage of epididymal sperm samples from red deer by comparing three options: storing the genital (testicles within the scrotum), diluting the semen in extender or diluting the semen in extender supplemented with an anti-oxidant. Twenty-nine pairs of testes were collected. Spermatozoa from one of each of the pairs were immediately recovered, and diluted to 400×10^6 sperm/ml in Tris-citrate-fructose with 20% egg yolk. Control group was stored as such, and Anti-oxidant group was supplemented with 0.8 mm vitamin C. The remaining epididymides and the diluted samples were stored at 5°C and spermatozoa were analysed at 0, 24, 96 and 192 h for: motility [computer-assisted semen analysis (CASA)], acrosomal integrity, sperm viability (eosine/nigrosine staining), normal tails and chromatin status [sperm chromatin structure assay (SCSA)]. In general, seminal quality decreased with storage time. Vitamin C supported progressive motility better at 24 h (median 42% vs 23% Control and 15% epididymis), reduced the incidence of tail abnormalities and protected chromatin. Storing the semen in the epididymis slowed down motility loss, but slightly increased the occurrence of tail abnormalities and viability was lower at 192 h. However, regarding chromatin status, sperm stored in the epididymis was protected similarly to those diluted in the medium supplemented with vitamin C. Although the differences between the three groups were small, there were some advantages in supplementing the extender with vitamin C. Besides, refrigerating the epididymis may be a good option when immediate processing is not available.

Introduction

The interest in preserving germ plasms of deer species has resulted in recent attention to the possible recovery, evaluation and preservation of sperms from the epididymides of dead animals (Zomborszky et al. 1999; Comizzoli et al. 2001; Hishinuma et al. 2003; Martinez-Pastor et al. 2006; Fernandez-Santos et al. 2007, 2007). Preservation of semen requires a reduction or arrest of the metabolism of spermatozoa, thereby prolonging their fertile life. This is commonly achieved by cryopreservation (Fernandez-Santos et al. 2006, 2007; Martinez-Pastor et al. 2006), but semen may also be stored in a liquid (unfrozen) state, using reduced temperatures or other means to depress metabolism (Maxwell and Salamon 1993). Despite the general agreement that only a few hours are enough to impair epididymal sperm, it has been shown that storage of epididymides at 5°C could be also a way of preserving sperm motility and fertilizing ability for several days (Kikuchi et al. 1998; Songsasen et al. 1998; An et al. 1999; Yu and Leibo 2002; Kaabi et al. 2003). Besides, there are several studies on this topic in red deer (Martinez-Pastor et al. 2005, 2005). Moreover, Soler et al. (2003) concluded that red deer spermatozoa recovered from epididymides that had been stored at 5°C were of good quality, provided that storage time was less than 48 h.

However, metabolism is not completely arrested during refrigerated storage, and spermatozoa may undergo irreversible reduction of motility, morphological integrity and fertility. These changes may be caused by the accumulation of toxic products of metabolism and, more importantly, of reactive oxygen species (ROS) formed through the univalent reduction of oxygen (e.g. superoxide anion, hydroxyl radical and hydrogen peroxide) (Misra and Fridovich 1972). This oxidative stress ends up causing structural damage to biomolecules and cellular components (Halliwell 1991). Oxidative damage may be increased in epididymal spermatozoa, since they are not exposed to the complex secretions of the accessory sex glands (seminal plasma), which are recognized as the predominant source of anti-oxidant protection for spermatozoa (Chen et al. 2003).

A wide array of enzyme scavengers and anti-oxidants has been used for blocking or preventing oxidative stress in a variety of cell systems. These anti-oxidants either scavenge ROS directly or prevent propagation of lipid peroxidation in cell membranes. A number of studies demonstrated the positive effect of adding ROS scavengers or anti-oxidants to liquid semen of ruminants for preservation at either 15 or 5°C (Maxwell and Stojanov 1996; Upreti et al. 1997, 1998). Vitamin C is a major chain-breaking anti-oxidant, being present in the extracellular fluid. It neutralizes hydroxyl, superoxide and hydrogen peroxide radicals and prevents sperm agglutination (Agarwal et al. 2004). Besides, Dawson et al. (1992) indicated an improvement in sperm viability, motility and maturity, along with a decrease in the percentage of abnormalities when increasing the dietary intake of ascorbic acid. Moreover, Vitamin C increases sperm counts in vivo in infertile male (Lewis et al. 1997), and it has been associated with fertility, may be having evolutionary significance (Millar 1992). Moreover, from a practical point of view, vitamin C is a component easy to find and cheaper than other anti-oxidants such as vitamin E or enzymatic ones (superoxide dismutase or catalase). Thus, it is necessary to investigate which of the two options, whether the addition of vitamin C to the liquid storage or the maintenance of sperm within epididymides at 5°C, would benefit the sperm preservation, in order to develop refrigeration protocols really adequate for red deer spermatozoa.

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The main objective of the present study was to evaluate the refrigerated storage of epididymal red deer sperm under different conditions. Spermatozoa from one of each of the pairs were immediately recovered, evaluated and put on storage (control group). The remaining epididymides were cooled to 5°C and stored for 24, 96 and 192 h (experimental groups), after which spermatozoa were collected and evaluated as in the control group. We have investigated the efficacies of three treatments; (1) Storing the genital (testicles and epididymes within the scrotum); (2) diluting the sperm sample in extender; (3) diluting the sperm sample in extender and supplementing with the anti-oxidant vitamin C.

Storing the testicles instead of collecting the sperm sample is a common strategy when it is not possible to freeze the sample immediately, for instance, when harvesting takes place in the wild. Besides, we have interest on testing diluted storage with anti-oxidant supplementation, because the alternative strategy would be to extract the sample in the field and keep it diluted in extender until freezing is possible. Moreover, there are no other studies on the effect of anti-oxidants on red deer spermatozoa, apart from the one study by our group on cryopreserved semen (Fernandez-Santos et al. 2007).

Materials and Methods

All chemicals were of reagent grade and were purchased from Sigma or Merck (both of Madrid, Spain).

Stags and testis collection

For this study, we used spermatozoa recovered from the epididymides of 29 mature stags (age > 4.5 years, weight > 130 kg) that were legally culled and hunted in their natural habitat during the rutting season (September–October). Gamekeepers collected the complete male genitalia and provided the hour of the death. Hunting was in accordance with the harvest plan of the game reserve, which made following Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, which conforms to European Union Regulation.

Immediately upon removal, the testes with attached epididymides were placed into plastic bags and transported to the laboratory at room temperature (approximately 22°C) within 2 h after being removed.

Experimental design

Samples were processed as soon as they arrived at the laboratory. Spermatozoa were immediately collected from one epididymis of each pair of testicles according to the method described by Soler et al. (2003). Briefly, testes and epididymides were removed from the scrotal sac. Cauda epididymides were separated and the sperm mass was collected performing several cuts. The sperm samples were evaluated and split into two aliquots. One of the aliquots (Control) was diluted ($\sim 400 \times 10^6$ sperm/ml) in a modified Salamon's solution (Tris-citrate-fructose-egg yolk, TCF; see below). The other aliquot was diluted ($\sim 400 \times 10^6$ sperm/ml) in the same extender and supplemented with 0.8 mM of vitamin C.

The remaining epididymides, still attached to the testicles, were put into plastic bags, and placed in beakers with water at 5°C. The testicles and the diluted aliquots were stored at 5°C in the dark and evaluated at 24, 96 or 192 h after sample processing. The aliquots were analysed at each sampling time; the stored testicles were divided in three groups, and each group was processed at one sampling time (24 h: eight testicles; 96 h: eight testicles; 192 h: 13 testicles), collecting the sperm and evaluating it.

Modified Salamon's solution contained Tris (0.22 M), citric acid (0.07 M) fructose (0.05 M) and clarified egg yolk (20% v/v) (Fernandez-Santos et al. 2006). Clarified egg yolk was prepared as described by Holt et al. (1996). Briefly, fresh hen eggs were manually broken. Yolks were separated from the albumen and were carefully rolled on a filter paper to remove chalazae and traces of albumen adhering to the vitelline membrane. The latter was then disrupted with a scalpel blade and yolk was collected with a sterile syringe. Then, whole egg yolk was diluted in redistilled water (1 : 3) and centrifuged in sterile tubes at 10 000 × g for 30 min. After centrifugation, the pellet (granules) at the bottom of the tube was discarded and the water-soluble clear fraction (plasma) was saved to prepare the clarified EY-TCF medium.

Sperm evaluation

Motility was assessed immediately after recovery, and those samples having less than 30% individual motility were discarded.

Sperm motility

Sperm motility was determined with a computer-assisted motility analyzer (CASA system) using an optical phase contrast microscope (Nikon Eclipse 80i), equipped with negative phase contrast objectives and a warming stage at 37°C, a Basler A302fs camera, and a PC with the Sperm Class Analyzer software (SCA2002; Microptic, Barcelona, Spain). A pre-warmed Makler counting chamber (10 μm depth) was loaded with 5 μl of sample. The proportions of total (MT, %) and progressively motile (MP, %) spermatozoa, as well as kinematic parameters were recorded. Kinematic parameters were: 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 head beat (BCF, Hz). Sample acquisition rate was 25 images/s, and progressivity was defined as VCL > 25 μm/s and STR > 80%. At least five fields per sample were recorded and analysed afterwards. We used VCL, LIN, ALH and BCF for this study.

Acrosomal integrity

Acrosomal integrity was evaluated after a 1 : 20 dilution in 2% glutaraldehyde, 0.165 M cacodylate/HCl buffer

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(pH 7.3). The percentage of spermatozoa with intact acrosomes (% NAR) was assessed by phase-contrast microscopy at ×400, after counting 100 cells.

Sperm viability

The sperm viability was also evaluated by using a nigrosine–eosine stain (NE). The NE stain was prepared as per method described Tamuli and Watson (1994). The diluted sperm (5 μl) was mixed with the NE stain (10 μl) at 37°C, incubated for 30 s, smeared and dried on a warm plate at 37°C. The samples were evaluated using bright field microscopy at ×400. Live spermatozoa remained unstained, whereas dead cells were dull pink. The percentage of live spermatozoa was expressed as viability (%).

Assessment of sperm chromatin stability

Chromatin stability was assessed by staining with the metachromatic fluorescent dye acridine orange (AO), whose use is based on the susceptibility of sperm DNA to acid-induced denaturation *in situ*. AO shifts from green (dsDNA) to red (ssDNA) fluorescence depending on the degree of DNA denaturation (Evenson et al. 1980; Januskauska et al. 2001). Samples were diluted in TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) to a final sperm concentration of 2×10^6 cells/ml in cryotubes. Samples were dropped into LN2 and then allowed to thaw at room temperature. This process was repeated two more times and then frozen samples were stored in an ultra cold freezer at −80°C until needed. For analysis, the samples were thawed on crushed ice. Acid-induced denaturation of DNA in situ was achieved by adding 0.4 ml of an acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4). After 30 s, the cells were stained by adding 1.2 ml of an acridine orange solution (0.1 M citric acid, 0.2 M Na2HPO4, 1mM EDTA, 0.15 M NaCl, 6 μg/ml acridine orange, pH 6.0). The stained samples were analysed by flow cytometry just 3 min after adding the acridine orange solution.

Samples were analysed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), equipped with standard optics and an Ar-ion laser tuned at 488 nm, and running at 200 mW. Calibration was carried out using standard beads (Fluoresbrite plain YG 1.0 μM, Polysciencies Inc., Warrington, PA, USA). Green fluorescence was detected using the FL-1 photodetector (530/30 band pass filter) and red fluorescence with the FL-3 photodetector (650 long pass filter). Data were collected from 10 000 events for further analysis with Cell-Quest software (Becton Dickinson). Sideward and forward scatter of light were recorded, so that only sperm cell-specific events were selected for analysis. Flow rate was 200 cells/s. At the beginning of each session, a standard semen sample was run through the cytometer, and settings were adjusted in order that mean fluorescence values (0–1023 linear scale) for FL-1 and FL-3 were 475 and 125 respectively.

Results of the DNA denaturation test were processed to obtain the DFI (DNA fragmentation index; formerly called αt) for each spermatozoa, that express the shift from green to red fluorescence, and is expressed as the ratio of red fluorescence to total intensity of the fluorescence [red/(red + green) × 100]. High values of DFI, indicates chromatin abnormalities. Flow cytometry data was processed with WinMDI software (Scripps Research Institute, La Jolla, CA, USA), and data was saved as tabbed text in order to obtain DFI and its derived indexes: standard deviation of DFI (SD-DFI), DFI% (% of spermatozoa with DFI > 25) HDS (High DNA Stainability; % of spermatozoa with green fluorescence higher than 600, of 1024 channels).

Statistical analysis

Statistical analyses were carried out using the *R* statistical package (http://www.r-project.org). Data were fitted to linear mixed-effect models by maximizing the log-likelihood (ML method) (Pinheiro and Bates 2000). Male was always included as random effect, and time (covariate) and treatment (factor with three levels: storage in the epididymis, in extender and in extender supplemented with vitamin C) were included as fixed factors, as expressed by the following formula:

$$Y_{ijk} = \mu + \mathrm{Time}_i + \mathrm{Treatment}_j + \mathrm{Time}_i \times \mathrm{Treatment}_j + \mathrm{Male}_k + \varepsilon_{ijk}$$

For comparing treatments at a given time or between 0 h and sampling times, we used the contrasts provided by the analysis (adjusting p-values by Holm's correction for multiple comparisons).

Results

Table 1 shows the coefficients of the models tested and their significations. These coefficients describe the linear model (intercept and slope) for the Control treatment (sperm diluted in TCF and stored at 5°C), and when the model is modified by the complementation with 0.8 mM vitamin C (vitamin C and time × vitamin C), or sperm is kept in the epididymis (epididymis and time × epididymis).

All parameters related to sperm motility (Fig. 1) decreased with incubation time. Storing the samples diluted with a vitamin C supplemented medium (VitC) did not improved total motility, but it was better preserved keeping them into the epididymis. In fact, the model showed that VitC slightly accelerated the loss of motility regarding to samples stored diluted without supplementation (Control), whereas it was slowed down when kept into the epididymis (EP). Interestingly, at 24 h motility was lower in samples from stored epididymes, but it was higher for these samples at 96 and 192 h. However, progressive motility was clearly higher for VitC at 24 h, as showed by the main effects value. Nevertheless, at 192 h there were very low progressive motility because of low total motility, velocity and linearity. For the rest of motility parameters we did not find any effect of treatments in the model. However, VitC seemed to better preserve linear movement at the

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Table 1. Coefficients for the tested models and their significations (see the model formula and explanation in the statistic analysis section). The Intercept represents the theoric value of the model for the Control treatment at 0 h, and time is the slope of the model for the control treatment along time. The values under Vitamin C and Epididymis would affect the value of the intercept for these treatments, while the interactions (time × vitamin C and time × epididymis) indicate if the parameter increased or decreased faster than the Control (affecting time, the slope of the model). The signification symbols indicate that the corresponding coefficient is statistically different from 0

Parameter	Intercept	Time	Vitamin C	Epididymis	Time × vitamin C	Time × epididymis
MT%	79.375***	−0.347***	3.042	−2.707	−0.069*	0.076*
MP%	23.122***	−0.118***	8.376***	−2.143	−0.049*	−0.004
VCL	91.889***	−0.269***	−3.512	−1.149	−0.044	0.036
LIN	40.123***	−0.070**	8.781	−2.109	−0.062	−0.036
ALH	4.018***	−0.010**	−0.528	−0.496	0.000	0.001
BCF	8.152***	−0.018***	−0.292	0.804	0.004	−0.012
NAR	95.718***	−0.026***	0.164	−0.030	−0.011	−0.016
Viability	91.374***	0.012	1.496	1.258	−0.009	−0.098***
Normal tail	92.177***	−0.063***	−0.171	2.803	0.036**	−0.044**
SDDFI	0.875	0.035***	0.340	1.305	−0.024**	−0.029***
DFI%	−0.181	0.052***	0.355	0.908	−0.039**	−0.046**
HDS%	6.214***	−0.023**	−2.287*	−3.749**	0.015	0.042***

Signification symbols indicate that the corresponding coefficient is different from 0: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

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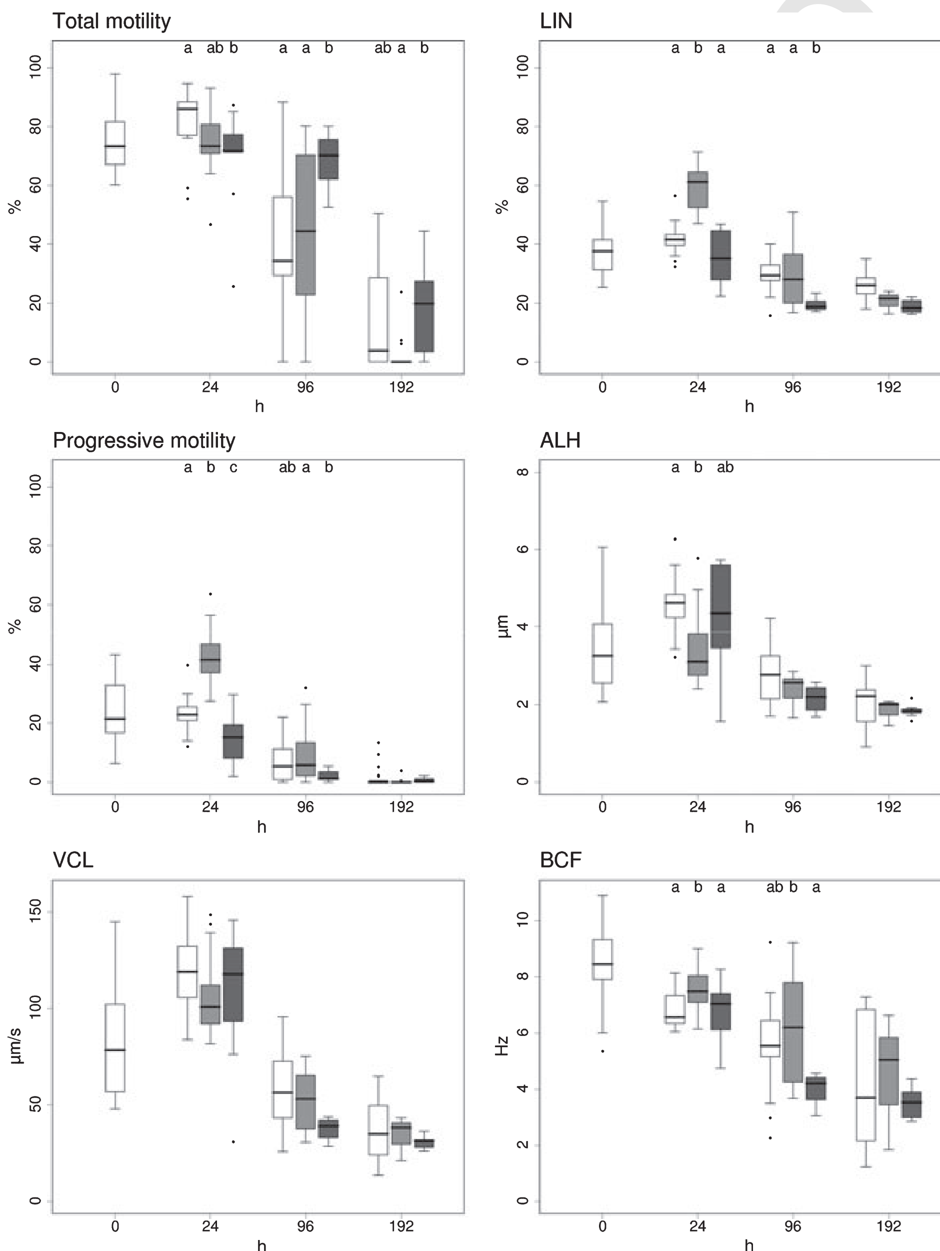

Fig. 1. Motility parameters assessed by CASA for the three treatments along time (white: Control; light grey: VitC; dark grey: EP). Different letters indicate significant differences ($p < 0.05$) after a pairwise comparison within each time. The boxes spread from the 1st to the 3rd quartiles, the whiskers extend to the interquartile range × 1.5, and the horizontal line indicates the median. Outliers are represented by dots

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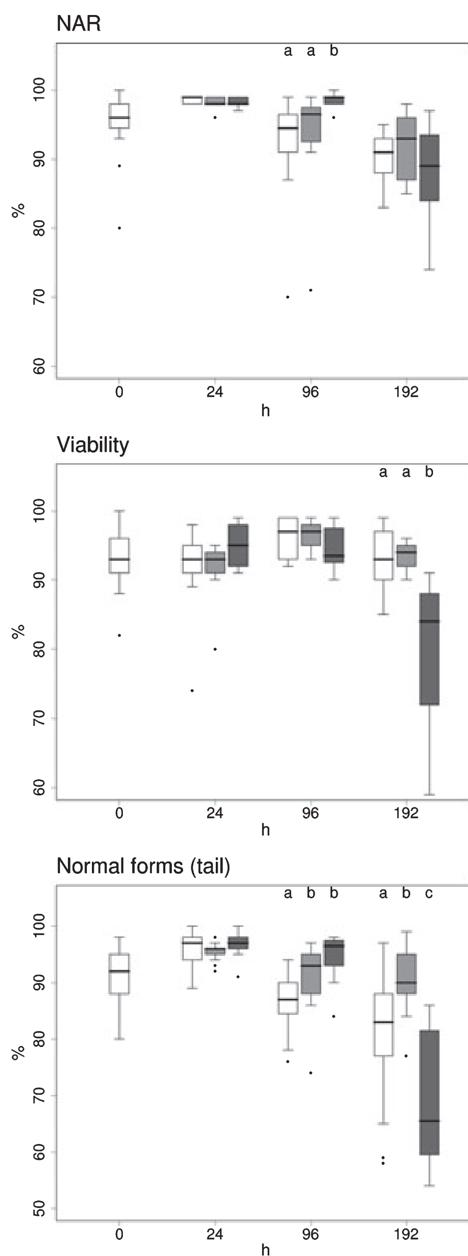

Fig. 2. Acrosomal status (NAR), viability and % of normal tails for the three treatments along time (white: Control; light grey: VitC; dark grey: EP). Different letters indicate significant differences ($p < 0.05$) after a pairwise comparison within each time. The boxes spread from the 1st to the 3rd quartiles, the whiskers extend to the interquartile range × 1.5, and the horizontal line indicates the median. Outliers are represented by dots

short-term (higher LIN and BCF, and lower ALH at 24 h), whereas EP seemed to present lower parameters at 96 h (LIN and BCF).

Microscopy-assessed parameters were differently affected by storage treatments (Fig. 2). Acrosomal status seemed not to be specially preserved by any treatment. There were not great changes in general for sperm viability as assessed by the eosine–nicrosine staining, as the model indicated no effect of time in this parameter, but for EP samples, for which a drop was significantly found at 192 h. Maybe related to that, we found a similar drop in normal forms for EP samples at the same sampling time. In this case, normal forms slowly decreased with time, but VitC partially prevented this decrease. Interestingly, storing the samples in the epididymis seemed to prevent too the occurrence of abnormal tails at least till 96 h sampling.

The SCSA analysis showed that both VitC and EP helped protecting chromatin in the long term (Fig. 3). SD-DFI and %DFI increased with time from nearly 0 (non significant intercept), but slowly. The interaction of VitC and EP with time was significant and negative, showing that this increase was even slower in these treatments. In fact, at 192 h, we found significant differences between Control and the treatments (at

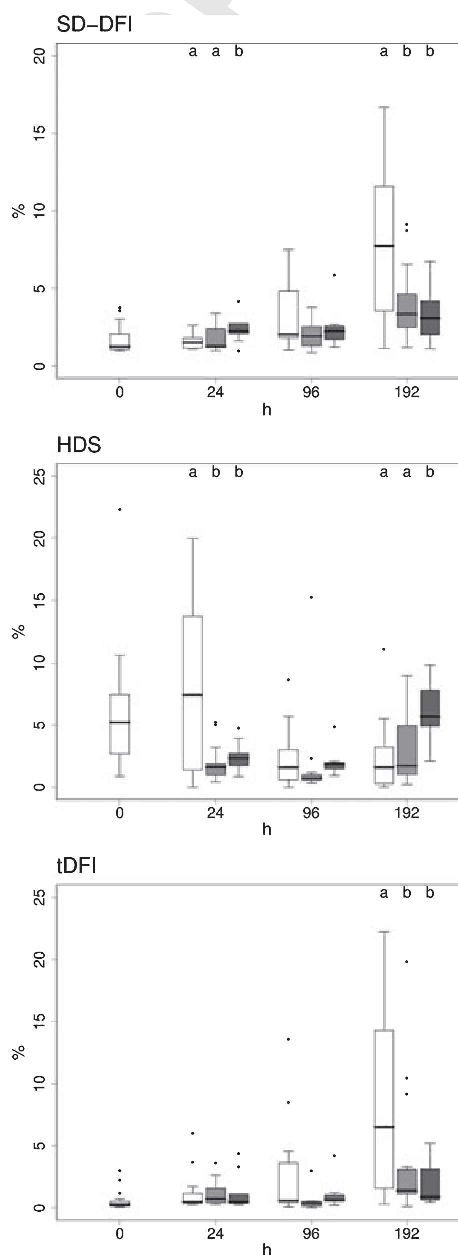

Fig. 3. Chromatin status (SCSA) for the three treatments along time (white: Control; light grey: VitC; dark grey: EP). Different letters indicate significant differences ($p < 0.05$) after a pairwise comparison within each time. The boxes spread from the 1st to the 3rd quartiles, the whiskers extend to the interquartile range × 1.5, and the horizontal line indicates the median. Outliers are represented by dots

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24 h there was a transient higher SD-DFI for EP samples). HDS was significantly higher at 24 h for Control, and its distribution became very wide, but at 96 h it returned to values similar to VitC and EP. HDS distribution was wider at 192 h for all treatments, and this parameter was significantly higher for EP at that time.

An important fact is that there were few differences between measures at 0 and 24 h. Only MP (higher for VitC and lower for EP), LIN (higher for VitC and lower for Control) and BCF (lower for EP) showed significant differences for that sampling time.

Discussion

The cauda epididymides of live animals provide an excellent environment for sperm storage. Sperm mature as they pass through the caput and corpus of the epidymis and, once in the caudal portion, sperm may be held naturally in this segment for extended times. In this situation, spermatozoa retain their motility and fertilizing capacity (Young 1931).

However, it is generally assumed that gametes within the body of animals degenerate quickly after death. However, many studies have demonstrated that spermatozoa recovered from post-mortem specimens, even may hours after death, retained their function (Sankai et al. 1997; An et al. 1999; Kaabi et al. 2003; Soler et al. 2005). Indeed, in early studies of our own group we noticed that when testes of red deer after death are conserved by refrigeration (5°C), sperm remain for extended periods viable and post-mortem changes are minimized by storage at this temperature (Soler et al. 2003). Although these earlier results are valuable for developing protocols to improve red deer spermatozoa survival after the animal death, there has been no other effort to find other alternatives to this situation. Moreover, sperm from epididymides are known to be susceptible to cold shock (White 1993).

One of the reasons storage temperature is critical may be due to the damage caused by reactive oxygen species (ROS), which leads to the event called ‘oxidative stress’ (Nichi et al. 2007). Epididymal samples are particularly susceptible to attack by ROS, as they are not exposed to the complex secretions of the accessory sex glands (seminal plasma), which are recognized as the prime source of anti-oxidant protection (Chen et al. 2003). Therefore, sperm characteristics after testes storage at 5°C could be better preserved if the effects caused by ‘oxidative stress’ are minimized. In this sense, many studies have noted the beneficial effects of vitamin C supplementation on sperm characteristics (Fraga et al. 1991; Yousef et al. 2003; Silver et al. 2005). However, only few studies have been aimed at investigating whether vitamin C supplementation *in vitro* could improve sperm survival during refrigeration (Donnelly et al. 1999; Ball et al. 2001).

In the present study, adding ascorbic acid helped to preserve linear movement at the short-term (higher progressive motility, LIN and BCF and lower ALH at 24 h). These findings agree with those obtained by Eskenazi et al. (2005) who demonstrated that the anti-oxidant oral intake is associated with better semen quality, in particular, motility. Our results also revealed that refrigerated storage of testicles better preserved total motility. In fact, after 192 h of incubation, there was no motility in VitC samples, but in Control and storage of testicles, most samples had some motility. This result is consistent with the observations of Ball et al. (2001), who demonstrated that supplementation with vitamin C did not improve the maintenance of motility of cooled equine spermatozoa during 96-h storage, although in that study, there were no previous evaluations to 96-h. The possible beneficial effect of ascorbic acid during storage could not be demonstrated in sperm characteristics unless long time storage aspects were tested because, as Kankofer et al. (2005) reported, the anti-oxidative activity in both native and extended semen are maintained over 24 h storage at 5ºC. Therefore, we could expect that exogenous anti-oxidant protection was not necessary during short term storage, but for longer times, anti-oxidant could avoid bad effects of oxidative stress.

The most important finding of our study is the relationship between ascorbic acid supplementation and sperm DNA integrity during refrigerated storage, showing that Vitamin C protected sperm chromatin *in vitro*. Increased DFI has been associated with increased risk of miscarriage and increased time to pregnancy in humans (Larson et al. 2000; Evenson et al. 2002) and bulls and mice (Evenson et al. 1980; Ballachey et al. 1988). However, whereas there is some evidence that vitamin C intake may reduce DNA strand breaks (Green et al. 1994) and DNA base oxidation (Duthie et al. 1996) in human lymphocytes, there has been little research on the protective effect of ascorbic acid on sperm DNA (Ball et al. 2001). We have to consider that increased oxidative damage to sperm DNA has been associated with low ascorbic acid concentrations in seminal fluid (Fraga et al. 1991, 1996), and infertile males were found to have low ascorbic acid levels in seminal plasma (Dawson et al. 1992). Interestingly, Song et al. (2006) found that patients with low levels of seminal ascorbic acid had increased sperm DNA damage. Moreover, Fraga et al. (1991) demonstrated that ascorbic acid protects against endogenous oxidative DNA damage in human sperm. These authors also indicated that low ascorbic acid was associated with increased DNA damage even though no effect on sperm quality was observed, agreeing with our results. However, most of the studies of ascorbic acid on sperm characteristics have consisted of dietary supplementation and at the moment there is little information about the effects of ascorbic acid supplementation *in vitro*. Hughes et al. (1998) reported that *in vitro* treatment of sperm with ascorbic acid (300 and 600 μM) reduced the magnitude of DNA damage as measured by the Comet assay, and agreeing with our results.

Interestingly, in our experiment, testes storage (EP) seemed to protect chromatin as effectively as VitC. The present work seems to indicate that the endogenous anti-oxidant system of epididymes is capable for sustaining DNA protection. These observations are in

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agreement with other studies which showed that the protective mechanisms are indeed present in the sperm cells as well as in the epididymal fluid (Hinton et al. 1995). Data available for epididymal spermatozoa demonstrate that they are equipped with enzymatic mechanisms that can dispose of potentially harmful ROS (Tramer et al. 1998).

Besides, we found differences between our treatments regarding HDS. HDS data is interesting, because this parameter has been related to sperm chromatin maturity (Evenson and Wixon 2006). Although HDS was high at 24 h in the Control samples, it lowered at subsequent times, whereas it remained low in VitC and EP samples, at least till 96 h. Since we were working with epididymal spermatozoa, it is possible that dilution and storage induced these changes in the chromatin, maybe related to maturation, thus the higher HDS at 24 h in the Control, while further incubation would reverse these changes. In contrast, an anti-oxidant environment or the epididymal environment would prevent them. The increase of HDS in VitC and EP observed at 196 h may indicate that these treatments simply delayed the changes observed in the Control at 24 h, and it is possible that anti-oxidant consumption or general decay in the epididymis triggered these events at advanced storage time.

In conclusion, our study showed that refrigerated storage of epididymal spermatozoa from red deer may be improved either by refrigerating the testicles and collecting the sample just before freezing, or by diluting the spermatozoa in a medium supplemented with vitamin C. These findings are especially interesting regarding its practical use in the field, when it is not possible to take up immediate freezing of post-mortem samples. It is important to note that motility was preserved better in VitC at 24 h, therefore, when freezing can be carried out within few hours after harvesting the genital, the better option would be extracting and diluting the sperm sample in a medium with anti-oxidants.

Nevertheless, none of the treatments was able to maintain sperm quality in prolonged storage. However, when a very valuable male dies and it is not possible to freeze the sperm until several days later, we have still the option of freezing the sample for trying *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). In this case, just storing the genital would be the more convenient option, since we have observed that chromatin status, possibly the most important characteristic when using *in vitro* techniques, is preserved as well as when diluting in an anti-oxidant-supplemented medium. Nevertheless, we must take into account that some parameters (viability, normal tails) were lower in epididymal samples at the most prolonged time, and the significance of HDS increase in these samples must be still assessed. Although there are some studies regarding post-mortem storage and IVF or fertility success (Kikuchi et al. 1998; Kishikawa et al. 1999), they have not been related to chromatin status at post-mortem times. Thus, a further step in our research would be to test if the differences we have found at prolonged storage times are indeed reflected in IVF or ICSI success.

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