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Functional Significance of the Sperm Head Morphometric Size and Shape for Determining Freezability in Iberian Red Deer (*Cervus elaphus hispanicus*) Epididymal Sperm Samples

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Abstract

In the present study, computer-automated sperm head morphometry of epididymal samples was used to determine if sperm head area and shape are useful measurements for separating "good" and "bad" Iberian red deer freezers. A microscope slide was prepared from single diluted sperm fresh samples collected from 38 mature stags. Slides were air-dried and stained with Hemacolor. The sperm head area and shape (length/width) for a minimum of 145 sperm heads were determined for each male by means of the Sperm-Class Analyser. The remainder of each sample was frozen. After thawing, sperm cryosurvival was judged in vitro by microscopic assessments of individual sperm motility and of plasma membrane and acrosome integrities. All sperm parameters evaluated at thawing were placed in a statistical database and a multivariate cluster analysis performed. Mean sperm parameters of the 2 clusters generated ("bad" and "good" freezers) were compared by ANOVA. Our results show that sperm quality at thawing for all sperm parameters evaluated was significantly higher ($P < .01$) for "good" freezers than for the "bad" ones (sperm motility index: 67.4±2.0 vs 57.1±2.8; NAR: 67.1±2.5 vs 54.5±3.5; viability: 68.8±2.0 vs 60.1±2.8; HOST: 71.3±2.2 vs 63.1±3.1). Additionally, differences ($P < .01$) in epididymal sperm head area and shape were found between "good" and "bad" freezers before freezing, with the smallest overall sperm head dimensions found in the "good" freezers group (area: 32.04 μm^2 vs 34.42 μm^2). Thus, the lower the

sperm head area in the fresh samples, the greater the sperm cryoresistance. Our results show that the 2 groups of males also differ in sperm head shape in fresh samples (good: 1.96 vs poor: 1.72; $P < .01$). It is possible that sperm head area and shape influence total sperm volume, thus causing differences in heat exchange as well as in movements of water, ions, and cryoprotectants and, in turn, on sperm freezability.

Key words: cryopreservation, morphology, postmortem recovery

The interest in preserving germplasm of wild deer species has resulted in rather recent attention to the possible recovery, evaluation, and cryopreservation of sperm from the epididymides of dead animals (Zomborszky et al, 1999; Comizzoli et al, 2001a,b; Hishinuma et al, 2003; Soler and Garde, 2003; Soler et al, 2003, 2005). However, little information is published on freezing and thawing methods for epididymal spermatozoa of this species, although there have been many studies of freezing of ejaculated semen from stags (Asher et al, 2000). Most procedures used to cryopreserve epididymal spermatozoa from red deer have been modified from those developed for ejaculated semen. This approach would not seem to be very appropriate, since it is well known that there are important differences in the physiological characteristics of epididymal vs ejaculated spermatozoa, especially in their membrane properties, that affect the sperm cell survival after freezing.

Variation between individuals in the extent to which their spermatozoa are damaged by freeze-thawing has been widely reported in several species. These differences in the resistance to thawing of the spermatozoa of different individuals have been observed for spermatozoa of domestic (Curry, 2000) and wild (Leibo and Songsasen, 2002) species. Within this context, semen donors have routinely been categorized as "good" or "bad freezers." The mechanisms underlying differences in cryosensitivity between different individuals have yet to be elucidated, but there is some evidence for physiological differences between spermatozoa from individuals of the same species. Recently it has been demonstrated that these consistent interindividual variations in sperm freezability are genetically determined (Thurston et al, 2002).

Appropriate, sensitive, and rapid methods of assessment are necessary for adequate evaluation of sperm function. Conventional sperm parameters are not sufficient to identify animals known as "poor freezers," whose sperm quality is greatly impaired by cryopreservation. The routine evaluation of semen, including normal sperm morphology assessment, has long been employed to evaluate the effects of freezing-thawing procedures on sperm cryosurvival. Poor semen morphology is an important indicator of decreased fertility in men (Kruger et al, 1993); stallions (Jasko et al, 1990), bulls (Sekoni and Gustafsson, 1987), and goats (Chandler et al, 1988). Although normal sperm morphology may be an indicator of the fertility potential of a given male, correlations have been based on subjectively performed analyses. However, large variations between technicians and laboratories in the subjective evaluation of semen characteristics are known to exist (Saacke, 1982; Baker and Clarke, 1987), making accurate interpretation of the resulting data difficult. The need for accurate objective assessment of sperm morphology has led to the development of computer-assisted sperm head morphometry analysis, ASMA (Katz et al, 1986; Davis et al, 1992; Pérez-Sánchez et al, 1994). The precision of the ASMA system has been utilized to detect morphometric differences in sperm head dimensions of fertile and subfertile males (Casey et al, 1997) as well as subtle changes in human sperm head morphometry due to toxicant exposure when no morphological differences were detected by manual assessment (Davis et al, 1993).

Previous studies utilizing ASMA have also demonstrated that cryopreservation affects head morphometry of bull (Gravance et al, 1998), human (Thompson et al, 1994), stallion (Arruda et al,

2002), dog (Rijsselaere et al, 2004), and red deer (Esteso et al, 2006) spermatozoa. Recent evidence suggests that in boar semen, the sperm head dimensions of individual sperm samples may be an indicator of sperm cryosurvival (Thurston et al, 2001; Peña et al, 2005), while no data exist regarding sperm morphometry and freezability in deer species.

Thus, adopting methods previously utilized in other species (Sancho et al, 1998; Buendía et al, 2002) and in red deer by us (Esteso et al, 2003, 2006), our objective was to determine if sperm head area and shape are useful measurements for predicting freezability in Iberian red deer epididymal sperm samples. To achieve this goal, first, frozen-thawed spermatozoa from a single sample collected from 38 stags were evaluated for sperm motility and for acrosome (NAR) and membrane integrities (HOST and viability). All data generated were used for a multivariate cluster analysis which objectively classified all sperm samples (stags) within a data set into one of 2 groups, categorized as "good" or "bad" according with their freezability. Second, we retrospectively compared the routine sperm parameters and the sperm head size and shape in freshly epididymal sperm samples between the 2 defined groups.

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With the exception of dibutyl phthalate xylene (Fluka, Madrid, Spain), all chemicals were of reagent grade and were purchased from Sigma or Merck (both of Madrid, Spain).

Preparation of Testes and Collection of Epididymal Spermatozoa

For this study, we used spermatozoa recovered from the epididymides of 38 mature stags (age > 4 years, weight > 140 kg) that were legally culled and hunted in their natural habitat during the rutting season (September–November). The hunting of stags was performed in accordance with the harvest plan of each game reserve. The harvest plans were 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 20 °C) within 2 hours after being removed. Samples were processed as soon as they arrived at the laboratory. For collection of epididymal spermatozoa, testes and epididymides were removed from the scrotal sac. Cauda epididymides, including about 5–10 cm of the proximal ductus deferens, were separated and transferred to 35-mm plastic dishes.

Spermatozoa were collected from the distal portion of the epididymis by repeated longitudinal and transverse cuts with a surgical scalpel and collecting the oozing sperm mass and placing it in 0.5 ml of Salamon modified solution (Fernandez-Santos et al, 2005); in particular, the base solution (Fraction A) containing Tris (2.70%, w/v), fructose (1%), citric acid (1.4%), glycerol (0%, v/v), and egg yolk (20%, v/v), with pH = 6.8 and osmolality of 300 mOsm. Epididymal contents from both testicles of an individual male were pooled for processing, because our previous observations showed that no differences appeared to exist in cell quality between testes belonging to the same individual (Garde et al, 1998).

Semen Processing and Cryopreservation

Then, the sperm mass was again diluted at room temperature to a final sperm concentration of ~400 x 10^6 sperm/ml with Fraction A of the extender. Sperm dilution was performed in a 2-step procedure at room temperature, first adding base extender up to 2 times the final desired sperm concentration and then a second extender (Fraction B) at a 1:1 ratio to achieve a final concentration ~200 x 10^6

spermatozoa/ml. Fraction B differed from the base diluent in the replacement of water (12%, v/v) with the same volume of glycerol (final concentration = 6%, v/v). This 2-step procedure was employed for sperm dilution to obtain the same final concentration of glycerol for each stag. At this point, subsamples were taken for sperm head morphometric dimensions evaluation. Then the sperm diluted was placed in a 15-ml centrifuge tube (Iwaki, Japan) and was slowly cooled to 5 °C. For this, the tubes were placed in a beaker with water (75 ml at room temperature) and transferred to a refrigerator at 5 °C. Cooling down to 5 °C lasted for about 1 hour, and extended samples were held for equilibration at that temperature for 2 hours more. After the equilibration of the diluted sperm samples, the extended sperm was loaded into 0.25-ml plastic straws. Immediately, they were frozen at 4 cm in nitrogen vapor (−120 °C) for 10 minutes before being immersed into liquid nitrogen (−196 °C) for storage. For freezing, straws were placed horizontally on a metal rack that was positioned into a freezing close container (Cryo Diffusion CD-45). The frozen straws remained for a minimum period of 1 month in liquid nitrogen before thawing was carried out.

Frozen semen was thawed in a water bath (37 °C) for 20 seconds and the content of the straws poured into a glass tube. Samples were evaluated for motility, viability, and acrosome and membrane integrities after 5 minutes of incubation at 37 °C, using the methods described below. At thawing, subsamples were also taken for sperm head morphometric dimensions evaluation.

Semen Evaluation

Sperm concentration and subjective scores of motility were assessed shortly after collection. Sperm concentrations of the original suspensions were determined using a hematocytometer. Percentage of individual motile sperm (motility) was noted, and quality of motility was assessed using a scale of 0 (lowest) to 5 (highest). A Sperm Motility Index was calculated = [% individual motility + (quality of motility x 20)] x 0.5. The sperm suspension was also used to assess acrosome integrity and viability. Acrosomal integrity was evaluated after a 1:1 dilution in 2% glutaraldehyde in 0.165 M cacodylate/HCl buffer (pH 7.3). The percentage of spermatozoa with intact acrosomes (% NAR) was assessed by phasecontrast microscopy.

In addition, samples were taken to assess the membrane integrity by means of the hypo-osmotic swelling (HOS) test. Plasma membrane functionality was assessed using an HOS test, as described by Garde et al (1998). The osmotic swelling technique consisted of mixing 0.01 ml of diluted sperm samples with 0.1 ml of hypo-osmotic solution (100 mOsmol/kg) and incubating the mixture at room temperature for 30 minutes. The samples were then fixed in 2% glutaraldehyde buffered solution and evaluated by phase-contrast microscopy at 400x. The sperm membrane was considered functional in cases where the sperm tail was coiled, and the result was expressed as HOS (%).

Membrane integrity (viability) was also evaluated by using a nigrosin-eosin stain (NE). The NE stain was prepared according to the method of Soler et al (2005). Sperm (5 µl) was mixed with the NE stain (10 µl) at 37 °C, incubated for 30 seconds, and smeared and dried on a warm plate at 37 °C. The samples were evaluated using bright field microscopy at 400x. Live spermatozoa remained unstained, while dead cells were dull pink. The percentage of live spermatozoa was expressed as viability (%).

For sperm assessments, 100−200 spermatozoa were counted in each preparation. Additionally, slides of extended sperm samples were prepared from each sample for sperm head morphometric characterization. For each sperm parameter evaluated, a ratio was calculated to assess the cryoprotective abilities of the different individual samples, as follows:

Cryoresistance ratio =

(Value after thawing/value in fresh sperm) × 100.

Morphometric Analysis of Sperm Heads

Microscopic slides were prepared from each fresh (upon dilution) sample by placing 5 µl of the sperm samples on the clear end of a frosted slide and dragging the drop across the slide. Semen smears were air-dried and stained using a Hemacolor (Merck) procedure, originally described for staining of ram (Sancho et al, 1998) and alpaca (Buendía et al, 2002) sperm heads and recently adapted by our group to red deer spermatozoa (Esteso et al, 2003). Stained sperm samples were permanently mounted to the slide with a coverslip and dibutyl phthalate xylene.

Stained slides were used to perform ASMA using the morphometry module of a commercially available system (Sperm-Class Analyzer, version 99 CASMA system, Microptic, Barcelona, Spain). The machine was equipped with a Nikon (Labophot-2, Tokyo, Japan) microscope with a 60 x bright-field objective and a Sony video camera (CCD AVC-D7CE, Sony Corporation, Tokyo, Japan) connected to a Pentium 950 MHz processor. The illumination source was centered, and the intensity of the bulb and the gain and offset of the camera were standardized for all samples. The configuration of the computer system included a PIP-1024 B video digitizer board (Matrox Electronic Systems Ltd., Quebec, Canada), the sperm image analysis software, and a high-resolution assistant monitor Sony Trinitron PVM-1443MD (Sony). The array size of the video frame recorder was 512 x 512 x 8 bits; digitized images were made up of 262 144 pixels (picture elements) and 256 grey levels. Resolution of images was 0.15 and 0.11 µm per pixel in the horizontal and vertical axes, respectively.

The morphometric dimensions for area (A) and shape factor (length/width) were acquired for 150—160 images. Acquiring 150—160 images assures that a minimum of 145 properly measured sperm heads are analyzed after improperly measured sperm heads are deleted from the analysis. The measurements of each individual sperm head from each stag and sperm treatment were saved in an Excel (Microsoft Corporation, Redmond, Wash)-compatible database by the software for further analysis.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows, version 11.5 (SPSS Inc, Chicago, Ill). The effects of cryopreservation on sperm motility, viability, and acrosome and membrane integrities were compared across stags by a general linear model analysis of variance (GLM-ANOVA) using a split plot design. Stags served as the main plot, and sperm cryopreservation step (prefreezing or postthawing) served as the subplot. Group differences were compared by Fisher's least significant differences test. Effects were considered significant at $P < .05$.

Data of postthaw sperm motility, sperm viability, and acrosome and sperm membrane integrities obtained from each of the 38 epididymal sperm samples were used to build a single data set. The data set was subjected to a multivariate analysis as described by Esteso et al. (2003) for head sperm morphometric patterns. This procedure uses the cluster analysis to classify the sperm samples, using all measured sperm variables within the data set, on a small number of groups. Two groups were finally obtained from the nonhierarchical clustering (K-means clustering) of the 38 stag sperm samples tested. The statistics of each sperm variable of the 2 groups of males were calculated, and compared using GLM-ANOVA. Percentage data of sperm fresh parameters and sperm head area and shape in fresh samples from sperm samples clustered following the multivariate analysis were also compared using ANOVA. Data that did not follow a normal distribution were transformed. A probability of $P < .05$ was considered to be statistically significant.

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Effects of Cryopreservation

In this work, epididymal spermatozoa from 38 stags were frozen and thawed. Because we used epididymal spermatozoa for this experiment, it was only possible to make single observations for each stag. After freezing and thawing, a decrease ($P < .0001$) in all routine sperm parameters was observed (Table 1). Thus, sperm motility index decreased from 83.16 ± 1.7% in extended fresh samples to 63.91 ± 1.7% in thawed samples. The results found for the other seminal parameters evaluated are similar if not identical (Table 1). Our results also showed differences among males for most of the seminal parameters evaluated immediately after thawing (Figures 1 and 2).

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Table 1. *Effects of cryopreservation on characteristics of red deer epididymal spermatozoa**†

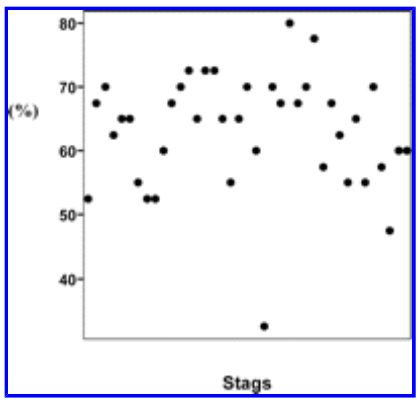

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Figure 1. Sperm motility index of thawed red deer spermatozoa collected postmortem from epididymides of different stags.

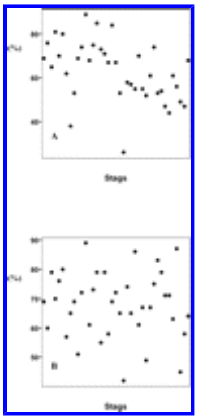

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Figure 2. Individual characteristics of thawed red deer spermatozoa collected postmortem from epididymides of different stags: **(A)** percentage of sperm cells with intact acrosomes (NAR), and **(B)** percentage of spermatozoa with intact plasma membrane (HOS).

Male Subpopulations Analysis

After the cluster analysis of frozen-thawed sperm quality, 2 groups of sperm samples (stags) were clearly identified. Those samples with the best frozen-thawed sperm characteristics were identified as "good," whereas the others represented samples showing considerably reduced frozen-thawed sperm characteristics and were considered as "bad" following a standard freezing protocol. Twenty-five samples were identified as "good" and 13 as "bad." Summary statistics for these 2 groups are shown in Table 2. Note the significant differences ($P < .01$) for all sperm parameters evaluated.

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Table 2. *Descriptors of frozen-thawed sperm characteristics in the two groups ("good" vs "bad") of single stag sperm samples defined after multivariate cluster analysis**

Sperm Quality Before Freezing

There was no significant variation between groups before freezing for all the sperm parameter evaluated (Table 3). Sperm samples from the 2 groups consisted of spermatozoa with high quality and would be expected to maintain this quality after cryopreservation.

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Table 3. *Descriptors of fresh sperm characteristics in the two groups ("good" vs "bad") of single stag sperm samples defined after multivariate cluster analysis**

Sperm Cryoresistance

As a consequence of the fact that differences in fresh sperm quality between stags exist, we also determined the cryoresistance ratios for all the sperm parameters evaluated (see "Materials and Methods"). The use of rates rather than absolute values allows for a direct comparison between males that differ in fresh semen parameters (Table 4). Note the significant differences present between groups ($P < .05$ to $P < .0001$) for all the cryosurvival ratios. These results demonstrate that the resistance to the cryopreservation was different between "good" and "bad" freezers. Good freezers had the highest ratios (range: 70%–83%) in all the parameters evaluated (sperm motility index, % endosmosis, and % intact acrosomes), with results that in all cases were significantly different from those seen in the "bad" group (range: 58%–70%).

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Table 4. *Sperm cryosurvival in the two groups ("good" vs "bad") of single stag sperm samples defined after multivariate cluster analysis**

Sperm Head Size and Shape in Fresh Samples

Data of sperm head area and shape from stags classified as "good" and "bad" freezers, evaluating 3406 and 1821 properly digitized spermatozoa respectively, revealed that the frozen-thawed sperm characteristics (base for the discrimination into 2 groups) were significantly affected by the sperm head size and shape (Table 5). There were no differences in the percentage of properly analyzed sperm heads between "good" and "bad" freezers (data not shown). Our results showed that the smallest overall sperm head dimensions in fresh samples were found in the "good" freezers group (area: 32.04 μm^2 vs 34.42 μm^2). Therefore, the lower the sperm head area in the fresh samples, the greater the sperm cryoresistance. Our results also show that the 2 groups of males differ in sperm head shape (good: 1.96 vs bad: 1.72; $P < .01$). Thus, the sperm heads in the fresh samples from the "good" freezers were more elongated and narrow than those from the "bad" group. In this sense, the sperm head length in the fresh samples from "good" freezers was approximately 2 times higher than the width. Figure 3 shows the sperm head area for each stag categorized as "good" or "bad" freezers.

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Table 5. *Sperm head area and shape factor for fresh sperm samples in the two groups ("good" vs "bad") of single stags defined after multivariate cluster analysis**†*

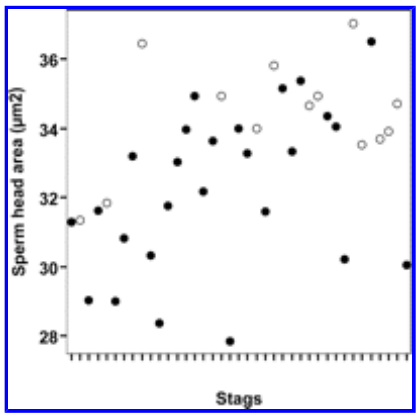

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Figure 3. Sperm head area (μm^2) for individual "good" (•) and "bad" freezers (○).

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Our previous studies utilizing ASMA have reported the normal sperm head dimensions for fresh and thawed red deer epididymal spermatozoa (Esteso et al, 2003; 2006). In the present study, the ASMA protocol used was useful to detect differences in sperm head area and shape between "good" and "bad" freezers before freezing. Thus, the lower the sperm head area in the fresh samples, the greater the sperm cryoresistance. Differences in the resistance to thawing of the spermatozoa of different individuals have been observed for spermatozoa of other domestic (Curry, 2000) and wild (Leibo and Songsasen, 2002) species. Within this context, semen donors have routinely been categorized as "good" or "bad freezers." Although similar experiences have been reported for several species, no explanations for these differences have been substantiated. The mechanisms underlying differences in cryosensitivity between different individuals have yet to be elucidated,

but there is some evidence for physiological differences between spermatozoa from individuals of the same species (Leibo and Bradley, 1999).

On the other hand, authors have been very interested in determining the relationship between single fresh semen quality characteristics such as motility, viability, morphology, or acrosome status and freezability. In any case, a single fresh semen characteristic could show a positive relationship with freezability, but the relationship was either of low significance or measurable in some males but not in others. Inclusion of several sperm variables, measured either by functional methods or by the combination of significant outcomes on a multivariate regression analysis, has been regarded as more discriminative, and in some cases even of predictive value. For more details see Rodriguez-Martinez (2003). Bearing this in mind, the present work aimed to group a series of postthaw sperm variables defining the survival status of red deer spermatozoa subjected to a conventional freezing protocol and, by using a multivariate cluster analysis as described by Esteso et al (2003) and used already by other authors to classify subpopulations of spermatozoa (Quintero-Moreno et al, 2003), defined 2 groups of stags as "good" and "bad" according to their freezability.

In this study, out of the 38 males, 25 were identified as "good" and 13 as "bad." To explore the differences between the 2 groups, conventional ANOVA analysis was undertaken, and significant differences were found for all parameters. The differences between stag groups were found for all quality parameters of thawed spermatozoa as well as the degree of declined cellular integrity by cryopreservation. However, there were no significant differences in routine semen quality between groups before freezing. On the other hand, differences in epididymal sperm head area and shape were found between "good" and "bad" freezers before freezing, with the smallest overall sperm head dimensions found in the "good" freezers group. Besides, the sperm heads in the fresh samples from the "good" freezers were more elongated and narrow than those from the "bad" group. In this sense, the sperm head length in the fresh samples from "good" freezers was approximately 2 times higher than the width. It is noteworthy that when comparisons are made among species for their ability to sustain cold shock, clear sperm differences are evident; the spermatozoa of those species less sensitive to cryopreservation are smaller (Garde et al, 2003). Obviously, many other factors are involved in cryoresistance, but we hypothesized that sperm head area and shape cause differences in heat exchange as well as in movements of water and ions. It is therefore plausible to think that spermatozoa may vary in their physical properties depending on their area and shape and that these variations are at least partially responsible for the interindividual resistance to the cryopreservation process.

This relationship between sperm head dimensions in fresh ejaculates and sperm freezability has been previously supported for boar spermatozoa (Thurston et al, 2001; Peña et al, 2005). The study of Thurston et al (2001) reported that the percentage of spermatozoa in the fresh ejaculates with slightly tapering heads was positively correlated with sperm quality after thawing. Thurston et al (2001) support the hypothesis that the interindividual variations in sperm morphology are genetically determined, and therefore those differences in sperm morphology in fresh samples that affect sperm freezability are indicative of the genetic variations responsible for the relative ability of spermatozoa to withstand freezing procedures. Here we present the hypothesis that these variations in sperm morphology among stags can influence per se biophysical characteristics of the spermatozoa that are essential for successful cryopreservation. Taken together, our results revealed that fresh spermatozoa from the "good" freezers had small and elongated (higher shape) sperm heads, whereas spermatozoa from "bad" freezers had large and wide (lower shape) sperm heads. However, there was no significant variation between stag groups before freezing for the routine semen parameters evaluated. Therefore, these 2 groups of males only differed in the dimensions of their fresh sperm heads, and it might be assumed that sperm head size and shape in spermatozoa of fresh samples were

good indicators of freezability. However, these results must be carefully interpreted, since there were sperm samples classified as "good" showing large sperm heads (>34 μm^2) and sperm samples categorized as "bad" with small sperm heads in the fresh samples (Figure 3). It seems reasonable, then, based on our results, that sperm head area and shape of fresh spermatozoa discriminate between 2 clear-cut populations whose sperm freezability is different. However, such a relationship, though it exists, is not enough to accurately estimate, much less predict, the sperm freezability of individual samples. More studies are needed in order to develop procedures for prospectively selecting sperm samples for cryopreservation.

The observation that sperm head size and shape are highly indicatives of stag sperm survival after cryopreservation is important for 2 reasons. First, it is possible that sperm head area and shape influence total sperm volume, thus causing differences in heat exchange as well as in movements of water, ions, and cryoprotectants and, in turn, in sperm freezability (Curry, 2000). Second, it suggests that sperm survival from individuals considered as "bad freezers" (with large and wide sperm heads) can be optimized by modifying either the cryoprotectant concentration or the cooling rates. Thus, previous works have reported that sperm characteristics like surface area and volume have important implications for determining optimum cooling and warming procedures for sperm cryopreservation (Curry et al, 1996). In this sense, improvements in boar sperm cryosurvival have been reported when spermatozoa were frozen at higher rates (Fiser and Fairfull, 1990). When cell suspensions are frozen, they are cooled at finite rates, often referred as slow or fast. A rate that is slow for one cell type may be rapid for a second type. The optimum cooling rate is the one at which maximum survival is observed. Thus, the optimum cooling rates for freezing human, boar, and ram spermatozoa are different (see Leibo and Bradley, 1999). Besides, sperm area and volume have important implications for determining optimum cooling procedures for cryopreservation (Curry et al, 1996). These sperm characteristics influence the rate at which the cell can lose water; and the rate at which a cell can lose water is a principal determinant of its optimum cooling rate. For reasons briefly described above, efforts to improve sperm freezing protocols have increased substantially during the past years. In this sense, we propose that sperm samples from the different stags require different cooling rates for optimal cryosurvival. Thus the results of the present study suggest that the freezability of the spermatozoa from individuals considered as "bad freezers" (with large and wide sperm heads) could be increased in the future using different cooling rates than those used in this work (20 °C/min). We base this hypothesis on the fact that cellular area and volume have important implications for determining optimum cooling rates for cryopreservation (Curry et al, 1996; Leibo and Bradley, 1999).

In summary, the results of the present study show that although a multivariate pattern analysis based on frozen-thawed red deer sperm quality attributes was able to separate "good" and "bad" freezers, and further that these populations could relate to sperm head area and shape in fresh samples, there were sperm samples outside this pattern. This situation confirms the need for caution when aiming at estimating the potential freezability of a given sperm sample by assessing only phenotypic variables of the spermatozoa. Future work will utilize ASMA to identify sperm morphometric subpopulations in epididymal fresh sperm samples and their possible relationships with freezability.

Footnotes

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