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Influence of Various Permeating Cryoprotectants on Freezability of Iberian Red Deer (*Cervus elaphus hispanicus*) Epididymal Spermatozoa: Effects of Concentration and Temperature of Addition

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With the aim of finding an ideal cryoprotectant (CPA) in a suitable concentration for red deer epididymal spermatozoa cryopreservation, we evaluated the effects of the 3 most commonly used CPAs, glycerol (GLY), ethylene glycol (EG), and propylene glycol (PG), on sperm cryoresistance. The aim of Experiment 1 was to evaluate the influence of 3 different final concentrations (3%, 6%, and 12%) of each CPA on sperm freezability. Sperm samples were diluted to a final sperm concentration of ~400 x 10^6 spermatozoa/mL with a Tris-citrate-fructose-EY extender (TCF) prior to freezing. Sperm cryosurvival was judged in vitro by microscopic assessments of individual sperm motility (SMI), viability, and plasma membrane (by means of the HOS test) and acrosome (NAR) integrities. Thawed samples were incubated at 37°C for 2 hours in the freezing medium. At the end of this incubation period, sperm suspensions were again assessed. Our results showed that 12% of any CPA was toxic to red deer epididymal spermatozoa membrane integrity ($P < .05$). Moreover, regardless of the level of CPA, results indicated that the cryoprotective effects on red deer epididymal spermatozoa of the 3 CPAs after thawing are in the following sequence: GLY > EG > PG (higher symbols mean $P < .001$). Furthermore, our results also showed an improvement in sperm parameters when the TCF diluent contained 6% of GLY. In Experiment 2 extenders were prepared using GLY 6%. This experiment was designed to investigate the effect of 2 different temperatures of GLY addition –22°C

(ambient temperature) and 5°C2 on sperm freezability. Our results showed a differential response ($P < .05$) of motility (SMI) to temperature of GLY addition before freezing, the best being 22°C (81.94 ± 2.4% vs 72.38 ± 2.4%). Although there were no statistically significant differences ($P > .05$) between the 2 temperatures of GLY addition after thawing in terms of sperm quality, after 2 hours of incubation, results tended to be better when CPAs were added at 22°C. In conclusion, our work showed the efficacy of a TCF diluent with 6% of GLY and its addition at 22°C, as an alternative to the more common 3%–4% of GLY and addition at 5°C, in red deer semen freezing protocols.

Key words: Ethylene glycol, glycerol, propylene glycol, sperm, cryopreservation

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The interest in preserving germplasm of wild deer species has resulted in 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, 2003b, 2005). However, little information has been 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. In addition, most procedures used to cryopreserve deer epididymal spermatozoa 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 membranes properties, that affect cell survival after cooling and freezing (Walton, 1930; Watson et al, 1987; Martinez-Pastor et al, 2006). Consequently, the improvement of existing protocols for cooling and freezing red deer epididymal spermatozoa is necessary.

Important factors in semen cryopreservation include cooling, freezing, thawing, and the addition of cryoprotectants (CPAs). No studies have been carried out to check the effects of different CPAs on red deer epididymal spermatozoa freezability, and the optimal CPA for these spermatozoa remains to be established. Cryoprotectants, although essential for protection against cryoinjury (Mazur, 1970), could be harmful even at relatively low concentration (Blackshaw, 1960). Many compounds have been tested for their efficacy as sperm CPAs (Molinia et al, 1994), but most semen preservation protocols still favor glycerol (GLY). In certain instances other CPAs are possibly better; for example, dimethyl sulphoxide (DMSO) was preferred for elephant spermatozoa (Jones, 1973). The choice of CPA seems to have been a matter of trial and error in nearly all investigations; this is partly because a complete and satisfactory explanation for the action of CPAs does not exist (Holt, 2000).

Cryoprotectants can also be added to the sperm samples in a separate diluent fraction (2-step dilution) or by a single addition of the medium containing the agent (one-step method). For GLY, initially it was found better to add the glycerolated diluent portion at 29 °C than at 5 °C (Blackshaw, 1960), but in a subsequent test, addition at 5 °C was more suitable (Colas, 1975). Colas (1975) suggested that GLY may be slightly toxic to spermatozoa even at a concentration of 4%, and its harmful effect is less when added at a temperature close to 0 °C. As can be seen, the effects of temperature of CPA addition on sperm quality have been studied with conflicting results.

There is currently a remarkable interest in the use of assisted reproductive technologies (ART) for the management of Iberian red deer (*Cervus elaphus hispanicus*) populations. Specifically, ART may play an important role for the purpose of ensuring genetic preservation and/or genetic progress. Both roles are becoming increasingly important as a result of the genetic isolation of wild populations within fenced game estates (Martínez et al, 2002). Deleterious effects of inbreeding have been found on some components of the fitness of hinds (Coulson et al, 1998), and also on male reproductive function in other ungulate species (Roldan et al, 1998; Gomendio et al, 2000). The

reasons stated above highlight the importance that ART may have in managing natural populations of reed deer, as is the case for Iberian red deer. Of the genetic material in cryobanks, the collection, storage, and subsequent use of spermatozoa have found the most widespread application (Watson and Holt, 2001). However, very little is known about the freezability of Iberian red deer epididymal spermatozoa, and hence the present work, as part of a larger study, was conducted to study the influence of different CPAs and factors related to them on the cryosurvival of these spermatozoa. The specific aims of the present study were to study: 1) the effects of different CPAs and concentrations on red deer epididymal sperm cryoresistance, and 2) the influence of adding GLY at 2 temperatures, 22 °C (ambient temperature) or 5 °C, on sperm freezability.

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

Stags and Preparation of Testes

For this study, we used spermatozoa recovered from the epididymides of 23 mature stags (age >5 years, weight > 145 kg) that were legally culled and hunted in their natural habitat. Sperm samples were collected from stags shot during the rutting season (September-October). Gamekeepers collected the complete male genitalia and provided the hour of death. Stags were legally culled and hunted in their natural habitat in accordance with the harvest plan of the game reserve. The harvest plans were made following Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, which conforms to European Union regulations.

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 hours after being removed. Samples were processed as soon as they arrived at the laboratory. Time elapsed between animal death and sperm recovery ranged from 3 to 6 hours, an adequate and reliable time interval for evaluating sperm parameters, because a decrease in the quality of sperm traits begins to take place 12 hours after the death of a male (Soler and Garde, 2003). 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 (Nunc, Denmark).

Collection, Processing, and Initial Evaluation of Epididymal Spermatozoa

Spermatozoa were collected from the distal portion of the epididymis according to the method used by Soler et al (2003a). After spermatozoa collection, a routine sperm evaluation was made. Sperm concentrations of the original suspensions were determined using a hematocytometer. Sperm motility and acrosomal status were assessed for each sample. The percentage of motile sperm in the sample was noted, and quality of motility was assessed using a scale of 0 (lowest: immobile or death) to 5 (highest: progressive and vigorous movement). A sperm motility index [SMI = % individual motility + (quality of motility x 20) x 0.5] was calculated following the method used by Comizzoli et al (2001a) for red deer semen. Acrosomal integrity was evaluated after a 1:20 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 phase-contrast microscopy at x400. Only samples with an initial sperm motility and NAR greater than 60%–65% were used for this study. Epididymal contents from both testicles of an individual male were pooled for processing.

The sperm samples were diluted to a final sperm concentration of ~400 x 10^6 sperm/ml with a Salamon

modified solution (Tris-citrate-fructose [TCF]-egg yolk diluent). The diluent was prepared in 2 fractions. Fraction A contained Tris (27.0 g/L), citric acid (14.0 g/L), fructose (10.0 g/L), and clarified egg yolk (20%, v/v), and had a pH = 6.8 and an osmolality of 300 mOsm/kg (Fernández-Santos et al, 2006). Clarified egg yolk was prepared as described in Holt et al (1996). Sperm dilution was performed in a 2-step procedure at room temperature, first adding Fraction A to reach up to twice the final desired sperm concentration and then a second diluent fraction (Fraction B) at a 1:1 ratio to achieve a final concentration ~400 x 10^6 spermatozoa/ml. Fraction B differed from the Fraction A in that water was replaced (0%, 6%, 12%, or 24%, v/v) with the same volume of each CPA tested (final concentration = 0%, 3%, 6%, or 12%, v/v, depending on the experimental design). This 2-step was employed to obtain the same final concentration of CPA for each stag. The second dilution was made at ambient temperature (approximately 22 °C) or at 5 °C, depending on the experimental design. When CPAs were added at 5 °C, diluted samples were previously refrigerated at 5 °C.

Cryopreservation and Evaluation of Frozen-Thawed Spermatozoa

A detailed description of methods used for sperm freezing and thawing can be found elsewhere (Fernández-Santos et al, 2006). Before freezing, sperm subsamples were assessed for sperm motility and acrosome integrity using the methods described above. 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 a HOS test as described by Garde et al (1998). The sperm membrane was considered functional in cases where the sperm tail was coiled, and the result was expressed as HOST positive (%).

Frozen semen was thawed in a water bath (37 °C) for 30 seconds and the content of the straws poured into a glass tube. Samples were evaluated for motility and membrane and acrosome integrities using the methods described above. Sperm viability was also evaluated by using a nigrosin-eosin (NE) stain. The NE stain was prepared as described in Tamuli and Watson (1994). The diluted 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 (%).

Thawed samples were incubated at 37 °C for 120 minutes without dilution (that is, in the same diluent they were frozen). At the end of this incubation, sperm suspensions were also assessed for motility, acrosome and membrane integrities, and viability as described above. Two hundred sperm cells were assessed in each sample and for each sperm evaluation technique.

Experimental Design

Experiment 1: Effects of CPA Type and Concentration on Red Deer Epididymal Spermatozoa Freezability— This experiment was conducted to evaluate the effects of the 3 most commonly used CPAs, glycerol (GLY), ethylene glycol (EG), and propylene glycol (PG), and their concentrations on red deer epididymal spermatozoa freezability. To evaluate the influence of CPA concentration, spermatozoa were frozen in 3%, 6%, and 12% final CPA concentrations. A medium without CPAs (control) was also included. The experiment was replicated with epididymal sperm samples from 13 mature stags.

Experiment 2: Effects of Temperature of Glycerol Addition on Red Deer Epididymal Spermatozoa Freezability— In Experiment 1, spermatozoa frozen in the diluent containing 6% of GLY had significantly higher post-thaw sperm parameters than those frozen in diluents containing other CPAs. Therefore, in Experiment 2 all extenders were prepared using 6% of GLY. This experiment was designed to evaluate the influence of adding penetrating CPAs at 2 temperatures, 22 °C (ambient temperature) and 5 °C, on sperm freezability. The experiment was replicated with epididymal sperm samples from 10

mature stags.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows, version 11.5 (SPSS Inc, Chicago, Ill). Data were analyzed by GLM-ANOVA procedures and were expressed as least-squares means ± SEM. Comparisons of means among treatments were performed using Duncan's multiple range tests. Differences were considered significant when *P* was less than .05.

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We obtained spermatozoa from epididymides of all stags. Thus, a total of 23 epididymal sperm samples were collected from 23 hunter-killed mature males. There were no significant differences among the different intervals elapsed between the male death and the sperm collection on sperm quality for any seminal parameter evaluated in the fresh samples (data not shown).

Experiment 1: Effects of CPA Type and Concentration on Red Deer Epididymal Spermatozoa Freezability

After cooling, SMI was higher for diluents containing 3% or 6% GLY than for the control diluent (Figure 1A). No significant differences in SMI were seen between the other diluents. At this stage, acrosome integrity was equally high in all diluents (Figure 1B), with the exception of diluent 12% GLY, which showed significantly lower values. Membrane integrity was significantly lower ($P < 0.05$) with all diluents containing 12% of each CPA (Figure 1C).

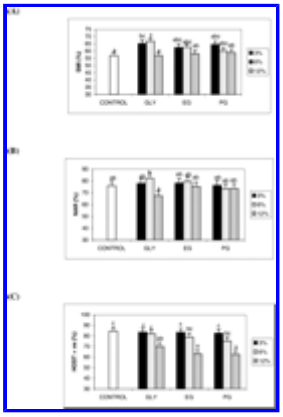

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Figure 1. Effects of type and concentration of CPA (control, 3%, 6%, and 12%) on epididymal red deer spermatozoa parameters before freezing. **(A)** Sperm motility index (SMI). **(B)** Acrosome integrity (% NAR) as the percentage of spermatozoa with intact acrosomes. **(C)** Membrane integrity (HOST +) as the percentage of spermatozoa with coiled tail (positive response) after HOS test. Values are means ± SEM (n = 13). Data are examined using ANOVA and Duncan post-hoc tests. Bars with different letters indicate statistically significant differences ($P < .05$).

After freezing and thawing, control diluent exhibited the lowest sperm parameters (Figure 2). However, diluent containing 6% GLY afforded better preservation than the other treatments. Sperm motility (SMI) was highest for 6% GLY (Figure 2A). Moreover, sperm viability (Figure 2B) and acrosome integrity (Figure 2C) were higher ($P < .05$) for diluents containing 3% or 6% GLY than for the control diluent. Membrane integrity was significantly higher ($P < .001$) with diluent containing 6% of GLY than with the control. No significant differences in membrane integrity were seen between 6% GLY, 12% GLY, and 6% EG (Figure 2D). In general terms, within each CPA an increase in CPA concentration from 3% or 6% to 12% in the TCF diluent resulted in a significant decrease in most of

the seminal parameters evaluated after thawing (Figure 2).

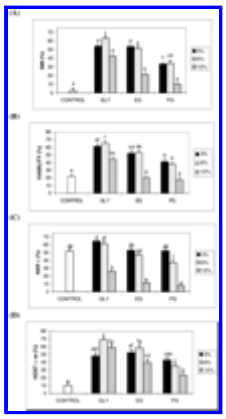

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Figure 2. Effects of type and concentration of CPA (control, 3%, 6%, and 12%) on epididymal red deer spermatozoa parameters post-thawing. **(A)** Sperm motility index (SMI). **(B)** Viability as the percentage of viable sperm. **(C)** Acrosome integrity (% NAR) as the percentage of spermatozoa with intact acrosomes. **(D)** Membrane integrity (HOST +) as the percentage of spermatozoa with coiled tail (positive response) after HOS test. Values are means ± SEM (n = 13). Data are examined using ANOVA and Duncan post-hoc tests. Bars with different letters indicate statistically significant differences ($P < .001$).

When spermatozoa were incubated for 2 hours at 37 °C, there was a decrease in most of the seminal parameters evaluated. At this stage, SMI was significantly higher ($P < .001$) for diluent containing 6% GLY than for the other diluents (Figure 3A). Sperm viability (Figure 3B) and acrosome integrity (Figure 3C) were higher ($P < .05$) for diluents containing 3% or 6% GLY than for the control diluent. Membrane integrity was significantly higher ($P < .001$) with diluents containing 6% or 12% GLY than with the control diluent (Figure 3D).

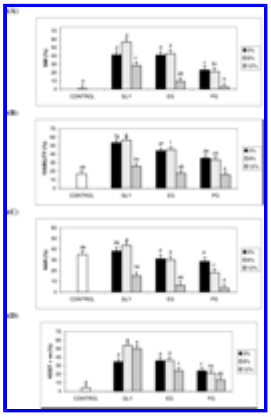

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Figure 3. Effects of type and concentration of CPA (control, 3%, 6%, and 12%) on epididymal red deer spermatozoa parameters after post-thawing incubation during 2 hours at 37°C. **(A)** Sperm motility index (SMI). **(B)** Viability as the percentage of viable sperm. **(C)** Acrosome integrity (% NAR) as the percentage of spermatozoa with intact acrosomes. **(D)** Membrane integrity (HOST +) as the percentage of spermatozoa with coiled tail (positive response) after HOS test. Values are means ± SEM (n = 13). Data are examined using ANOVA and Duncan post-hoc tests. Bars with different letters indicate statistically significant differences ($P < .001$).

In summary, the diluent containing 6% of GLY afforded the best sperm cryopreservation for red deer epididymal spermatozoa, with the opposite being true for diluents containing 12% of the different CPAs and for the control (without CPAs).

At the 3 stages of the freezing-thawing procedure, there were no significant ($P > .05$) interactions between CPA type and concentration in any of the tested parameters. Thus, the results for each CPA type tested were similar; the higher concentration of CPA evaluated (12%) yielded lower sperm

parameters than the other 2 (3% or 6%), as can be seen in Figures 2 and 3 for all the semen parameters evaluated after thawing and after 2 hours of post-thawing incubation at 37 °C, respectively. Thus, Figure 4 shows the results of the influence of the different CPAs on the parameters evaluated upon thawing (Figure 4A) and after 2 hours of post-thawing incubation at 37 °C (Figure 4B), regardless of the 3 different final concentration of CPAs tested. Overall, our results indicate that GLY afforded better cryopreservation than the other CPAs tested. Therefore, we report that the cryoprotective effects on red deer epididymal spermatozoa of the 3 CPAs tested are in the following sequence: GLY > EG > PG (higher symbols mean $P < .001$).

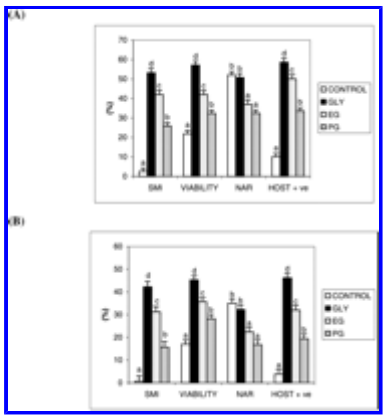

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Figure 4. Effects of type of CPA (control without CPA, GLY, EG, and PG) on epididymal red deer spermatozoa parameters. **(A)** After thawing. **(B)** After 2 hours of incubation at 37°C after thawing. Values are means ± SEM (n = 13). Data are examined using ANOVA and Duncan post-hoc tests. Bars with different letters indicate statistically significant differences ($P < .001$).

Experiment 2: Effects of Temperature of Glycerol Addition on Red Deer Epididymal Spermatozoa Freezability

In Experiment 1, the diluent containing 6% of GLY afforded the best sperm cryopreservation for red deer epididymal spermatozoa. Therefore, this experiment was designed to investigate how 2 different temperatures of GLY addition 222 °C (ambient temperature) and 5 °C2 affected the post-thaw quality of cryopreserved red deer epididymal spermatozoa. The results of the ANOVA (Figure 5) showed that there was no effect ($P > .05$) of temperature of GLY addition in most of the seminal parameters evaluated, except for the SMI ($P < .05$) before freezing (Figure 5A). Thus, there was no effect of treatment on sperm parameters immediately after thawing (Figure 5B). Besides, there was no effect of treatment on sperm parameters after 2 hours of incubation at 37 °C, although there was a trend ($P = .08$) towards better sperm cryopreservation when GLY was added at 22 °C.

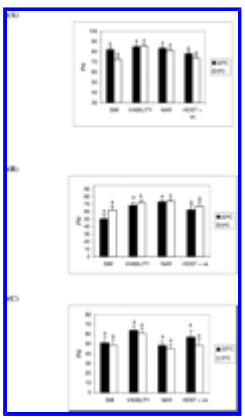

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Figure 5. Effects of temperature of CPA addition (22°C and 5°C) on epididymal red deer spermatozoa parameters. **(A)** Before freezing. **(B)** Post-thawing. **(C)** After 2 hours of incubation at 37°C after thawing. Values are means ± SEM (n = 10). Data are examined using ANOVA and Duncan post-hoc tests. Bars with different letters indicate statistically significant differences ($P < .01$).

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Permeating CPAs, although essential for protection against cryoinjury, could be harmful even at relatively low concentration (Watson, 1995). Causes of CPA-induced injury to biological systems could be osmotic, biochemical, or physicochemical. In addition, it has recently been demonstrated that red deer spermatozoa from different sources (epididymis vs ejaculate) may have different responses to permeating CPAs (Martinez-Pastor et al, 2006). Thus, in a recent work (Martinez-Pastor et al, 2006) we have noticed some interesting differences regarding extender suitability for red deer sperm cryopreservation, depending on sample source (ejaculate or epididymis). Although these earlier results are valuable for developing new protocols for the cryopreservation of red deer epididymal spermatozoa, there has been no other effort to evaluate the efficacy of different CPAs or CPA concentrations on red deer epididymal spermatozoa cryosurvival. To our knowledge, this study represents the first systematic effort to evaluate the effects of different permeating CPAs and factors related to them on red deer epididymal spermatozoa cryosurvival.

The type and amount of permeating CPAs are well known to affect the outcome of spermatozoa cryopreservation (Salamon and Maxwell, 1995, 2000; Leibo and Bradley, 1999; Watson and Holt, 2001) and thus deserve consideration. Among CPAs, GLY is considered as the most effective for ruminants (Leibo and Songsasen, 2002). For ungulates, reported concentrations range between 4% to 8% in diluents employed (Holt, 2001; Leibo and Songsasen, 2002), but results seem to depend rather on other components or on the followed protocols than on GLY concentration. We sought to improve freezing protocols by modifications of a TCF diluent employed previously in this species (Fernández-Santos et al, 2005, 2006), evaluating the type and concentrations of CPAs, or modifying the temperature of GLY addition. The results of this study are discussed here.

The results of this study indicate that GLY yields the best cryoprotection for red deer epididymal spermatozoa. Ethylene glycol is significantly worse in efficiency than GLY but better than propylene glycol. Propylene glycol resulted in the poorest protection for spermatozoa among these 3 CPAs. These findings are basically consistent with those of De Leeuw et al (1993) for bull spermatozoa. Similar results have also been recorded for ram thawed spermatozoa (Salamon and Maxwell, 1995). In summary, several agents have been examined for their cryoprotective action on most mammalian spermatozoa, but none has proved better than GLY (Watson, 1995). Rabbit (Wales and O'Shea, 1968) and elephant (Jones, 1973) spermatozoa are 2 of the few exceptions in which DMSO and not GLY is the preferred CPA. Furthermore, Mantovani et al (2002) have suggested ethylene glycol as an alternative to the more common GLY as a CPA for stallion semen cryopreservation, when used in conjunction with a milk-egg yolk extender (Palmer, 1984).

The mechanisms of permeation of CPAs into sperm are still not known. According to Gilmore et al (1997), the optimal CPA is one that can permeate the cell in the shortest period of time, causing the least amount of volume excursion during its addition and removal. Therefore, the cryoprotective action of a CPA depends on its permeability coefficient. According to the study of Gao et el (1995), approximately 60% of human spermatozoa exposed to GLY will lose their motility when their volume

decreases to 0.68 times or exceeds 1.38 times their iso-osmotic volume. However, permeability to CPAs is likely to be different among species, since it depends on the structure and composition of the membrane and in fact, while DMSO had a lower permeability than GLY in human and boar spermatozoa (Gilmore et al, 1995, 1998), its results were similar to those of GLY in the dog (Thirumala et el, 2003). No studies have been carried out to evaluate the permeability of different CPAs in epididymal red deer spermatozoa.

In the present study, viability was damaged dramatically in spermatozoa cryopreserved with propylene glycol (Figure 4), but this damage was less compared with the damage of motility and plasma membrane (as measured using the HOS test). This finding implies that apart from sperm viability, other components of sperm having an effect on sperm motility, such as tail membrane and mitochondria, may be damaged more severely. Another surprising finding is that there were no significant differences in acrosomal integrity for spermatozoa cryopreserved either with propylene glycol or ethylene glycol. This finding indicates that the sperm acrosome is the most resistant to freezing compared with motility or plasma membrane; meanwhile, it indicates indirectly that sperm motility is the most convincing parameter for evaluation of sperm function.

Another critical factor in freezing mammalian spermatozoa is the cell's tolerance to the cryoprotective levels of CPAs (Holt, 2000, 2001). In this study, we have evaluated the influence of 3 different final concentrations (3%, 6%, and 12%) of each CPA on sperm freezability. The results from the 3 CPAs tested are similar; the highest CPA levels (12%) yielded the lowest sperm freezability (Figure 2). It is well known that these adverse effects of CPAs need not necessarily be immediately apparent. The results of sperm incubation after thawing (Figure 3) clearly confirm the above idea. Our results show that sperm parameters decreased as a result of incubation for 2 hours at 37°C, especially when spermatozoa were frozen with 12% of each CPA (Figure 3). These results confirm that CPAs when used at high concentrations have a toxic effect on sperm cells (Blackshaw, 1960). On the other hand, in the present study the concentration of the different CPAs in the TCF diluent was found to affect sperm freezability, with better results obtained with 3%–6% than with 12%, although no effect of CPA concentration was seen for all sperm parameters evaluated. In addition, our results show that the diluent containing 6% GLY afforded better preservation than the other treatments.

In relation with the effects of GLY concentration on epididymal red deer spermatozoa characteristics after thawing, our results show that sperm freezability was significantly higher in sperm samples frozen with 6% of GLY than in those frozen with 3% or 12%. When spermatozoa were incubated for 2 hours at 37°C, these differences were maintained (Figure 3). The great differences found in the comparison of the 3 GLY concentrations indicate that epididymal red deer spermatozoa have a narrow margin of tolerance to GLY. These results are contradictory to published data on red deer semen, both from epididymal and ejaculated sources, which have reported that successful cryopreservation is possible using a wide range of GLY concentrations (from 3% to 10%) (Zomborszky et al, 1999; Soler et al, 2003a; Cheng et al, 2004; Martinez-Pastor et al, 2006). Recently, Cheng et al (2004), working with electroejaculated semen from *Cervus nippon* and *Cervus unicolor*, tested 5 different diluents with GLY concentrations ranging from 5% to 8%, and their results suggested that the efficacy of each extender was rather due to the presence of specific components and their interactions than to GLY concentration alone. Hence, spermatozoa from different species and sources may have different responses to these permeating CPAs. Additionally, different freezing techniques may interfere with different CPAs. From these points, we can see that results of sperm cryopreservation in red deer might vary greatly because of variation among semen donor individuals and different semen cryopreservation methods. This might be one of the reasons why it is not easy to repeat the results of other teams.

In our study we have analyzed extenders varying solely in CPA types and concentrations in order to isolate these factors. It seems that, considering epididymal spermatozoa, effectively 6% GLY yielded higher sperm viability at thawing. Taking into account the importance of this parameter, high GLY concentrations may be adequate in formulations similar to ours. Nevertheless, as the study of Cheng et al (2004) suggests, GLY effects may be modulated by other components of the extender, making it necessary to test not only a wider range of GLY concentrations but also its effect when used in different kind of extenders.

The other factor we studied, temperature of GLY addition, is of great importance for sperm cryopreservation. It seems that, effectively, epididymal spermatozoa from red deer can be cryopreserved almost equally well when GLY was added at 5 °C or at 22 °C (ambient temperature), the later being preferred attending to its tendency to improve all post-thaw sperm parameters after 2 hours of incubation at 37 °C. It was expected that exposure of red deer epididymal spermatozoa to GLY at ambient temperature would markedly reduce sperm motility and acrosomal integrity post-thawing. However, there were no differences between addition of GLY at ambient temperature or at 5 °C on red deer spermatozoa freezability. Permeability of GLY at ambient temperature is relatively high, and it may be that damage induced by GLY entering sperm cells at ambient temperature is only slightly confounded by prolonged exposure. Regardless, this unexpected result was favorable for field-friendly red deer sperm cryopreservation systems, where samples may need to be diluted with GLY at ambient temperature before the initiation of the cooling process.

Previous studies have also not found differences between temperatures of CPA addition on sperm quality (Maxwell and Salamon, 1979; Cochran, 1984). However, it has been reported in several species that the temperature of the addition of the CPAs can affect post-thaw sperm quality (Salisbury et al, 1978; Parks and Lynch, 1992; Sieme et al, 1998; McGonagle et al, 2002). As can be seen, the effects of temperature of CPA addition on sperm quality have been studied with conflicting results. These contradictory results could be due to at least 2 factors. Firstly, these differences could be due to species differences in resistance of sperm to CPA addition at different temperatures. Another possible factor responsible for the differences above cited is the composition of the extender employed. In general, when the extender used is an egg yolk-Tris diluent, the best results were obtained when CPAs were added at room temperature, the opposite being true when sperm was diluted in an egg yolk-citrate medium (Foote, 1982; Thun et al, 2002). Perhaps if we had used other extenders, we would have obtained other results.

In conclusion, the results of this study demonstrate that a Tris-egg yolk diluent containing 6% of GLY significantly improves post-thaw epididymal red deer sperm parameters. Additionally, there were no differences between addition of GLY at ambient temperature and at 5 °C on red deer spermatozoa freezability. Therefore, we assert that the knowledge gained in this study was valuable for improving new protocols for the cryopreservation of red deer epididymal spermatozoa, especially under field conditions where it is not always possible to optimally freeze epididymal spermatozoa from Iberian red deer due to a lack of technicians and equipment. Nevertheless, further studies must be carried out in order to confirm with artificial insemination trials the results here reported, especially those related to the temperature of GLY addition. Our group is carrying out further experiments in order to assess the effects of GLY addition at ambient temperature on the fertility of thawed red deer epididymal spermatozoa.

Footnotes

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