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Effects of Thawing Procedure on Postthawed In Vitro Viability and In Vivo Fertility of Red Deer Epididymal Spermatozoa Cryopreserved at −196°C

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ABSTRACT: In this study, we have determined the effects of individual factor and thawing procedure on in vitro viability and in vivo fertility of frozen-thawed red deer epididymal spermatozoa. The spermatozoa that were collected from 13 Iberian deer stags were diluted at room temperature in a Triladyl®-20% egg yolk medium and frozen in nitrogen vapors. In the first experimental series, sperm samples were collected from 10 mature stags. For thawing, the frozen straws were subjected to 3 different procedures: I (37°C for 20 seconds), II (60°C for 8 seconds) and III (70°C for 5 seconds). Sperm cryosurvival was judged in vitro by microscopic assessments of individual sperm motility (SM) and of plasma membrane and acrosome (NAR) integrities. Statistically significant variations were found ($P < .05$) between stags for most of the seminal parameters evaluated. The thawing procedure did not have an effect on the seminal characteristics evaluated after this process, except for SM ($P < .05$), with the best overall recovery rates after freezing and thawing found with the use of protocol I. Our results also show a differential resistance to return to isosmotic conditions of spermatozoa thawed using the different thawing protocols. In the second experimental series (insemination artificial trial), with spermatozoa from 3 stags, results of fertility were statistically higher (69.7% vs 42.4%, $P = .014$) when spermatozoa were thawed at 37°C for 20 seconds than were warmed at 60°C for 8 seconds. Therefore, thawing protocol I, which provides slow thawing rates, was the most beneficial for epididymal spermatozoa thawing of the cervid subspecies analyzed in this study. In summary, high in vitro survival and in vivo fertility of frozen-thawed deer epididymal spermatozoa were dependent on warming rates, but each stag exhibited its own sensitivity to cryopreservation.

Key words: *Cervus elaphus hispanicus,* freezing, membrane integrity, sperm motility, postmortem.

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Cryopreservation of gametes and embryos and the development of Genetic Resource Banks (GRB) allow us to have a gene resource for an indefinite time (Watson and Holt, 2001). These assisted reproductive technologies (ART) are potentially capable of improving the propagation and conservation of wild and endangered species (Wildt et al, 1997). Of the genetic material in cryobanks, the collection, storage, and subsequent use of spermatozoa has found the most widespread application (Watson and Holt, 2001). According to this, cryopreservation of spermatozoa combined with artificial insemination (AI) has been the method of ART that has been most extensively applied to deer species (Asher et al, 2000).

Actually, there is a remarkable interest in the use of ART for the management of Iberian 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 games states (Martinez 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). In this situation, semen conservation of Iberian deer offers the possibility of genetic variability preservation via biotechnological reproduction programs. Thus, the global objective of our investigations is to create the potential for the establishment of a GRB in Iberian deer.

Studies by Wildt et al (1986) revealed that postmortem seminal recovery of functional sperm from the epididymides is quite useful, particularly in those species in which semen collection involves several difficulties. Furthermore, in many cervid species, hunter-killed males may represent a readily available source of sperm for captive breeding programs and conservation of the genetic variability (Hishinuma et al, 2003). Such is the case of the Iberian deer, which is a subspecies of red deer inhab-

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iting only areas of Spain and Portugal. The preservation of the subspecies is of great interest to the wildlife conservationists. In addition, in the wild, threatened or endangered species of animals may die unexpectedly, some naturally and others from poaching. Recovery and cryopreservation of their epididymal spermatozoa would be one useful way to rescue the germplasm of dead animals and use it to preserve endangered species.

As a consequence of the references cited above, 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, 2003a). There have been many studies of freezing ejaculated semen from stags (Asher et al, 2000), but few have been reported for epididymal spermatozoa. At present, most investigators who freeze deer semen do so in straws of 0.25 or 0.5 mL, subsequently thawing these at temperatures that vary between 35°C to 65°C and times from 6 to 60 seconds (Jacobson et al, 1989; Haigh and Bowen, 1991; Haigh et al, 1993; Monfort et al, 1993; Zomborszky et al, 1999). Most procedures used to cryopreserve epididymal spermatozoa from deer have been modified from those developed for ejaculated semen. This approach would not seem to be very appropriate because it is well known that there are important differences in the physiological characteristics of epididymal vs ejaculated spermatozoa, specifically in their membrane properties, which affect the cell survival after cooling and freezing (Hammond, 1930; Walton, 1930; Watson and Morris, 1987; Rath and Niemann, 1997; Gilmore et al, 1998a; Lambrechts et al, 1999). Furthermore, epididymal spermatozoa have not been exposed to the complex secretions of the accessory sex glands; these secretions alter both the chilling sensitivity and the freezing resistance of ejaculated spermatozoa (Viruela and Rajaniemi, 1983; Schmehl et al, 1986). Consequently, the improvement of existing protocols for freezing and thawing deer epididymal spermatozoa is necessary.

No studies have been carried out on semen thawing in deer, and the optimal thawing procedure for epididymal deer spermatozoa frozen in straws remains to be established. The thawing procedure is important for the successful cryopreservation of mammalian spermatozoa. The temperature and osmotic variations involved in the thawing of frozen semen inevitably reduce the proportion of viable sperm and cause ultrastructural, functional, and biochemical damage (Mazur, 1984). The resistance of spermatozoa to the thawing process is dependent on the basic extender used and the concentration of cryoprotectant as they interact with the freezing and thawing rates (Curry and Watson, 1994; Curry 2000). This sensitivity of the spermatozoa to thawing temperatures appears to differ between species (Fiser et al, 1981; Arav et al, 1994; Söderquist et al, 1997; Younis et al, 1998; Eriksson et al, 2000; Sukhato et al, 2001; Soler et al, 2003b) and between reproductive tract sources as has been shown in African buffalo sperm samples (Lambrechts et al, 1999). The effect of reproductive tract source (ejaculated vs epididymal) on sperm resistance to the thawing process might be related to different environment conditions between epididymal and ejaculated spermatozoa. Thus, the medium in the caudal portion of the epididymis is characterized by low pH (Carr et al, 1985), hyperosmotic pressure of the fluid (Cooper, 1998; Liu and Foote, 1998), and low oxygen tension (Free et al, 1976). This emphasizes the need for developing specific protocols for thawing deer epididymal spermatozoa, applicable to both laboratory and field conditions, that would substantially increase utilization of the technology.

One aspect of sperm cryobiology that is different from other types of cells is the fact that spermatozoa from different individuals may exhibit significantly different responses to the same freezing treatment (Holt, 2000). The males exhibited different susceptibilities to freezing and thawing procedures, independent from initial quality. These differences in the resistance to thawing of the spermatozoa of different individuals have been observed for spermatozoa of domestic (see Curry, 2000) and wild (see Leibo and Songsasen, 2002) species. The mechanisms underlying differences in cryosensitivity among different individuals have yet to be elucidated, but there is some evidence for physiological differences between spermatozoa from individuals of the same species. However, results reported for boar spermatozoa (Rath and Niemann, 1997) have demonstrated that such individual differences in sperm cryoresistance were evident for ejaculated semen but not for epididymal samples.

Therefore, the objective of the present study was to evaluate the effects of individual factor and thawing procedure on resistance to cryopreservation of epididymal deer spermatozoa obtained postmortem from Iberian stags. To achieve this goal, we evaluated the effects of various thawing procedures on in vitro sperm characteristics and on in vivo fertility.

Methods

All chemicals were of reagent grade and were purchased from Sigma or Merck (both in Madrid, Spain). Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation, RD223/1988, which conforms to European Union Regulation 86/609 and adheres to guidelines established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the American Society of Andrology.

Stags and Preparation of Testes

For this study, we used spermatozoa recovered from the epididymides of 13 mature stags (age > 4.5 years, weight > 130 kg) that were 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 the death and a tooth to assess the age of the individuals. 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 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 (Nunc, Glastrup, Denmark).

Collection and Initial Evaluation of Epididymal Spermatozoa

After spermatozoa collection, a routine semen evaluation was made. Sperm concentrations of the original suspensions were determined using a hematocytometer. Sperm motility and acrosomal status were assessed for each sample. Percentage of individual sperm motility (SM) was noted, and quality of motility was assessed using a scale of 0, lowest, to 5, highest. Acrosomal integrity was evaluated after a 1:10 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 ×400. Only samples with an initial sperm motility and NAR greater than 65–70% were used for freezing.

Spermatozoa were collected from the distal portion of the epididymis as described by Soler et al (2003a). The sperm mass was diluted to a sperm concentration ~400 × 10^6 sperm/mL with Triladyl® (Minitüb, Tiefenbach, Germany)—20% egg yolk medium (containing 6% glycerol) rewarmed to room temperature. Samples obtained from different epididymides from the same individual were mixed and manipulated jointly.

Cryopreservation and Assessment of Frozen-Thawed Spermatozoa

The diluted sperm suspension in a 15-mL centrifuge tube (Iwaki, Tokyo, Japan) was placed in a beaker with water (75 mL at room temperature) and transferred to a refrigerator at 5°C. Diluted samples were refrigerated slowly at 5°C for 1.5 hours, equilibrated at that temperature for 2 hours, and loaded into 0.25 mL straws (100 × 10^6 sperm/straw). The straws were frozen in nitrogen vapors 4 cm above the surface of the liquid nitrogen for 10 minutes and then plunged into liquid nitrogen. The straws remained for a minimum period of 1 year in liquid nitrogen before thawing was carried out.

The straws were thawed in a water bath and contents poured into a glass tube and assessed for SM and acrosome integrity, as described above. Samples were also taken to assess membrane integrity by means of the hypo-osmotic swelling test (HOST).

Plasma membrane integrity was assessed using a HOST 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 ×400. The sperm membrane was considered intact in cases in which the sperm tail was coiled.

Thawed samples were incubated at 37°C for 120 minutes without dilution (ie, in the diluent). At the end of this incubation, sperm suspensions were again assessed for motility (SM_{120}), acrosome integrity (NAR_{120}), and membrane integrity ($HOST_{120}$), as described above. In addition, upon thawing, semen was diluted in a modified Tyrode medium. SM was also determined as indicator of sperm quality. For that, 25 μL of thawed semen were diluted with 75 μL of the medium whose composition was: 120 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 20 mM HEPES, 5 mM glucose, 21.7 mM sodium lactate, 1 mM sodium pyruvate, 20 μg phenol red, and 5 mg bovine serum albumin (290 ± 5 mOsm). The medium had a pH 7.55 (adjusted with NaOH) at room temperature. A rate that would yield information on survival after dilution of thawed spermatozoa in Tyrode medium was calculated: dilution survival rate = value after dilution in Tyrode medium/value of thawed sample × 100.

Artificial Insemination Trial

The trial was conducted at the experimental farm of the ETSIA in Albacete (Spain) with insemination carried out during late September. Sixty-six Iberian deer hinds (4–6 years old; 75–101 kg live weight, mean 88.4 ± 6.3 kg) were housed in outdoor enclosures that provided exposure to natural fluctuations in light and temperature. The estrus of hinds was synchronized as described by Fennessy et al (1990). Briefly, single controlled internal drug releasing (CIDR) devices (type G, 330 mg progesterone per device, InterAg Effective Agricultural Systems, Hamilton, NZ) were inserted intravaginally for a total period of 12 days. The devices were replaced with a new one in each animal on day 9 to ensure that progesterone concentration remained high throughout the CIDR device insertion period. At CIDR withdrawal the hinds received 225 IU equine chorionic gonadotropin (Folligon, Intervet, Salamanca, Spain) intramuscularly. The hinds were inseminated with 100 × 10^6 total spermatozoa directly into the uterus at around 54 hours after CIDR devices withdrawal using laparoscopy.

The inseminations were performed as described by Haigh and Bowen (1991). For laparoscopic inseminations, the hinds were sedated with an intravenous injection of xylazine hydrochloride (Rompun, Bayer, Madrid, Spain; 0.8 mg/kg live weight) and ketamine hydrochloride (Imalgene, Leti & Merieux, Madrid, Spain; 2.0 mg/kg live weight). Following insemination, sedation was reversed with an intravenous injection of yohimbine hydrochloride (Sigma, Madrid, Spain; 0.3 mg/kg live weight). The total procedure, from administration of sedatives to complete reversal, lasted 5–7 minutes per hind. Fertility (proportion of hinds that calved) was calculated for each stag and thawing procedure from

Table 1. *Effects of thawing procedure on characteristics of cryopreserved epididymal red deer spermatozoa after thawing or a 120-min incubation in the freezing diluent**

Thawing Procedure	Assessed Parameters†					
	‡SM	‡NAR	‡HOST	‡SM_{120}	‡NAR_{120}	‡$HOST_{120}$
37°C/20 sec	76.8 ± 1.8[a]	79.5 ± 1.6[a]	62.7 ± 2.8[a]	58.6 ± 2.9[a]	61.7 ± 2.7[a]	45.1 ± 2.8[a]
60°C/8 sec	69.5 ± 1.8[b]	77.3 ± 1.6[a]	58.8 ± 2.8[a]	51.0 ± 2.9[a]	59.0 ± 2.7[a]	37.0 ± 2.8[a]
70°C/5 sec	70.6 ± 1.8[b]	79.1 ± 1.6[a]	60.3 ± 2.8[a]	50.8 ± 2.9[a]	58.2 ± 2.7[a]	38.8 ± 2.8[a]

* Data are expressed as least squares means ± SEM.
† Values with different superscripts within each column are significantly different ([ab]$P < .05$).
‡ See "Methods" for details of frozen-thawed spermatozoa assessment.

the calving records of individual hinds obtained the following spring.

Thawing Procedures and Experimental Design

In the first experimental series, 90 straws of 10 stags (i.e., 9 straws/stag) were thawed at either of 3 warming procedures: slow (I: 37°C for 20 seconds), intermediate (II: 60°C for 8 seconds), or rapid (III: 70°C for 5 seconds), and reached the following thawing rates respectively: I) 1359°C/minute, II) 1891°C/minute and III) 2715°C/minute. Thawing was carried out by placing the straws in water baths containing water at different temperatures. After thawing, the straws were dried and the samples were poured into previously warmed test tubes (37°C) and the sperm was allowed to equilibrate for 5 minutes before immediately post thaw evaluation. Three straws from each epididymal specimen (i.e., stag) were used, one for each of the described thawing protocols. The entire experiment was repeated 3 times; thus, triplicate independent straws of spermatozoa from each stag for each thawing procedures were assessed for motility, acrosome status, and membrane integrity. Two hundred sperm cells were assessed in each sample and for each sperm evaluation technique.

In the second experimental series, we developed an artificial insemination trial with sperm samples from 3 stags that were processed as described above and thawed using 2 different protocols. The purpose of this experiment was to compare the fertility of cryopreserved spermatozoa thawed at either of 2 warming procedures: slow (I: 37°C for 20 seconds) or intermediate (II: 60°C for 8 seconds). Sperm motility in semen samples obtained from the same stags and straws as used for AI was estimated. Fertility was assessed by inseminating cryopreserved spermatozoa into estrus synchronized hinds. We processed the semen using a split-sample design to provide an equal distribution of the two different thawing procedures (i.e., I and II) for each stag (A, B, C). Prior to insemination, the straws of each stag were immersed into water baths at either of 2 temperatures (37°C or 60°C). The hinds were inseminated at random, and we attempted to use an equivalent number of AIs/stag and thawing protocol.

To monitor the thawing temperature inside the straw during and after the thawing procedures, a type-IT23 copper-constantan thermocouple (0.07 mm, Physitemp Instruments Inc, Clifton, NJ) was placed in the center of a straw and connected to a temperature recorder (DuaLogR model 91100-50, Cole-Parmer Instrument Co, Vernon Hills, Ill) during freezing and thawing. The temperature of the semen inside the straw during thawing was recorded several times for each thawing procedure. The average temperature registered in the straws in the thermal bath was 23°C, 25°C, and 24°C when using thawing procedures I, II, and III, respectively. Additionally, the temperature in the straw continued to increase once taken out of the bath until it reached 32°C, 32°C, and 28°C, respectively, for these thawing protocols. These temperature recordings were highly repeatable.

Statistical Analysis

The statistical analysis was performed with the use of the General Linear Model procedures of the SPSS version 10.0 (SPSS Inc, Chicago, Ill). Data were expressed as least squares means ± SEM. In the first experimental series, an ANOVA tested the effects on sperm characteristics of thawing procedure (I, II, and III), male (1, 2, 3, 4, 5, 6, 7, 8, 9, and10) and interaction between male and thawing procedure. Comparisons of means among treatments were performed using the Duncan's multiple range tests, except for the sperm motility determinations that were analysed by the Least Significant Differences test. In the second experimental series, fertility data were analyzed by an ANOVA. The statistical model included the effects of stag (A, B, C), thawing procedure (I, II) and interaction between male and thawing procedure.

Results

Experimental Series 1

In this experimental series, epididymal spermatozoa from 10 stags were frozen and thawed at 3 different warming rates. Because we used epididymal spermatozoa for these experiments, it was only possible to make single observations for each stag with triplicate straws subjected to each treatment. The data of SM and NAR of deer spermatozoa after different stages of cryopreservation are presented in Figure 1. Percentage of motile sperm was 92.0% ± 3.8% in fresh samples, reduced ($P < .05$) to 72.0% ± 2.9% after freezing and thawing, and then to 53.5% ± 3.9% after a 120-minute incubation at 37°C. Spermatozoa with normal acrosomes decreased ($P < .05$) after freezing and thawing and again decreased after the sperm incubation.

The results of the ANOVA showed significant differences ($P < .01$) among males for most of seminal param-

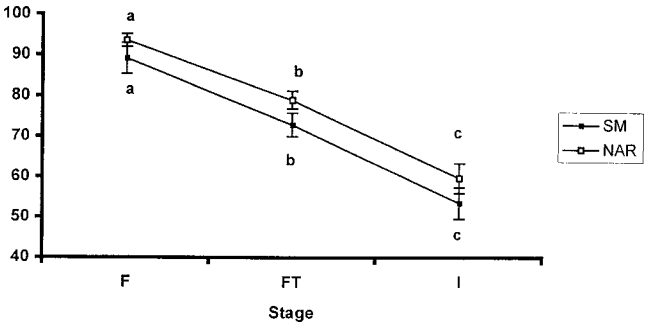

Figure 1. Changes (mean percentage ± SEM) in percentages of SM and normal acrosomes of epididymal red deer (n = 10) spermatozoa after recovery (fresh = F), freezing, and thawing (FT) and after a 120-minute incubation at 37°C (I). Means with different letters differ significantly ($P < .05$).

eters evaluated immediately after thawing (Figures 2 and 3) and after a 120-minute incubation at 37°C (Figure 4). However, our results showed that no significant differences were found ($P > .05$) in the seminal parameters for the different thawing procedures tested, except for the SM, after thawing ($P < .05$) and dilution in Tyrode medium ($P < .0001$) and for the dilution survival rate ($P < .0001$), in which the best overall recovery rates were found with the use of protocol I. Finally, the interaction between male and thawing procedure did not significantly affect ($P > .05$) sperm characteristics assessed.

Regardless of the thawing protocol, the results of the influence of the individual factor on the parameters evaluated upon thawing sperm samples are shown in Figures 2 and 3. For each parameter, bars represent means (least squares means ± SEM) from each male for the 3 thawing protocols tested. SM (Figure 2A and B) and especially the dilution survival rate (Figure 2C) from different stags were much more variable than membrane (Figure 3A) and acrosome (Figure 3B) integrities. In this sense, NAR varied only from ~ 70 to ~ 85%. In contrast, SM and dilution survival rate oscillated significantly among the 10 stags. Thus, after dilution in Tyrode medium, individual sperm motility percentages varied from ~20 to ~60%. Similarly, dilution survival rate varied from ~28 to ~81%. The results found after a 120-minute incubation at 37°C (Figure 4) are similar if not identical; for most of the seminal parameters evaluated after incubation, significant differences among stags were seen. Such differences between males were higher for SM than for membrane or acrosome integrities.

The effects of the thawing procedure on sperm characteristics are shown in Table 1. The percentages of membrane and acrosome integrities at thawing were slightly superior although nonsignificant ($P > .05$), when the slow procedure was used (37°C for 20 seconds). Moreover, significant differences ($P < .05$) among the 3 thawing procedures were found for SM, with best overall recovery

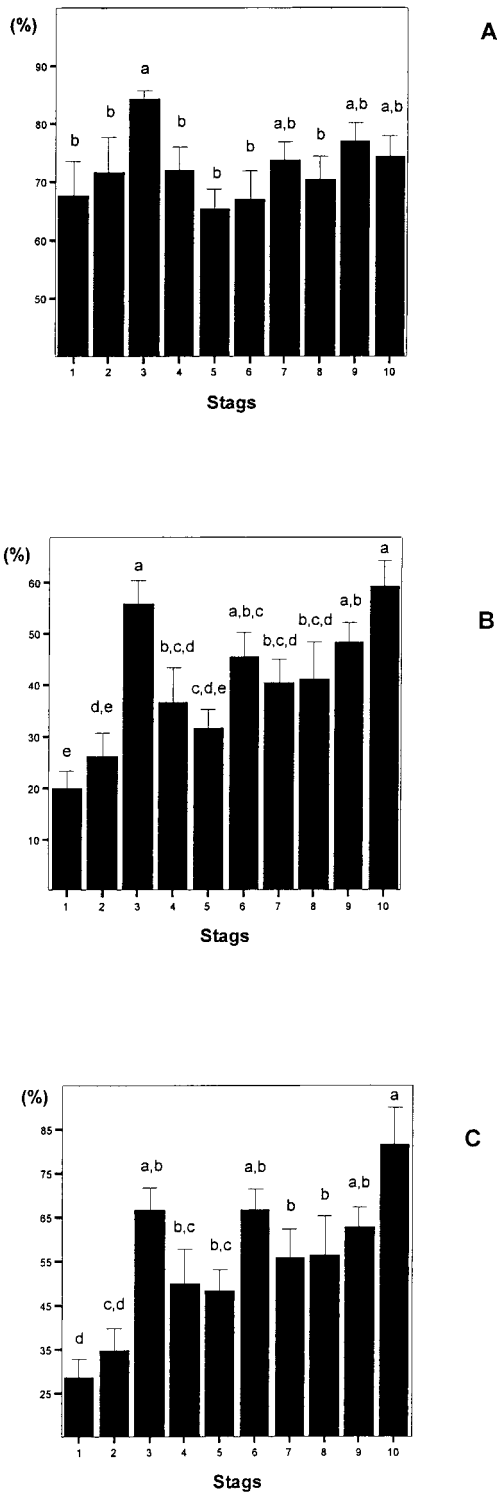

Figure 2. Freezability (mean percentage ± SEM) of epididymal sperm samples of 10 stags after their spermatozoa were cooled, stored in LN_2 and then thawed at each of 3 procedures. Triplicate independent straws of spermatozoa from each stag for each thawing procedures were assessed. Columns represent for each parameter the average of the 3 different thawing procedures. **(A)** Percentage of motile sperm after thawing in the freezing diluent. **(B)** Percentage of motile sperm after dilution of thawed sperm in Tyrode medium. **(C)** Diultion survival rate for the percentage of motile sperm. For each panel, bars with different letters indicate statistically significant differences among stags ($P < .01$).

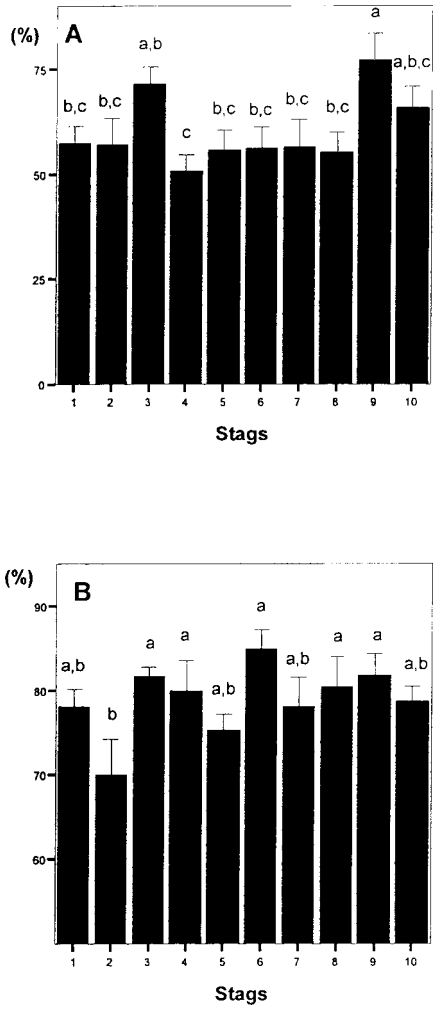

Figure 3. Freezability (mean percentage ± SEM) of epididymal sperm samples of 10 stags after their spermatozoa were cooled, stored in LN_2 and then thawed at each of 3 procedures. Triplicate independent straws of spermatozoa from each stag for each thawing procedures were assessed. Columns represent for each parameter the average of the 3 different thawing procedures. **(A)** Membrane integrity after thawing. **(B)** Acrosome integrity. For each panel, bars with different letters indicate statistically significant differences among stags ($P < .01$).

rates after freezing and thawing also found with the use of protocol I (slow). When spermatozoa were incubated for 120 minutes at 37°C, there was a marked loss in sperm parameters in all cases ($P < .01$). No differences were seen between thawing procedures, although sperm parameters were slightly superior when the slow procedure was used.

On thawing, spermatozoa were diluted in a modified Tyrode medium and SM evaluated. Results (Table 2) revealed that for all 3 treatments, there was a significant decrease ($P < .0001$) in SM on dilution in Tyrode medium. Comparison between the dilution survival rates revealed that spermatozoa thawed using protocol I showed much higher ($P < .05$) values than those thawed using protocols II or III and thus were able to withstand dilution.

Finally, the results of the ANOVA procedure revealed that thawed sperm parameters were not significantly ($P > .05$) affected by the interaction between individual factor and thawing procedure. Overall, the results from the 10 stags showed a similar pattern of response, with the best recovery rates with the use of protocol I.

Experimental Series 2

The purpose of this series was to examine the effect of thawing procedure used to thaw frozen deer semen on fertility after intrauterine insemination of hinds. We compared the fertility rates of 2 different procedures: 37°C for 20 seconds (the most beneficial for epididymal spermatozoa thawing determined by in vitro evaluation) vs 60°C for 8 seconds (one of the least beneficial). Table 3 shows the average of the fertility rates obtained for each thawing protocol and for each stag. Percentages of individual sperm motility for spermatozoa thawed by protocols I and II were 75.5% and 68.5%, respectively. The respective individual sperm motility for stags A, B, and C were 65, 72 and 80%. The results from the 3 stags showed a similar pattern of response, with the best SM values obtained using protocol I. In our study, the average fertility rate was 56.0%, with a wide variation, although no significant, between thawing procedures within stags and between stags (Table 3). However, the results of the ANOVA procedure revealed that fertility was significantly ($P = .014$) affected by the thawing procedure (Table 3), with the best overall rates after freezing and thawing found with the use of protocol I (37°C for 20 seconds). The interaction between male and thawing procedure did not affect fertility significantly. Thus, the results from the 3 stags are similar; slow thawing yielded higher fertility rates than intermediate thawing (Table 3).

Discussion

The present results show that cryopreservation, as was expected, substantially reduced deer sperm viability (Figure 1) and that similar protection was obtained in comparison with prior studies (see Asher et al, 2000). Following, rather than discussing the results of the individual experiments reported here, the discussion will be concerned with the overall results.

First, the most obvious result of these experiments was the clear demonstration of stag-to-stag differences in response of their spermatozoa to freezing and thawing. Thus, our results show that regardless of the thawing procedure, in vitro sperm characteristics evaluated in thawed samples showed considerable differences among males. However, fertility rates of hinds inseminated with sper-

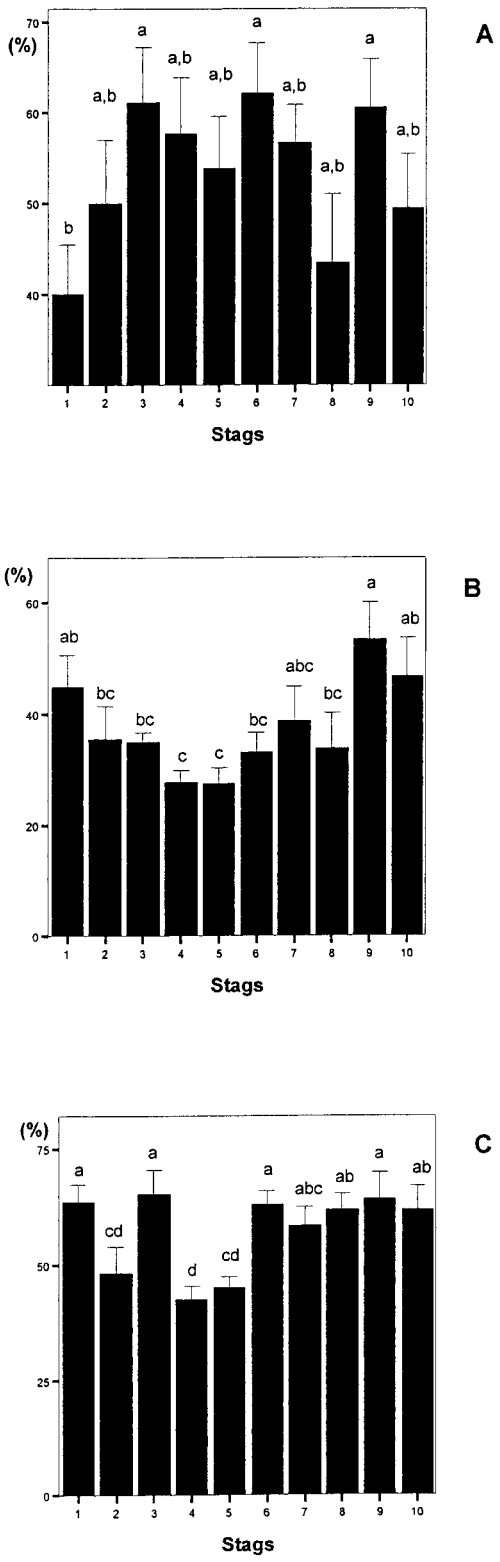

Figure 4. Freezability (mean percentage ± SEM) of epididymal sperm samples of 10 stags after their spermatozoa were cooled, stored in LN_2, thawed at each of 3 procedures and then incubated for 120 minutes the freezing diluent. Triplicate independent straws of spermatozoa from each stag for each thawing procedures were assessed. Columns represent for each parameter the average of the 3 different thawing procedures. **(A)** Percentage of motile sperm after a 120-minute incubation in the freezing diluent. **(B)** Membrane integrity. **(C)** Acrosome integrity. For each panel, bars with different letters indicate statistically significant differences among stags ($P < .01$).

matozoa from the different stags were not significantly different, although as can be see in the Table 3, the females that calved after AI with spermatozoa from stags A (52.9%) and B (43.7%) were lower than those inseminated with spermatozoa from stag C (63.6%). These differences in fertility among stags may be due to a variable susceptibility of their spermatozoa to cryoinjury that affects postthaw fertility. Similar results have been previously recorded for thawed bull spermatozoa (Beatty et al, 1976).

In our study, stags were not preselected for freezability. However, quality parameters of thawed spermatozoa as well as the degree of declined cellular integrity by preservation showed considerable differences among males. The differences among individuals were found for most parameters examined. The males showed different susceptibilities to freezing and postthawing incubation, independently from initial quality. These differences in the resistance to thawing of the spermatozoa of different individuals have been observed for spermatozoa of other domestic (see Curry, 2000) and wild (see 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 among spermatozoa from individuals of the same species. Ejaculated spermatozoa are known to acquire cold shock sensitivity as they transverse the epididymal tubule. But this does not explain male-to-male differences of epididymal spermatozoa. One possible explanation may be that such differences reflect properties of sperm membranes that are determined genetically.

A role for a genetic component in the overall effect is suggested by the strain differences in cryosensitivity reported for mice (Nakagata and Takeshima, 1993). A similar hypothesis has been supported for bull (Beatty et al, 1976) and boar spermatozoa (Thurston et al, 2001). Furthermore, in the last species, it has been described that interejaculate differences in cryosusceptibility are considerably lower than interboar differences, indicating that this is a characteristic of individuals rather than of the ejaculates (Medrano et al, 2002). These observations confirm the hypothesis that consistent interindividual variation in sperm freezability exists and may be genetically

Table 2. *Percentages of motile sperm after thawing in the freezing diluent or after dilution in Tyrode's medium. Dilution Survival Rate indicates value after dilution in Tyrode's medium/value of thawed sample × 100.**

Thawing Procedure	Same Diluent†‡	Dilution in Tyrode†‡	Dilution Survival Rate†
37°C/20 sec	76.8 ± 1.8^{aA}	45.8 ± 1.6^{aB}	59.9 ± 2.6^{a}
60°C/8 sec	69.5 ± 1.8^{bA}	36.8 ± 1.6^{bB}	52.8 ± 2.6^{b}
70°C/5 sec	70.6 ± 1.8^{bA}	37.0 ± 1.6^{bB}	52.3 ± 2.6^{b}

* Data are expressed as least-squares means ± SEM.

† Values with different superscripts within each column are significantly different ($^{ab}P < .05$).

‡ Values with different superscripts within each row are significantly different ($^{AB}P < .0001$).

Table 3. *Effects of thawing procedure on fertility (%) after intrauterine insemination of cryopreserved epididymal red deer spermatozoa**

Stags	Thawing procedure		Overall Fertility
	37°C/20 sec	60°C/8 sec	
A	62.5	44.4	52.9
B	75.0	12.5	43.7
C	70.5	56.2	63.6
Overall fertility†	69.7^{a}	42.4^{b}	56.0

* Hinds were inseminated with thawed sperm samples from 3 stags that were equally distributed at either of 2 warming procedures evaluated.

† Overall mean values with different superscripts are significantly different (n = 66; $^{ab}P = .014$) between thawing procedures.

determined. This observation can be very useful for future studies of cryopreservation of semen in farm animals because the males of these species can be selected based on its sperm freezability. However, the practical application of the previous observations for the creation of a GRB for endangered or wild species is very scarce because in that situation, what is of interest is to conserve the biggest possible genetic variability (Johnston and Lacy, 1995). For it, in our context, the objective is the cryopreservation of semen of the biggest number of males. In this circumstance, it becomes necessary the implementation of cryopreservation protocols for epididymal deer spermatozoa with the object of being able to increase the results of viability to the thawing and most possible of ''bad freezers'' it happens to ''good freezers.'' Of the variables that may influence survival of mammalian spermatozoa when cryopreserved, thawing rate is known to be especially critical (Mazur, 1984).

The other principal finding of these experiments was the clear demonstration of the effect of the thawing procedure on epididymal deer spermatozoa freezability. Thus, our results revealed that fertility was significantly ($P = .014$) affected by the thawing procedure. Fertility results were significantly higher (69.7% vs 42.4%) when spermatozoa were thawed at 37°C for 20 seconds than were warmed at 60°C for 8 seconds. Moreover, the interaction between male and thawing procedure did not significantly affect fertility. Thus, the results from the 3 stags are similar; slow thawing yielded higher fertility rates than intermediate thawing (Table 3). To the best of our knowledge, this is the first report demonstrating in wild ungulates that fertility after AI is affected by the procedure employed to thaw frozen epididymal spermatozoa. However, our results show that no significant differences were found ($P > .05$) in most of the seminal parameters for the 3 different thawing procedures tested, except for the SM, after thawing ($P < .05$) and dilution in Tyrode medium ($P < .0001$) and for the dilution survival rate ($P < .0001$), in which the best overall recovery rates were also found with the use of protocol I (37°C for 20 seconds). Similar results have been previously observed in deer spermatozoa ejaculated by other authors. Thus, Soler et al (2003b) reported that the use of thawing protocols that provide intermediate (50°C for 8 seconds) or slow (37°C for 20 seconds) thawing rates were the most beneficial for semen thawing of different cervid species.

Dissimilar results have been observed in ram spermatozoa by other authors: Pontbriand et al (1989) reported that sperm motility was similar when straws of 0.5 mL were thawed at 37°C or 60°C for 20 and 8 seconds, respectively. Similarly, Fiser et al (1986) and Söderquist et al (1997) observed that after thawing ram semen the percentage of motility was higher for rapid warming rates than for slower ones. The study of Fiser et al (1986) assessed a wide range of thawing rates (1, 2, 20, 80, 450, and 960°C/min). Results very similar to those obtained for ram semen have recently been described for thawed semen of buffalo, evaluating 2 (200 and 1000°C/min) very different thawing rates (Sukhato et al, 2001). The differences found between the results reported in previous studies and those in the present work could be due to the fact that the range of thawing rates studied was much wider than ours. Probably, if a wider range of thawing rates had been studied, some would be suboptimal for the thawing of deer spermatozoa. This did not happen in our study because we chose 3 thawing rates in a close range.

Alternatively, the differences between previous results and ours could also be due to differences in the composition of the extenders used. Fiser et al (1981) found an interaction between the osmolality of the diluent and the thawing rate used so that the freezing of ram semen in hypertonic medium combined with rapid thawing rates provided the highest percentages of sperm survival. The extender we employed was a simple medium with no additional cryoprotectant sugars, but that employed by Söderquist et al (1997) contained lactose, a light hyperosmotic extender. Therefore, the presence of sugar in the freezing medium might protect spermatozoa against damage occurring at a high thawing rate.

Finally, the differences between previous results and ours could also be due to differences in the source of the spermatozoa. For ungulates, there seems to be only a previous study (Lambrechts et al, 1999), in which these species have been used as a model for the analysis of the possible effects of thawing procedure on characteristics of epididymal spermatozoa. The referred work demonstrated that the effect of cryopreservation on sperm characteristics of epididymal African buffalo spermatozoa was independent of the thawing procedure (35°C for 30 seconds vs 80°C for 5 seconds). These results differ from previous studies using ejaculated spermatozoa from ungulates species (Söderquist et al, 1997), in that ultrarapid thawing procedures are required to successful cryopreservation in those species.

Because the assessment of motility alone is inadequate for the evaluation of sperm survival after thawing, we also assessed the functional status of sperm membrane (using the HOST) and acrosome integrity of spermatozoa. There were no statistically significant differences for the acrosome and membrane integrities among the different thawing procedures. These results agree with those obtained for boar (Eriksson et al, 2000), ram (Söderquist et al, 1997), and buffalo sperm samples (Sukhato et al, 2001). Finally, in the present study, the sperm parameters evaluated after 120 minutes of incubation at 37°C were not significantly different among the different thawing procedures tested.

In some species, the motility of recently thawed samples is not a good indicator of the fertility of those samples, whereas sperm motility evaluation after sperm dilution in an isotonic medium is a more reliable measure to estimate the sperm's ability to survive in similar conditions to that of the female reproductive tract (Morrier et al, 2002). Therefore, we also assessed SM after thawed sperm were diluted in Tyrode medium. Upon sperm dilution, we determined SM only because it has been recently demonstrated that sperm motility is a more sensitive indicator of osmotic stress than membrane integrity (Songsasen et al, 2002). In that study, the susceptibility of dog spermatozoa to osmotic injury was detected only when motility was evaluated and not when membrane integrity was assessed (Songsasen et al, 2002). In our conditions, SM was also lower ($P < .0001$) for thawing carried out with procedures II (36.8%) and III (37.0%) than with procedure I (45.8%). The same was true when dilution survival rates (ie, the ability of thawed sperm to resist dilution in Tyrode medium) were compared. These rates reveal that spermatozoa thawed using protocol I (slow) showed much higher ($P < .0001$) values than those thawed using protocols II (intermediate) or III (rapid) and thus were better able to withstand dilution.

Taken together, our results indicate that the higher incidence of fertility rates for spermatozoa thawed at 37°C was reflected only in higher SM (both after thawing as after dilution in Tyrode medium) and higher dilution survival rates. However, the higher fertility rate obtained for spermatozoa thawed at 37°C was not reflected in a higher incidence of spermatozoa with intact plasma membrane or higher NAR. The reason this better fertility seen when spermatozoa were thawed at 37°C is also not reflected in a better sperm membrane results is difficult to understand. One explanation could be that these sperm parameters do not provide a reliable prediction of the potential fertility of the semen. However, the discrepancy found between the in vivo and in vitro studies emphasizes the importance of controlled field trials for the ultimate estimation of effects on fertility.

Other important finding was the differential resistance of sperm membranes to return to isosmotic conditions. We compared the osmotic tolerance of spermatozoa frozen in Triladyl and thawed using 3 different thawing protocols to rapid removal of glycerol upon thawing. The 1-step decrease of glycerol by dilution with Tyrode medium resulted in a decline in SM for the 3 different procedures as has been reported previously by Guthrie et al (2002) for bovine spermatozoa. Osmotic stress after the rapid removal of glycerol is related to differences in the relative permeability of glycerol and water across the sperm membrane (Gao et al, 1997). Mammalian spermatozoa appear to behave as linear osmometers, and cell death occurs if the spermatozoa swell or shrink beyond species-specific osmotic tolerances (Gilmore et al, 1998b). In our conditions, sample osmolality increased approximately 1300 mOsm when spermatozoa were suspended in Triladyl extender, containing 6% glycerol. The replacement of glycerol with isosmotic Tyrode medium returned the spermatozoa to an environment in which the osmolality was approximately 290 mOsm. The difference in motility response subsequent to return of sperm to isosmotic conditions may therefore reflect differences in the relative tolerance to osmotic stress of membranes of spermatozoa thawing by the 3 different procedures. Our results thus indicate that the spermatozoa thawed by protocol I, in addition to being the ones that better resist the freezing process (determined by SM), are also those with a higher resistance to the osmotic change caused by the rapid removal of the glycerol (determined by dilution survival rate), with the opposite being true for protocols II and III.

These observations indicate that thawing at the rapid rates appears to induce a semilethal lesion in the sperm membrane in that, although the thawed sperm membranes are viable, its response to osmotic stress is compromised. In this sense, it has been reported that sperm membranes suffer alterations when the duration of the thawing is insufficient for egress of excess cryoprotectants from the cell and the cells swell and lyse as the medium becomes abruptly diluted by the melting of extracellular ice (Ma-

zur, 1984). Moreover, Curry and Watson (1994) observed that ram sperm is damaged by the rate at which water enters the cell as dictated by the osmotic difference across the membrane. These subtle changes in sperm membranes originated by rapid thawing rates were extensively evidenced when sperm motility was evaluated after spermatozoa were returned to isosmotic conditions after freezing and thawing. In this sense, our results therefore indicate that semen evaluation after dilution in an isotonic solution should be considered as a method capable of detecting subtle changes in sperm function after freezing and thawing.

Finally, the average fertility rate obtained in our study was 56.0%. These results indicate that timed AI with frozen–thawed epididymal deer spermatozoa following estrus synchronization can result in acceptable pregnancy rates in Iberian deer hinds. Similar fertility rates have been registered in red deer hinds by other authors (Fennessy et al, 1990; Haigh and Bowen, 1991) although as can be see in in the previous works, we used a higher number of spermatozoa per female (100×10^6 total spermatozoa) than the cited authors. However, in our experimental conditions the sperm concentration used provided strong fertility differences between the 2 thawing procedures tested. These differences should not have been reported if the number of spermatozoa had been too elevated so as to mask the effects of the different thawing procedures. On the contrary, our results showed that even when a relatively high number of spermatozoa were deposited in the uterus near the site of fertilization, there were highly significant fertility differences among the thawing procedures tested.

To our knowledge, such clear effects of thawing procedure on in vitro cryoresistance and in vivo fertility has not been shown previously in deer epididymal spermatozoa. Fertility and sperm motility were significantly higher when spermatozoa were thawed using a slow procedure than were warmed using a rapid protocol. Based on the results of the present study, we suggest that slow warming rates should be used to thaw cryopreserved epididymal spermatozoa. Additionally, our results show that the resistance to cryopreservation of epididymal deer spermatozoa was also affected by the individual factor. However, this work should be completed in the future with more artificial insemination trials since our results are based on so few specimens. In addition, further studies are required to evaluate the effects of lower thawing temperatures (25°C–30°C) on cryoresistance of epididymal deer spermatozoa.

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