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Comparative semen cryopreservation in ferrets (*Mustela putorius furo*) and pregnancies after laparoscopic intrauterine insemination with frozen–thawed spermatozoa

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Summary. A study was conducted to determine an optimum technique for semen cryopreservation and the biological competence of frozen–thawed ferret spermatozoa. Fifty-two fresh electroejaculates from 4 males were evaluated for sperm percentage motility, forward progressive motility, motility index (SMI) and acrosomal integrity. To determine the optimum temperature for maintaining sperm motility *in vitro* and the influence of glycerol on sperm motility, seminal aliquants were diluted in TEST diluent (containing either 0 or 4% glycerol) and maintained at 25° or 37°C. For cryopreservation, semen was diluted in each of 3 cryodiluents (TEST, PDV, BF5F), cooled for 30 min at 5°C and pelleted on solid CO_2 or frozen in 0·25 ml straws (20°C/min to −100°C). Following thawing, SMI and acrosomal integrity were determined. Ten females with maximum vulval swelling were given 90 i.u. human chorionic gonadotrophin and laparoscopically inseminated *in utero* with spermatozoa previously frozen using the optimum diluent and freeze–thaw method. The maintenance temperature of 25°C was superior ($P < 0·05$) to 37°C for sustaining sperm motility, and glycerol did not influence ($P > 0·05$) motility for up to 11 h of culture. After thawing, motile spermatozoa were recovered in all treatment groups, but sperm motility and normal acrosomal ratings were highest using the PDV diluent, the pelleting method and thawing at 37°C ($P < 0·05$). Seven of the 10 ferrets (70%) inseminated with spermatozoa frozen by this approach became pregnant and produced 31 kits (mean litter size 4·4; range 1–9 kits). These results illustrate the sensitivity of ferret sperm motility and acrosomal integrity to different cryopreservation conditions; and demonstrate the biological competence of frozen–thawed ferret spermatozoa.

Keywords: ferret; sperm; semen cryopreservation; artificial insemination

Introduction

The reproductive cycle and the physiological mechanisms controlling seasonal variation in spermatogenesis and circulating testosterone profiles have been characterized in male ferrets (*Mustela putorius furo*) (Allanson, 1932; Baum & Goldfoot, 1974; Walton, 1976; Erskine & Baum, 1982; Sisk & Desjardins, 1986; Kastner & Apfelbach, 1987). Reproductive activity in this species is stimulated by a long-day photoperiod, most breeding occurring from March until August in the northern hemisphere (Neal *et al.*, 1977). In our laboratory, the domestic ferret is being studied as a model for the endangered black-footed ferret (*Mustela nigripes*), a species considered extinct until a small

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population was rediscovered in Wyoming in 1981 (Clark, 1987). Artificial breeding strategies for this species and other rare mustelids would be valuable for: (i) propagating individuals which fail to mate because of a sexual incompatability or physical handicap; (ii) safely distributing gametes between different captive breeding sites without risking the transport of live animals; and (iii) storing germ plasm for use in subsequent animal generations, thereby offering a method of sustaining genetic diversity. The domestic ferret may serve as a model for developing artificial breeding techniques, because the species is genetically related to the black-footed ferret (O'Brien *et al.*, 1989) and is readily available.

Recently, we reported that ferrets chronically maintained under a 16 h light:8 h dark cycle were able to sustain spermatogenesis for as long as 65 weeks (Wildt *et al.*, 1989). We determined that vaginal deposition of ferret spermatozoa was ineffective (0 pregnancies in 10 attempts) in females induced to ovulate with human chorionic gonadotrophin (hCG). However, 17 of 24 ferrets inseminated laparoscopically with fresh semen into the uterine horns became pregnant and delivered live young.

The objectives of the present study were to: (i) examine methods for sustaining motility of ferret spermatozoa *in vitro*; (ii) study the sensitivity of these spermatozoa to freeze–thawing by comparing various cryodiluents, freezing techniques and thawing temperatures; and (iii) assess the ability of thawed spermatozoa to fertilize ova *in vivo* and result in live offspring.

Materials and Methods

Semen collection and analysis. Four adult, proven breeder males (24–36 months old; 1·2–1·5 kg body wt) were maintained at the National Institutes of Health (NIH) Animal Center in Poolesville, MD, USA. Males were housed in individual cages (91 cm deep × 87 cm wide × 79 cm high) containing a nest box and a 60-W light bulb. Each male was exposed to an artificial photoperiod of 16 h light:8 h dark. All animals had *ad libitum* access to water and a commercial dry mink chow (Milk Specialities Co. Dundee, IL, USA).

For electroejaculation, males were anaesthetized at intervals of 1–2 weeks with an intramuscular injection of tiletamine-zolazepam (Telazol®: A. H. Robbins, Richmond, VA, USA; 13 mg/kg). Semen was collected by rectal-probe electroejaculation using techniques described previously (Wildt *et al.*, 1989). Overall, 52 ejaculates were collected from 4 males (13 ejaculates/male). Seminal drops from each series were collected from the tip of the glans penis by capillary action into a warmed (37°C) glass pipette. Each ejaculate was transferred into a 1·5 ml microcentrifuge tube (Fisher Scientific, Pittsburg, PA, USA) containing either 120 µl TEST (Graham *et al.*, 1972), PDV (Nagase & Niwa, 1963; Platz & Seager, 1977) or BF5F (Howard *et al.*, 1981) diluent at 37°C.

The constituents of the diluents were as follows. TEST consisted of 20% egg yolk, 4·83% tes-n-tris, 1·15% tris, 0·4% glucose and 4% glycerol. PDV consisted of 20% egg yolk, 11% lactose and 4% glycerol. BF5F, a modified BF5 diluent (Pursel & Johnson, 1972), consisted of 20% egg yolk, 1·6% glucose, 1·6% fructose, 1·2% tes-n-tris, 0·2% tris, 4% glycerol and 0·5% surfactant mixture of sodium and triethanolamine lauryl sulphate, commonly referred to as Orvus ES Paste (Equex®: Nova Chemical Sales, Inc., Scitaute, MA, USA). Each millilitre of diluent contained 1000 i.u. penicillin G and 1000 µg streptomycin sulphate (Sigma Chemical Co., St. Louis, MO, USA). The justification for examining the diluent TEST was based on its previous use in domestic ferrets and its ability to sustain sperm motility of fresh semen for artificial insemination (Wildt *et al.*, 1989). PDV was assessed because this diluent has been used for freezing carnivore semen, including dog and cat spermatozoa (Platz & Seager, 1977; Platz *et al.*, 1978). Diluent BF5F was tested because of its ability to provide maximum sperm motility after thawing and acrosomal protection in several nondomestic species (Howard *et al.*, 1981, 1986b).

Immediately after collection, ejaculate volume and sperm concentration/ml of ejaculate were determined using procedures described previously (Wildt *et al.*, 1989). Diluted semen was assessed for percentage sperm motility and progressive motility (0–5 scale; 0 = no forward motility, 5 = rapid forward progressive motility). To determine an overall sperm assessment rating with equal emphasis on both sperm percentage motility and progressive motility, a sperm motility index (SMI) was calculated: SMI = [(% sperm motility) + (progressive motility × 20)/2] (Howard *et al.*, 1990). Sperm morphology and acrosomal integrity were assessed by fixing an aliquant (2 µl) of each sample in 0·3% glutaraldehyde followed by phase-contrast microscopic examination of 200 spermatozoa/aliquant at × 1575 (Howard *et al.*, 1990). Cells were classified as normal or having a tightly coiled flagellum, bent midpiece with cytoplasmic droplet, bent midpiece without droplet, bent flagellum, residual proximal or distal droplet or an abnormal acrosome. The acrosomal ultrastructure of each spermatozoon was categorized into 1 of 4 classes: normal apical ridge (NAR), damaged apical ridge (DAR), missing apical ridge (MAR) or loose acrosomal cap (LAC) (Howard *et al.*, 1981).

Semen processing. Diluted semen was assigned to 1 of 3 studies to determine the influence of maintenance temperature and addition of glycerol on longevity of sperm motility *in vitro* (Study 1); the impact of cryodiluent, freezing

method and thawing temperature on post-thaw sperm motility and acrosomal integrity (Study 2); and the biological competence of frozen–thawed ferret sperm (Study 3).

Because each cryodiluent to be examined in Study 2 contained glycerol, it was important to determine in Study 1 whether this cryoprotectant had a detrimental effect on sperm motility. Additionally, maintenance at 37°C vs 25°C was compared to determine the appropriate condition for sustaining maximum sperm activity. To accomplish the objectives of Study 1, a total of 8 ejaculates from 4 males (2 ejaculates/male) was collected. Each ejaculate was split into 2 aliquants and each aliquant was diluted in 120 µl TEST diluent containing either 0 or 4% glycerol. Each sample was split again into 2 aliquants and maintained at 37 or 25°C. Sperm motility, progressive motility and SMI were determined hourly for 11 h.

In Study 2, 36 ejaculates from 4 males (9 samples/male) were collected for comparison of cryopreservation techniques. Seminal samples were diluted in either 120 µl TEST, PDV or BF5F containing 4% glycerol. Therefore, the assessment of each cryodiluent was based on the total of 12 ejaculates from 4 males (3 replicates/male/cryodiluent). All aliquants were cooled for 30 min at 5°C and either pelleted on solid CO_2 or frozen in plastic straws. The pellet method (Nagase & Niwa, 1963; Howard *et al.*, 1986a, b) involved pipetting single drops (~30 µl) of cooled, diluted semen into 3 × 4 mm indentations in a block of solid CO_2. After a 3 min freezing interval, the solid CO_2 block was inverted, depositing the frozen pellets in a liquid nitrogen bath. The pellets were spoon-transferred into sterile, labelled vials (Nalgene®: Sybron Corp., Rochester, NY, USA) and stored in a container of liquid nitrogen. The straw freezing method involved manually filling diluted seminal samples into 0·25 ml French straws (I.M.V. International Corp., Minneapolis, MN, USA). Each straw initially was filled with 100 µl cryodiluent containing no semen, followed by an air space and then the diluted semen (100 µl/straw). Straws were heat-sealed, cooled for 30 min at 5°C and transferred to a programmable, controlled-rate freezing unit (Planer Products Kryo-10, TS Scientific, Perkasie, PA, USA) preset in a holding phase at 5°C. Straws were frozen to −100°C at 20°C/min, plunged into liquid nitrogen and stored.

Assessment of thawed spermatozoa. To compare 2 thawing methods, semen frozen in pellets and straws was thawed at either 37°C for 30 sec or 60°C for 3 sec. Three pellets from each aliquant were thawed rapidly in a warmed, sterile 12 × 75 mm glass culture tube containing 220 µl phosphate-buffered saline. Aliquants in straws were thawed by placing in a waterbath before transferring semen to a glass culture tube. Within each cryodiluent-freezing method treatment group, assessment of thawing method was based on split samples from the same ejaculate thawed at 37 and 60°C. Immediately after thawing, an aliquant from each sample was fixed in 0·3% glutaraldehyde for later assessment of sperm acrosomal integrity. All aliquants were maintained at 25°C and examined for sperm percentage motility, progressive motility and SMI at 0, 30 and 60 min after thawing to determine the longevity of sperm motility *in vitro*.

Artificial insemination. Using the optimum freeze–thawing technique detected in Study 2, 8 additional ejaculates from 4 males were collected for cryopreservation and laparoscopic intrauterine insemination of 10 females (Study 3). Two of these 8 ejaculates were of sufficient volume for inseminating 4 females (2 inseminations/ejaculate). After thawing, seminal samples were removed from the waterbath, evaluated for percentage sperm motility, progressive motility and SMI, and then maintained at 25°C for 20 min before artificial insemination (AI). The total number of motile sperm inseminated in each female was determined.

Female ferrets (24–36 months old; 0·9–1·3 kg body wt) were maintained at Path Valley Farm (Willow Hill, PA, USA), 63 km from the NIH site. All females were exposed continuously to a 16 h light:8 h dark cycle and had produced at least one litter within 6 months of AI. Ferrets were monitored daily for vulval tumescence, and those with maximum vulval swelling were considered in oestrus. On the day of AI, ferrets were transported to the NIH Animal Center. Each female was anaesthetized with Telazol (10 mg/kg), surgically prepared and then subjected to laparoscopic AI using techniques described earlier (Wildt *et al.*, 1989). In brief, each uterine horn was identified and cannulated for intrauterine deposition of semen using a sterile, feline indwelling catheter (Sovereign®, Sherwood Medical, St. Louis, MO, USA; 20 gauge, 5 cm long). Each catheter was inserted percutaneously into the uterine lumen proximal to the uterine bifurcation under laparascopic observation. Sterile, polyethylene tubing (PE-10, Intramedic®, Clay Adams, Parasippany, NJ, USA) containing 100 µl frozen–thawed semen was passed through the catheter into the lumen of each uterine horn for insemination. An intramuscular injection of 90 i.u. hCG (Sigma Chemical Co., St. Louis, MO, USA) was given at the time of AI to induce ovulation.

Statistical analysis. Values are reported as means ± standard error of the means (s.e.m.). The influence of temperature and glycerol on sperm motility and the effect of cryopreservation treatment on sperm survival after thawing were assessed statistically by the analysis of variance (ANOVA) in a randomized complete block design using a computerized statistical program (Statistical Analysis System; SAS, 1986). Because of variation in ejaculate characteristics among individuals, each animal was considered as one complete replicate (block). When a significant F value was calculated ($P < 0·05$), differences among means for selected pairwise comparisons were determined by a Duncan's multiple range test (SAS, 1986). Differences in sperm traits and number of motile spermatozoa inseminated between pregnant and nonpregnant females were analysed using Student's *t* test.

Results

Semen containing motile spermatozoa was obtained from each ferret at each electroejaculation. Based on a total of 52 electroejaculates, mean ejaculate characteristics are provided in Table 1.

Seminal traits including sperm motility, progressive motility, motility index and morphology were not influenced ($P > 0.05$) by dilution in TEST, PDV or BF5F diluent. Fewer than 33% of all spermatozoa were pleiomorphic, and the most prevalent abnormality included cells with a residual cytoplasmic droplet (Table 1). Microscopic assessment of the ultrastructure of spermatozoa revealed a prominent acrosome, which could be identified readily and classified by phase-contrast microscopy (Fig. 1). A high proportion of spermatozoa had a normal apical ridge (Table 1).

Table 1. Ejaculate traits, sperm morphology and acrosomal integrity in the ferret ($n = 52$ ejaculates)

Ejaculate volume (μl)	50·1 ± 3·7
Sperm concentration ($\times 10^{-6}$/ml)	706·1 ± 50·5
motility (%)	80·7 ± 1·0
progressive motility	3·3 ± 0·1
motility index	73·6 ± 1·0
Motile spermatozoa/ejaculate ($\times 10^{-6}$)	27·7 ± 2·8
Structurally normal spermatozoa (%)	67·3 ± 1·3
Abnormal spermatozoa (%)	
Abnormal acrosome	7·6 ± 0·3
Tightly coiled flagellum	0·7 ± 0·1
Bent midpiece with droplet	2·7 ± 0·2
Bent midpiece without droplet	0·8 ± 0·2
Bent flagellum	1·8 ± 0·2
Proximal cytoplasmic droplet	8·7 ± 0·5
Distal cytoplasmic droplet	10·4 ± 0·8
Acrosomal integrity (%)	
Normal apical ridge	92·4 ± 0·5
Damaged apical ridge	4·7 ± 0·4
Missing apical ridge	0·8 ± 0·1
Loose acrosomal cap	2·1 ± 0·3

Values are means ± s.e.m.

In Study 1, regardless of glycerol treatment, maintenance at 25°C was better ($P < 0.05$) than at 37°C for sustaining sperm motility *in vitro* (Fig. 2). Following dilution in TEST egg-yolk diluent, motility of spermatozoa was maintained for more than 11 h using the 25°C holding temperature. Within temperature treatments, the presence of glycerol had no effect ($P > 0.05$) on *in vitro* sperm motility.

In Study 2, there was no difference ($P > 0.05$) in prefreeze % sperm motility, progressive motility and SMI after dilution among the TEST (83·6 ± 1·3, 3·4 ± 0·1, 76·0 ± 0·9, respectively), PDV (79·0 ± 1·0, 3·1 ± 0·1, 70·5 ± 1·0, respectively) and BF5F (76·7 ± 1·6, 3·6 ± 0·1, 74·0 ± 1·3, respectively) diluents. Motile spermatozoa were recovered after cryopreservation in all treatment groups, but post-thaw sperm viability was influenced ($P < 0.01$) by cryodiluent, freezing method and thawing temperature. Maximum post-thaw survival, based on the maximum motility index, was achieved with PDV, pelleting and thawing at 37°C (Fig. 3).

Cryopreservation technique also influenced ($P < 0.01$) acrosomal integrity after thawing (Fig. 4). The mean, ± s.e.m., proportion of spermatozoa with normal apical ridges (NAR) after thawing ranged from 10·5 ± 1·9% to 41·0 ± 5·3% (Fig. 4). Freeze–thawing technique also affected ($P < 0.01$) the DAR, MAR and LAC values (Fig. 4). Overall, PDV-pellets thawed at 37°C provided maximum acrosomal protection as demonstrated by the highest incidence of NAR (Fig. 4).

The longevity of post-thaw sperm motility *in vitro* also was highly dependent ($P < 0.01$) on cryotechnique at 30 and 60 min after warming. Compared with the other treatments at the same interval, pelleted semen diluted in PDV and thawed at 37°C demonstrated the greatest SMI ratings at 60 min after thawing (Fig. 5).

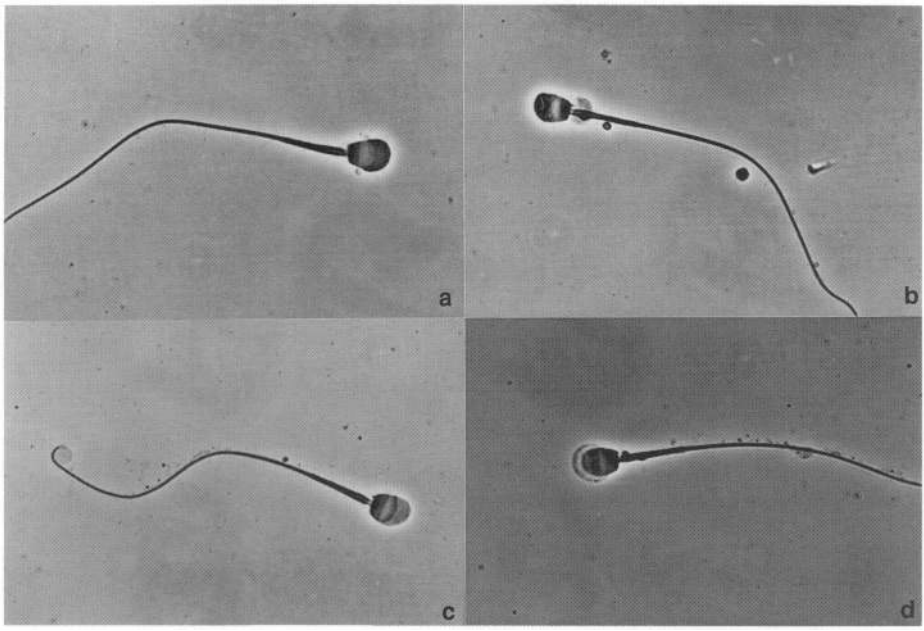

Fig. 1. Acrosomal morphology of ferret spermatozoa exhibiting a normal (a), damaged (b), or missing apical ridge (c) or loose acrosomal cap (d); (× 1575).

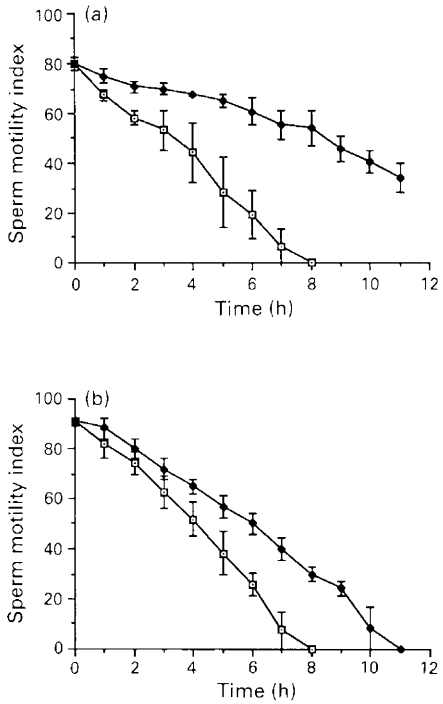

Fig. 2. Influence of maintenance temperature (25°C (◆) vs 37°C (□)) without (a) or (b) with 4% glycerol on longevity of ferret sperm motility *in vitro*. Sperm motility index is a mean of percentage sperm motility and sperm progressive motility × 20; $n = 8$ total ejaculates.

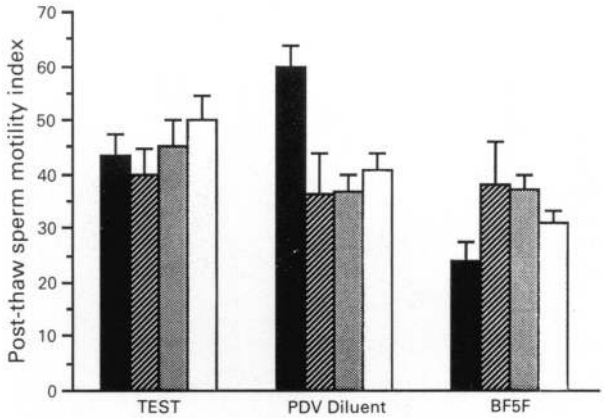

Fig. 3. Influence of cryodiluent, freezing in pellet or straw container and thawing temperature on ferret sperm motility index after thawing ($n = 36$) ejaculates; (■) pellet, 37°C; (▨) pellet, 60°C; (⊡) straw, 37°C; (□) straw, 60°C.

Table 2. Results of laparoscopic artificial insemination ($n = 10$ females) with frozen–thawed ferret sperm

Mean sperm motility index* after thawing	58·8 ± 2·2 (50·0–72·5)
Mean number of motile sperm inseminated ($\times 10^{-6}$)	3·5 ± 0·6 (1·4–6·0)
Mean percentage morphologically normal sperm inseminated	68·3 ± 2·1 (52·5–75·5)
Mean number of inseminated motile sperm with normal apical ridge ($\times 10^{-6}$)	1·5 ± 0·5 (0·5–3·1)
Proportion of anovulatory females after treatment with human chorionic gonadotrophin	1/10
Total number of pregnant females (% pregnant)	7 (70·0%)
Mean gestation (days)	42·9 ± 0·59 (41–45)
Mean number of kits/litter	4·4 ± 1·0 (1–9)

Values in parentheses represent ranges.
*[% sperm motility + (progressive motility × 20)]/2.

Because PDV-pellets thawed at 37°C provided the greatest cryoprotection in all categories, this method was used for the fertility study (Study 3). Table 2 provides relevant information on the number and quality of thawed sperm inseminated and the pregnancy results. Seven of 10 females conceived after laparoscopic AI and produced live young (70% pregnancy/offspring). There was no difference ($P > 0·05$) in motility traits or number of spermatozoa inseminated between pregnant and nonpregnant females (data not shown). Of the 3 ferrets which failed to become pregnant, 1 female did not ovulate after hCG administration and remained in oestrus following AI. The remaining 2 females ovulated, as evidenced by vulval detumescence, but neither became pregnant. In the 2 cases in which the ejaculate from 2 males was frozen–thawed and split for AI of 2 females, all 4 females became pregnant. The maximum length of time semen was maintained frozen before thawing and successful AI was 10 months.

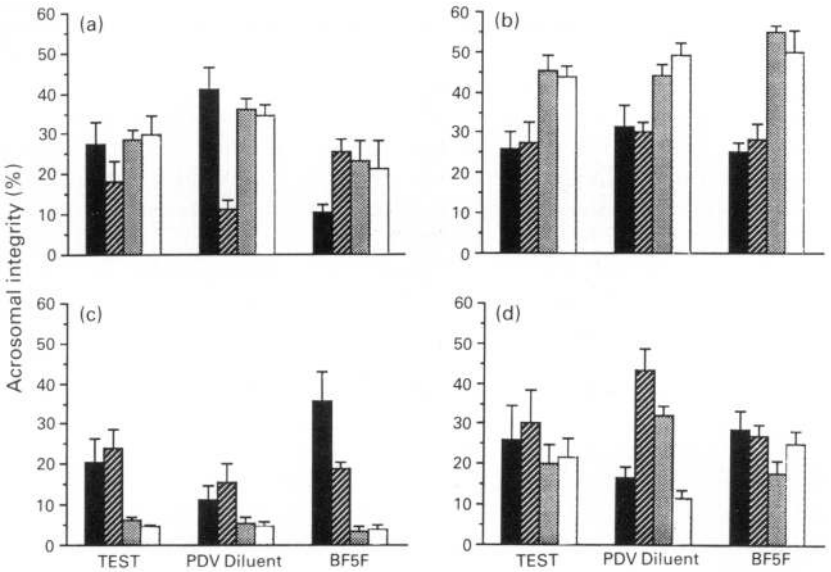

Fig. 4. Influence of cryodiluent, freezing in pellet or straw container and thawing temperature on ferret acrosomal integrity after thawing (n = 36 ejaculates); (a) normal, (b) damaged and (c) missing apical ridge and (d) loose acrosomal cap. See Fig. 3 for key.

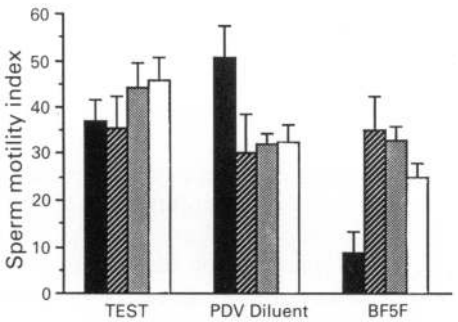

Fig. 5. Ferret sperm motility index *in vitro* at 60 min after thawing; symbols as in Fig. 3.

Discussion

The successful freezing of carnivore semen and its use for producing offspring have been limited to the dog (Platz & Seager, 1977), cat (Platz *et al.*, 1978), fox (Aamdal *et al.*, 1972) and wolf (Seager *et al.*, 1975). To our knowledge, this is the first attempt to determine an optimum method for cryopreserving ferret spermatozoa and assessing its biological competence. The use of a multitrait analysis system allowed the assessment of sperm motility immediately after thawing and the longevity of sperm motility in addition to categorizing the acrosomal damage induced by cryopreservation. Studying the viability of spermatozoa *in vitro* in a comparative fashion before attempting artificial insemination probably accelerated our ability to produce ferret offspring efficiently. A particularly important observation was the close relationship between post-thaw motility ratings and acrosomal integrity. Identifying PDV as the superior diluent was facilitated by in-vitro studies demonstrating that both sperm motility ratings and number of normal acrosomes

were highest using this diluent. Pursel *et al.* (1972) demonstrated that motility alone is an inadequate index of sperm survival after thawing, as highly motile cells can be afflicted with a severely damaged acrosome. During freezing of the spermatozoon, the acrosome can deteriorate progressively so that the apical ridge can become damaged or lost or the acrosomal cap can become loosened. Because acrosomal integrity has been correlated closely with fertility (Pursel *et al.*, 1972), this characteristic is now used as a complementary indicator of survival after thawing in domestic (Pursel *et al.*, 1972, 1978; Berndtson *et al.*, 1981) and nondomestic (Howard *et al.*, 1981, 1986b; Schiewe *et al.*, in press) species. In ferrets, assessing acrosomal integrity before and after freezing was an extremely useful criterion for comparing cryopreservation techniques. The prominent acrosome of the ferret spermatozoon appeared more similar to cattle (Saacke & Marshall, 1968) and pigs (Pursel *et al.*, 1972) than to domestic and nondomestic felids (Wildt *et al.*, 1983, 1986, 1988; Howard *et al.*, 1990), equids (Howard *et al.*, 1981) or non-human primates (Leverage *et al.*, 1972). Although Giemsa stain has been used to evaluate the integrity of acrosomes in domestic ferrets and black-footed ferrets (Curry *et al.*, 1989), we determined that phase-contrast microscopy of glutaraldehyde-fixed spermatozoa allowed easy assessment and classification of acrosomal damage. Likewise, phase-contrast microscopy was effective for determining structural morphology of ferret spermatozoa including defects in the midpiece and flagellum. The total number of normal spermatozoa observed in ferrets (67·3%) was similar to that in normospermic domestic felids (71·6%), but the proportion of proximal and distal cytoplasmic droplets was greater in the ferrets than in domestic cats (Howard *et al.*, 1990). Although the aetiology of specific spermatozoal defects is unknown, the electroejaculation technique does not appear to influence the incidence of abnormal spermatozoa, since certain carnivores trained to ejaculate into an artificial vagina produce numbers of defective spermatozoa similar to those in electroejaculates (Wildt *et al.*, 1983; Durrant *et al.*, 1985; Howard *et al.*, 1990).

The time that spermatozoa remain viable *in vitro* is a critical factor for in-vitro gamete interaction studies or artificial breeding. The present study demonstrated that sperm viability during incubation is influenced by maintenance temperature, the duration of sperm motility being extended significantly by maintaining sperm at 25 rather than 37°C. This observation, which is probably mediated by a reduction in metabolic rate, is similar to recent findings in other carnivore species, including the domestic cat (Goodrowe *et al.*, 1989). Because poor sperm survival during incubation *in vitro* is associated with a lower sperm-fertilizing ability in certain domestic species (Larsson, 1976; Pursel *et al.*, 1978), it also appears that longevity of sperm survival is an important criterion for determining optimum cryopreservation techniques. In this study, there also was a significant interaction between the longevity of post-thaw sperm motility *in vitro* and cryodiluent/freeze–thaw method. The PDV/pellet/37°C method, which resulted in the highest SMI ratings immediately after thawing, also sustained maximum sperm viability after thawing, including for at least 60 min *in vitro* and apparently even longer *in vivo*. Because ovulation in ferrets occurs 24–36 h after an hCG injection (Chang & Yanagimachi, 1963; Chang, 1965) and because these females were administered hCG at the time of AI, it is reasonable that the fertilizable life-span of the thawed spermatozoa was maintained for at least 24 h in the female reproductive tract. This interval was similar to the in-vivo longevity exhibited by fresh ferret spermatozoa used in an earlier laparoscopic insemination study (Wildt *et al.*, 1989).

The utility of the PDV-pelleting technique for cryopreserving ferret spermatozoa was similar to earlier reports involving domestic dogs and cats (Platz & Seager, 1977; Platz *et al.*, 1978). Interestingly, the straw container technique provided results inferior to those obtained after pelleting. Comparative cryopreservation studies in other species, including the African elephant (*Loxodonta africana*), have demonstrated that pelleting is superior to the straw method for sperm freezing (Howard *et al.*, 1986b). The cooling rate during freezing is one of the most important factors controlling the life or death of cells (Mazur, 1985). Most sperm damage during freezing occurs from −3 to −20°C for boar sperm (Pursel & Park, 1985) and from −10 to −35°C for bull sperm (O'Dell *et al.*, 1958; Parkinson & Whitfield, 1987). The marked loss of motility and increased

acrosomal derangement during this time appears to be related to the onset of ice crystal formation in the cryodiluent (Niwa & Taguchi, 1981). Compared with the straw freezing rate used in this study, the pellet method may have provided faster freezing (Nagase & Niwa, 1963), resulting in a shorter duration of the freezing point plateau and reduced sperm damage.

This study proved that thawed ferret spermatozoa were biologically competent, as demonstrated by a 70% pregnancy rate after AI. The incidence of pregnancy in this study was similar to that in an earlier study (70·8%) in which ferrets were inseminated by the same approach but using freshly ejaculated spermatozoa (Wildt *et al.*, 1989). Our study was not designed to determine the minimum number of thawed, motile sperm necessary to achieve pregnancy. Nevertheless, conceptions occurred using as few as $1{\cdot}4 \times 10^6$ motile sperm. Although ferrets produce a low-volume ejaculate, we have demonstrated in an earlier study (Wildt *et al.*, 1989) that the ejaculate of a single male can be divided and used to produce dual pregnancies using as few as $1{\cdot}6 \times 10^6$ sperm/female. The pelleting method of sperm freezing proved to be equally effective in maximizing the usefulness of the small-volume ejaculate. On both occasions when thawed ejaculates were split and used to inseminate 2 ferrets, all females became pregnant.

In summary, these results demonstrated that domestic ferret sperm responded differently to variations in cryodiluent, freezing technique and warming procedure. The PDV diluent and pelleting technique provided the overall highest post-thaw survival characteristics for ferret spermatozoa. Most importantly, this study demonstrated the potential of using a multitrait assessment strategy combined with comparative freezing and in-vitro testing trials for formulating effective sperm-freezing protocols. Such a pre-emptive approach appears particularly appropriate if large-scale, germ plasm banking and AI are planned for a related wild and/or rare species. In this case, we speculate that the described techniques can be applied to improving the captive management of the endangered black-footed ferret. The PDV-pellet method is currently being used to cryopreserve black-footed ferret semen.

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