

Sensitivity of Domestic Cat (*Felis catus*) Sperm from Normospermic versus Teratospermic Donors to Cold-Induced Acrosomal Damage¹

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ABSTRACT

Freeze-thawing cat sperm in cryoprotectant results in extensive membrane damage. To determine whether cooling alone influences sperm structure and viability, we compared the effect of cooling rate on sperm from normospermic (N; > 60% normal sperm per ejaculate) and teratospermic (T; < 40% normal sperm per ejaculate) domestic cats. Electroejaculates were divided into raw or washed (Ham's F-10 + 5% fetal calf serum) aliquots, with the latter resuspended in Ham's F-10 medium or Platz Diluent Variant Filtered without glycerol (20% egg yolk, 11% lactose). Aliquots were 1) maintained at 25°C (no cooling; control), 2) cooled to 5°C in a commercial refrigerator for 30 min (rapid cooling; ~4°C/min), 3) placed in an ice slush at 0°C for 10 min (ultrarapid cooling; ~14°C/min), or 4) cooled to 0°C at 0.5°C/min in a programmable alcohol bath (slow cooling); and aliquots were removed every 4°C. All samples then were warmed to 25°C and evaluated for percentage sperm motility and the proportion of intact acrosomes using a fluorescein-conjugated peanut agglutinin stain. In both cat populations, sperm percentage motility remained unaffected ($p > 0.05$) immediately after exposure to low temperatures and after warming to 25°C. However, the proportion of spermatozoa with intact acrosomes declined ($p < 0.05$) after rapid cooling (~4°C/min) to 5°C (N, 65.6%; T, 27.5%) or ultrarapid cooling (~14°C/min) to 0°C (N, 62.1%; T, 23.0%) in comparison to the control value (N, 81.5%; T, 77.5%). Transmission electron microscopy of cooled sperm revealed extensive damage to acrosomal membranes. In contrast, slow cooling (0.5°C/min) to 5°C maintained ($p > 0.05$) a high proportion of spermatozoa with intact acrosomes (N, 75.5%; T, 68.3%), which also remained similar ($p > 0.05$) between cat populations (N, 64.7%; T, 56.8%) through continued cooling to 0°C. Results demonstrate that 1) rapid cooling of domestic cat sperm induces significant acrosomal damage without altering sperm motility, 2) spermatozoa from teratospermic males are more susceptible to cold-induced acrosomal damage than normospermic counterparts, and 3) reducing the rate of initial cooling markedly decreases sperm structural damage.

INTRODUCTION

It is well known that the viability of sperm after freezing and thawing is directly related to subsequent fertility [1]. It is evident that during the cryopreservation process, a significant proportion of spermatozoa undergo marked membrane damage that reduces subsequent fertilizing ability. Ever since the serendipitous discovery of the cryoprotective characteristics of glycerol in the late 1940s [2], sperm from a variety of species have been shown to survive a cryo-

preservation stress on the basis of some degree of postthaw motility. However, almost a half century later, cryopreserved semen is routinely used for practical breeding purposes in only a few species, largely livestock (especially cattle [3, 4]), and recently for assisted reproduction in infertile men and cancer patients [5]. Hence, more studies are needed to develop suitable sperm cryopreservation technology that allows routine offspring production.

One of our laboratory's long-term goals is developing artificial insemination (AI) with cryopreserved sperm as a tool for managing rare species (such as endangered cats) and genotypes (including animal models for human diseases) [6, 7]. Organized banking of germ plasma from such species and populations could facilitate exchange of genetic material and overcome sexual incompatibility problems common in such efforts. Successful cryopreservation of domestic cat sperm was first reported in the early 1970s using methods previously developed for dog sperm [8]. Briefly, this involved pelleting semen extended in a cryodiluent (containing 4% glycerol) on dry ice, followed by plunging into liquid nitrogen. Vaginal insemination of queens with thawed sperm resulted in ~10% pregnancy rate [8]. Laparoscopic intrauterine insemination improved these results to ~50% pregnancy rate [9]. Further, a few pregnancies have resulted in wild felids (leopard cat, *Prionailurus bengalensis*; ocelot, *Leopardus pardalis*; cheetah, *Acinonyx jubatus*) using this sperm cryopreservation method in conjunction with laparoscopic intrauterine insemination [10]. However, overall sperm quality after thawing does not appear consistent with an ability to routinely expect pregnancy after AI. On the contrary, the advent of available staining techniques to assess membrane damage has made it clear that this sperm cryopreservation method, the only one available for felids, causes extensive membrane disruption [11–14]. The most logical approach is to systematically assess those factors that perhaps influence cryopreservation sensitivity in felid spermatozoa.

As a first step, the present study was conducted to examine the sensitivity of felid spermatozoa to various rates of cooling to temperatures above freezing and prior to the actual cryopreservation event. Of additional interest was the variability in cold-stress sensitivity between two distinctly different domestic cat populations, one that normally ejaculates high proportions of structurally normal sperm and another that routinely produces many malformed sperm types (males classified as teratospermic). This comparison was important because 20 of 28 species of wild felids commonly studied can be characterized as teratospermic [15]. Our extensive studies have revealed that malformed sperm, and even some structurally normal sperm, from these teratospermic donors fail to participate in fertilization, largely due to subcellular and molecular dysfunction [16–20]. Thus, an additional objective was to determine whether a propensity to produce malformed spermatozoa also was related to a tendency for increased membrane disruption upon cold exposure.

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MATERIALS AND METHODS

Animals

Adult male domestic cats (*Felis catus*) were maintained under 12 h per day artificial lighting in individual cages at the National Institutes of Health Animal Center, Poolesville, MD, or the National Zoological Park, Washington, DC. All were provided dry cat food (Purina Cat Chow; Ralston-Purina Co., St. Louis, MO) and water ad libitum. Each male was designated as normospermic (N; > 60% structurally normal sperm; $n = 3$) or teratospermic (T; < 40% normal sperm; $n = 3$) on the basis of at least three semen evaluations during the previous 6 mo. All investigations were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

Semen Collection, Analysis, and Processing

Two semen samples (2 wk apart) were collected from each male using a standardized electroejaculation procedure [21]. Briefly, each male was anesthetized with an i.m. injection of tiletamine hydrochloride-zolazepam (Telazol; 4.5 mg/kg; A.H. Robins, Richmond, VA) and electroejaculated with a 60-Hz sine wave stimulator (P.T. Electronics, Boring, OR) after insertion of a rectal probe (1-cm diameter) with three longitudinal electrodes (P.T. Electronics). Ejaculates then were immediately examined for volume, sperm concentration, percentage motility, and forward progressive motility (scale, 0 to 5; 5 = best) and divided into raw or washed aliquots [22]. The aliquot designated for washing was further processed in two steps. First, the semen was diluted with an equal volume of Ham's F-10 medium (Irvine Scientific, Santa Ana, CA) containing 5% fetal calf serum (FCS; HyClone Laboratories, Logan, UT); it was then divided equally into two tubes and centrifuged at $300 \times g$ for 8 min to obtain a sperm pellet. After removal of supernatant, the sperm pellet from one tube was resuspended in Ham's F-10 containing 5% FCS, whereas the other was resuspended in Platz Diluent Variant Filtered (PDVF) through a 0.2- μ m filter. The latter is the standard diluent used for cryopreserving cat sperm [8, 13], but without the cryoprotectant glycerol. PDVF was prepared in our laboratory by mixing 20% egg yolk with 11% lactose in deionized water (pH 7.0; 420 mOsm). For morphological examination, a 10- μ l aliquot of the raw ejaculate was fixed in 0.3% glutaraldehyde in PBS (pH 7.4) and evaluated (200 sperm per aliquot) by phase-contrast microscopy ($\times 1000$) [22].

Cooling

Experiment 1. Aliquots (125 μ l) of raw semen and washed spermatozoa resuspended in Ham's F-10 versus PDVF were transferred into 0.5-ml microcentrifuge tubes and 1) maintained at 25°C (control), 2) rapid cooled ($\sim 4^\circ\text{C}/\text{min}$) to 5°C for 30 min by placing the tube in a commercial refrigerator, or 3) ultrarapid cooled ($\sim 14^\circ\text{C}/\text{min}$) to 0°C for 10 min by immersing the tube in an ice-slush water bath. After each equilibration period, tubes were warmed slowly to 25°C by placing in a water bath (25°C). Subsequently, 250 μ l of Ham's F-10 at 25°C was slowly added to the sperm aliquots and centrifuged ($300 \times g$; 8 min), supernatant was discarded, and pellets were resuspended in 50 μ l of fresh culture medium. Percentage sperm motility and percentage intact acrosomes (see below) were determined

at 0, 60, 180, and 360 min; 0 min is the time after the samples were rewarmed to 25°C.

Experiment 2. Sperm aliquots resuspended in PDVF were rapid cooled ($\sim 4^\circ\text{C}/\text{min}$) in a commercial refrigerator (5°C), and temperature decline in the sample was monitored using a digital thermocouple (Omega Engineering, Inc., Stamford, CT). An aliquot (20 μ l) was removed every 4°C drop in temperature (at 25°, 21°, 17°, 13°, 9°, and 5°C) and slowly diluted with 250 μ l of Ham's F-10 at the corresponding temperature. After a brief equilibration (10 min) at 25°C, samples were centrifuged ($300 \times g$; 8 min), supernatant was discarded, and pellets were resuspended in 50 μ l of fresh culture medium. Sperm motility and acrosomal integrity (see below) were evaluated at 0, 60, 180, and 360 min, with 0 min being the time after the samples were rewarmed to 25°C.

Experiment 3. Sperm aliquots resuspended in PDVF were slow cooled ($0.5^\circ\text{C}/\text{min}$) from 25°C to 0°C in a programmable alcohol bath (Bio-Cool; FTS Systems, Stone Ridge, NY). An aliquot (20 μ l) was removed every 4°C, slowly diluted with 250 μ l of Ham's F-10 at the corresponding temperature, and then placed in a 25°C water bath for a minimum of 10 min. After this equilibration, samples were centrifuged ($300 \times g$; 8 min), supernatant was discarded, pellets were resuspended in 50 μ l of fresh culture medium, and acrosomal integrity (see below) was evaluated.

Acrosomal Evaluation Using Fluorescein-Conjugated Peanut Agglutinin

To evaluate acrosomal membranes, washed sperm were stained using fluorescein-conjugated *Arachis hypogaea* (peanut) agglutinin (FITC-PNA; Sigma Chemical Co., St. Louis, MO). Briefly, FITC-PNA was dissolved in PBS at 1 mg/ml and stored frozen at -70°C . Before use, FITC-PNA stock solutions were diluted to 100 $\mu\text{g}/\text{ml}$ in PBS, and a 15- μ l aliquot was applied to the air-dried sperm samples. Slides were placed in a humidified chamber at 4°C for 15 min. Excess stain was washed three times with PBS, and slides were mounted in an antifade solution (Vectashield; Vector Laboratories Inc., Burlington, VT) and examined on a Leitz Ortholux epifluorescent microscope ($\times 1000$) [17]. At least 200 spermatozoa were examined for each aliquot. Spermatozoa exhibiting uniform bright staining over the acrosomal region were classified as acrosome intact, and those exhibiting a fragmented appearance or bright staining in the equatorial segment alone were classified as acrosome damaged [17].

Ultrastructural Analysis by Transmission Electron Microscopy

Washed sperm pellets were resuspended in PDVF at room temperature and maintained at room temperature (control) or rapid cooled ($\sim 4^\circ\text{C}/\text{min}$) to 5°C for 30 min in a commercial refrigerator (as described for experiment 1). After warming to 25°C, both samples were washed by centrifugation (as above), fixed in 2.5% glutaraldehyde for 2 h, and then postfixed in 1% osmium tetroxide for 1 h [17]. Fixed sperm then were stained with 2% uranyl acetate for 1 h and dehydrated through a series of ethyl alcohols. Dehydrated sperm were embedded in Spurr's resin, and sections were stained with lead acetate and examined under a Zeiss EM10 CA transmission electron microscope (Carl Zeiss, Inc., Oberkochen, Germany).

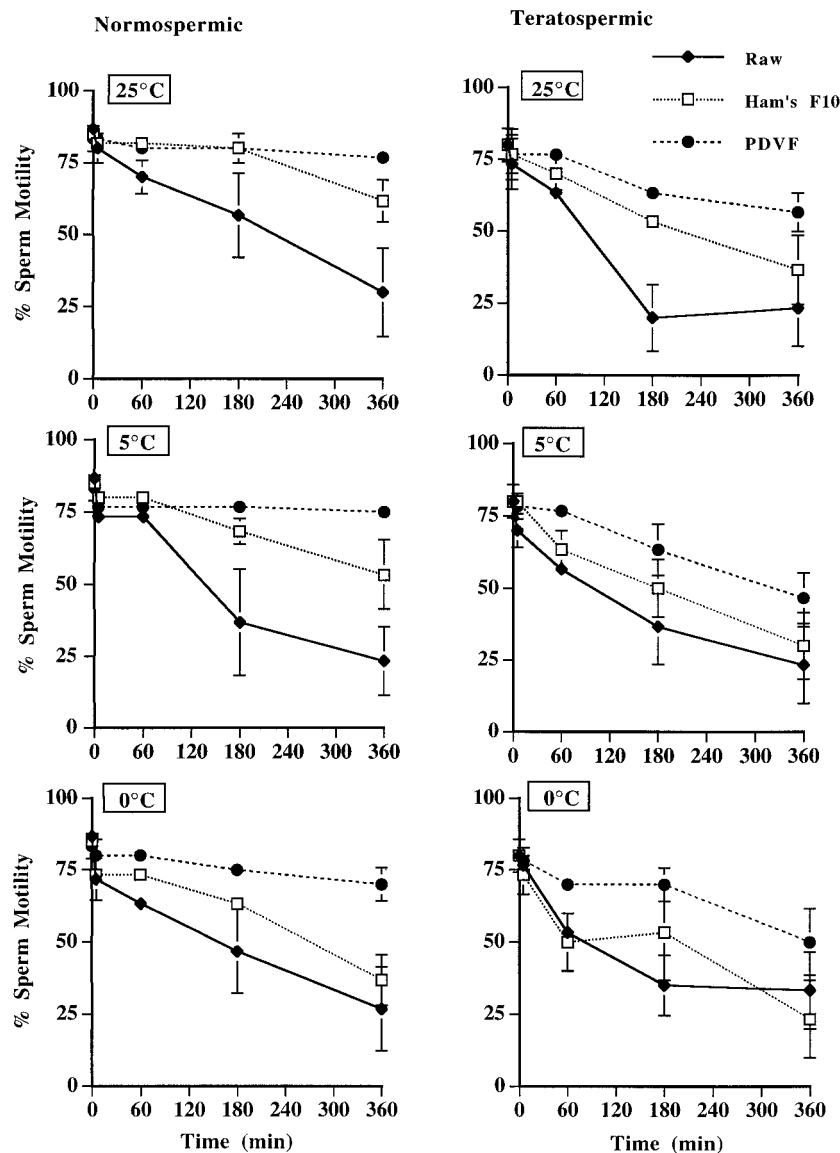


FIG. 1. Effect of cooling on domestic cat sperm motility. Electroejaculates from normospermic ($n = 3$ males; 2 ejaculates per male) and teratospermic ($n = 3$ males; 2 ejaculate per male) cats were maintained 1) raw, 2) washed and resuspended in Ham's F-10 containing 5% FCS, or 3) washed in Ham's F-10+FCS and resuspended in PDVF containing no glycerol. Samples were maintained at room temperature (25°C), rapid cooled ($\sim 4^{\circ}\text{C}/\text{min}$) to 5°C in a refrigerator for 30 min (5°C), or ultrarapid cooled ($\sim 14^{\circ}\text{C}/\text{min}$) in an ice-water bath for 10 min (0°C). After cooling, samples were diluted and rewarmed to 25°C for evaluation of percentage sperm motility at 0, 60, 180, and 360 min. Values are expressed as means \pm SEM.

Statistical Analyses

Sperm motility, forward progressive motility, and intact acrosomes were analyzed by using ANOVA after angular transformation of the data (SAS Institutes, Cary, NC). Statistical differences were determined by the least-significant difference multiple comparison procedure [23]. Means are presented \pm SEM.

RESULTS

Experiment 1: Effect of Rapid Cooling on Sperm Motility and Acrosomal Integrity

No reduction ($p > 0.05$) in sperm percentage motility was observed immediately after exposure to low temperatures (5°C or 0°C ; data not shown) or after warming to 25°C (Fig. 1; 0 min, 5°C and 0°C panels) in both cat populations. Also, no differences ($p > 0.05$) were seen in sperm progressive motility measures (data not shown). However, sperm motility, as well as progressive status, declined ($p < 0.05$) rapidly over time in both the raw ejaculate and washed samples upon prolonged storage at 25°C after prior cooling to 5°C or 0°C (Fig. 1, 60 to 360 min, 5°C and 0°C

panels). A similar decline also was observed in noncooled samples maintained at 25°C (Fig. 1, 25°C panels). Both rapid and ultrarapid cooling of normospermic domestic cat sperm in the presence of PDVF (an egg yolk buffer) maintained high levels of sperm percentage motility up to 360 min (Fig. 1). Furthermore, the decline in sperm percentage motility of the raw ejaculate maintained at 25°C was more rapid ($p < 0.05$) for teratospermic donors as compared to normospermic counterparts.

Raw ejaculate from both cat populations contained a high proportion of spermatozoa with intact acrosomal membranes (N, $85.3 \pm 2.1\%$; T, $81.5 \pm 3.8\%$; $p > 0.05$). However, rapidly cooling the extended semen (from both donor types) in a laboratory refrigerator or by immersion in an ice slush had a detrimental effect on acrosomal integrity. The proportion of intact acrosomes in normospermic males declined ($p < 0.05$) from $81.5 \pm 3.5\%$ (25°C) to $65.6 \pm 3.1\%$ (5°C) and $62.1 \pm 2.4\%$ (0°C) in the cooled groups. Sperm from teratospermic cats were even more susceptible ($p < 0.001$) to cold-induced membrane damage. In these donors, percentage intact acrosomes declined from $77.5 \pm 4.4\%$ (25°C) to $27.5 \pm 3.3\%$ (5°C) and $23.0 \pm 3.4\%$ (0°C), respectively.

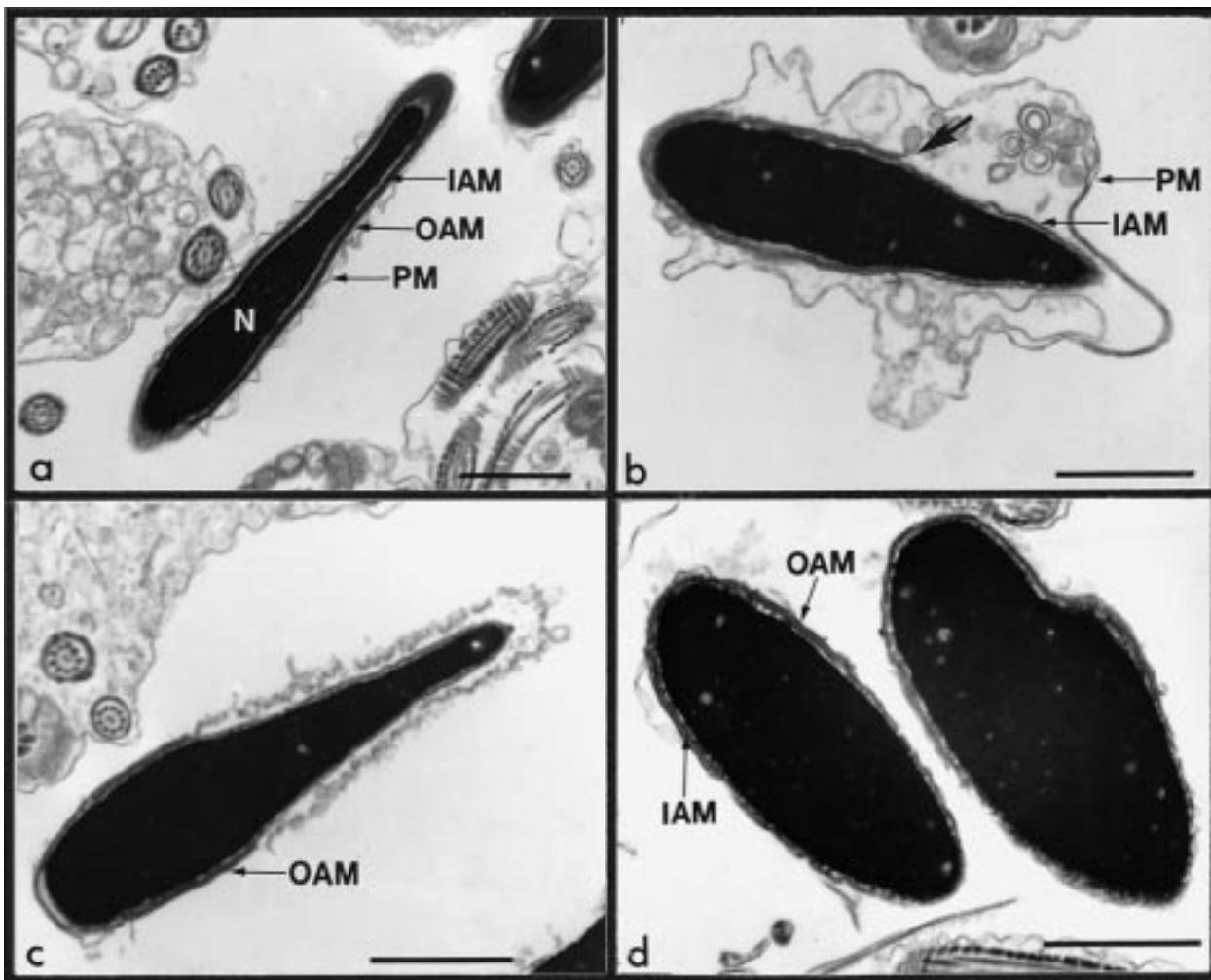


FIG. 2. Ultrastructural examination of cold-induced damage in cat spermatozoa ($\times 16\,000$). **a)** Normal spermatozoon with intact plasma and acrosomal membranes; N, nucleus; PM, plasmalemma; OAM, outer acrosomal membrane; IAM, inner acrosomal membrane. **b)** Spermatozoon with intact but distended plasmalemma and several internal vesicles; PM, plasmalemma; IAM, inner acrosomal membrane. Arrow, break in the outer acrosomal membrane. **c)** Spermatozoon with multiple areas of acrosomal vesiculation and complete absence of the plasmalemma; OAM, outer acrosomal membrane. **d)** Spermatozoa with complete loss of plasma and outer acrosomal membranes and acrosomal contents; OAM, outer acrosomal membrane; IAM, inner acrosomal membrane. Bar = $1\ \mu\text{m}$.

Ultrastructural examination of the noncooled aliquots revealed spermatozoa with intact membranes (Fig. 2a). After cooling, the sperm's plasmalemma appeared intact but distended, with the outer acrosomal membrane vesiculated and disintegrating (Fig. 2b). The inner acrosomal membrane remained fairly intact. A second damage category included extensive vesiculation of the plasma and outer acrosomal membranes, while the inner acrosomal membrane remained in close proximity to the sperm nucleus (Fig. 2c). Some spermatozoa experienced a complete loss of both the plasmalemma and outer acrosomal membrane, with only the inner acrosomal membrane covering the nuclear envelope (Fig. 2d).

Experiment 2: Kinetics of Rapid Cooling-Induced Acrosomal Damage

When sperm aliquots were assessed during rapid cooling ($\sim 4^\circ\text{C}/\text{min}$) in a commercial refrigerator, the proportion of intact acrosomes in normospermic males was higher ($p < 0.01$) than in teratospermic counterparts (Fig. 3). Percentage intact acrosomes declined gradually from $84.4 \pm 1.6\%$ (25°C) to $58.6 \pm 2.4\%$ (5°C) in ejaculates from normo-

spermic males versus $79.5 \pm 3.4\%$ (25°C) to $30.7 \pm 4.3\%$ (5°C) in teratospermic males. However, the greatest decline in acrosomal integrity occurred from 25°C to 17°C in both populations.

Experiment 3: Effect of Slow Cooling on Sperm Motility and Acrosomal Integrity

Controlled slow cooling ($0.5^\circ\text{C}/\text{min}$) of cat spermatozoa in a programmable unit from 25°C to 5°C and 0°C did not ($p > 0.05$) affect sperm percentage motility or sperm forward progressive motility in either sperm donor group (data not shown). Over a temperature range of 25°C through 9°C , the proportion of intact acrosomes was unaffected ($p > 0.05$), with a gradual decline thereafter in both cat groups (Fig. 4). Slow cooling markedly reduced ($p < 0.01$) acrosomal damage in teratospermic cats compared to rapid cooling. After cooling to 5°C , $75.5 \pm 5.0\%$ of normospermic and $68.3 \pm 3.9\%$ of teratospermic sperm exhibited intact acrosomes ($p > 0.05$). At 0°C , $64.7 \pm 7.7\%$ of sperm from normospermic and $56.8 \pm 3.6\%$ of sperm from teratospermic males contained intact acrosomes ($p > 0.05$).

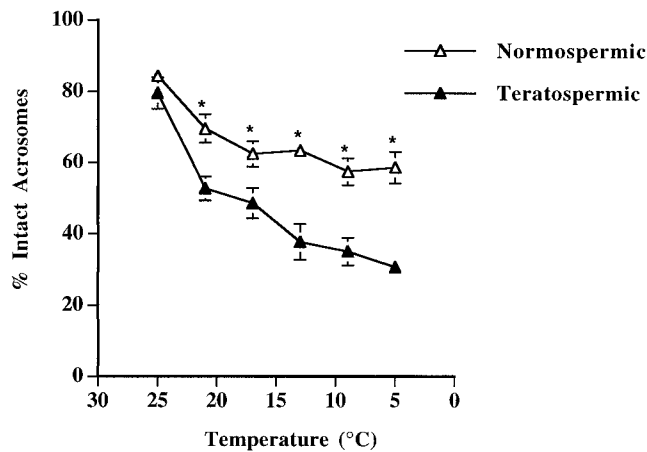


FIG. 3. Kinetics of acrosomal damage induced by rapid cooling ($\sim 4^\circ\text{C}/\text{min}$) of spermatozoa in a commercial refrigerator. Sperm from normospermic ($n = 3$ males; 2 ejaculates per male) versus teratospermic ($n = 3$ males; 2 ejaculates per male) cats were washed, and the sperm pellet was resuspended in PDVF. Aliquots were placed in a commercial refrigerator (5°C); change in temperature was monitored using a thermocouple. At indicated temperatures, aliquots were removed for acrosomal assessment using FITC-PNA stain. Values are expressed as means \pm SEM. At a given temperature, an asterisk indicates differences ($p < 0.01$) between the two cat populations.

DISCUSSION

The simple cooling of domestic cat spermatozoa caused massive acrosomal membrane damage without a concomitant loss in motility characteristics. Acrosomal disruption has been reported previously for cooled, frozen-thawed felid sperm [13, 14], but the damage was always assumed to have originated with actual cryopreservation events (crystallization and cell lysis) and not preliminary cooling equilibration. Although cat sperm motility appeared resilient to cold shock, acrosomal membrane integrity was highly sensitive to commonly used cooling rates. The second important finding from this study was that there were population-specific differences in sperm tolerance to cold exposure. Sperm from cats that routinely produced more malformed spermatozoa suffered more acrosomal damage than sperm from normospermic counterparts. Nevertheless, the extent of temperature-induced damage was significantly mitigated by slow cooling ($0.5^\circ\text{C}/\text{min}$), even in teratospermic ejaculates.

In most mammals studied to date (bull, ram, goat, pig), cooling spermatozoa rapidly to temperatures above zero can lead to an irreversible loss of motility, or cold shock. Glover and Watson [24] suggested that cat sperm collected by an artificial vagina are sensitive to cold shock. In contrast, sperm from our electroejaculates expressed normal motility traits despite cooling. Perhaps the conflicting findings are due to variations in seminal fluid arising from semen collection by an artificial vagina versus electroejaculation [25]. However, cat sperm motility is sustained within the epididymides or vasa deferentia stored (postcastration) at 5°C for extended periods [12], providing further evidence of a resilience to cold-induced depression in sperm motility. Recently, Hay et al. [26] reported that dog sperm, like cat sperm, seem to tolerate cooling better than that of many other mammalian species, suggesting that this may be a trait of the carnivores. In any case, sperm cooled in the presence of 20% egg yolk maintained higher sperm percentage motility and forward progression for longer intervals than raw ejaculate or washed sperm resuspended in

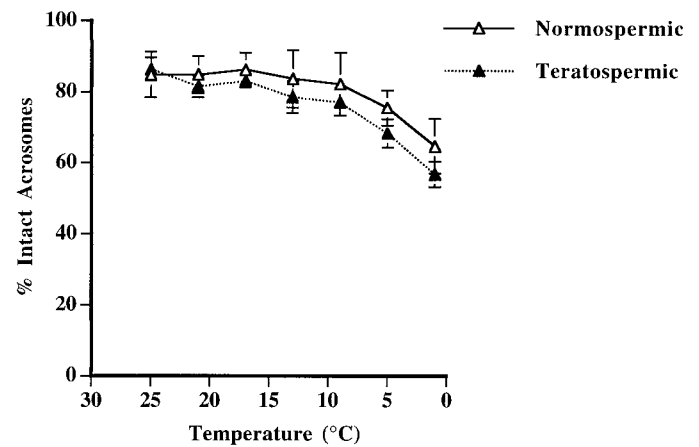


FIG. 4. Maintenance of cat sperm acrosomal integrity by slow cooling ($0.5^\circ\text{C}/\text{min}$). Sperm samples from normospermic ($n = 3$ males; 2 ejaculates per male) versus teratospermic ($n = 3$ males; 2 ejaculates per male) cats were washed, and the sperm pellet was resuspended in PDVF. Aliquots were placed in a programmable alcohol bath and cooled to 5°C . At indicated temperatures, aliquots were removed for acrosomal assessment using FITC-PNA stain. Values are expressed as means \pm SEM.

culture medium, demonstrating that cat sperm clearly have an affinity for egg yolk diluents. Although several mechanisms have been proposed to explain the value of egg yolk, the exact mode of action, including that for cooled cat sperm, remains to be elucidated [27].

There is increasing evidence in mouse [28] and pig [29] spermatozoa that cooling induces capacitation-like changes. These studies examined the effects of cooling on motility, plasma membrane intactness, acrosomal integrity, and fertilization rates in vitro. Capacitation-like changes were described as expression of the B staining pattern (using chlorotetracycline fluorescence staining) by sperm with an intact acrosome. Examination of spermatozoa in our studies, however, revealed a partial or complete loss of acrosomal integrity, changes that occurred not from capacitation events but rather from serious structural damage to sperm membranes. Byers et al. [11] determined that frozen-thawed tiger sperm more readily penetrated zona-free hamster eggs than freshly collected sperm. The interpretation was that cryopreservation was accelerating the capacitation process. However, based on present data, it is more likely that the tiger sperm experienced acrosomal disruption. Because sperm do not require acrosomal contents to penetrate zona-free oocytes [30], the cold-induced loss in acrosomal integrity may have facilitated higher penetration by mimicking a capacitated, acrosome-reacted state. It is worth noting that cooled domestic cat epididymal sperm coincubated with conspecific oocytes have an increased ability to attach to the zona but not to penetrate or fertilize [12]. Additional studies are needed to clarify the influence of cooling temperatures (above 0°C) on felid sperm capacitation.

Previous studies of bull and boar spermatozoa have demonstrated that rapid dilution in cryodiluent containing glycerol and/or rapid dilution combined with warming causes visible acrosome damage [31, 32]. These investigators indicated that both the dilution and warming components appear to be important factors influencing acrosomal stability, largely because high glycerol concentrations exert severe osmotic effects. Our results suggested that acrosomal disruptions can be unlinked to cryoprotectant/osmotic effects, simply because 1) we added no glycerol and 2) all samples were slowly diluted with diluent at the corresponding tem-

perature and gradually warmed. Obviously, the structural damages observed were due primarily to cooling injury alone.

Sperm from teratospermic cats were clearly more susceptible to cold-induced acrosomal damage than sperm from normospermic cats. We know from previous studies that even normal-appearing sperm from teratospermic ejaculates are compromised in the ability to capacitate and undergo the acrosome reaction in vitro [17]. Thus, it is likely that there are differences in sperm membrane composition between the two populations. Hoshi et al. [33] have reported that sperm with a high cholesterol:phospholipid ratio capacitate at a slower rate. Likewise, sperm from human patients with unexplained infertility also have a high cholesterol:phospholipid ratio, which is solely due to a lower phospholipid content [34]. Given this finding, determining the relative amounts of phospholipids and cholesterol may help explain observed variations in cooling sensitivity [27]. Such differences in sperm membrane primary structure could influence fluidity properties and phase-transition characteristics. Cooling and reheating of sperm membranes also have been shown to significantly alter fluidity patterns and affect the onset of lipid phase shifts, at least in boar sperm head membranes [35]. Such changes could alter the structural organization of membranes and subsequently influence sperm functionality [36]. However, no information is currently available on the primary composition or the phase-transition properties of sperm membranes from our two cat populations; such work is currently in progress.

Finding that sperm from teratospermic ejaculates were highly sensitive to cooling was relevant because teratospermia is a condition often seen in a variety of endangered felids [21, 37]. A high incidence of acrosomal membrane damage has been observed after cooling combined with freeze-thawing of sperm from these wild felid species [14]. Such precooled, prefrozen ejaculates also consistently contain a high proportion of sperm with morphologically deformed acrosomes. These anomalies could further increase susceptibility to irreversible membrane damage after cooling and/or rewarming. A preliminary report on normospermic domestic cats has demonstrated that slow cooling ($< 0.2^{\circ}\text{C}/\text{min}$) significantly reduces the proportion of sperm with damaged acrosomal membranes and, in turn, promotes an in vitro fertilization success rate of $\sim 75\%$ [38]. Integrated together, all findings suggest that a slow rate of cooling is an essential first step to ensuring preservation of acrosomal integrity in felid spermatozoa. Future studies will focus on characterizing the biophysical properties of felid sperm and the relationship to sperm survival and normal function after a cryostress.

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