

Sperm Capacitation and the Acrosome Reaction Are Compromised in Teratospermic Domestic Cats¹

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ABSTRACT

The efficiency of sperm capacitation and of the acrosome reaction was studied in the teratospermic domestic cat to evaluate further the etiology of compromised zona pellucida penetration and oocyte fertilization. Specific objectives were to compare normospermic and teratospermic cat ejaculates for 1) the kinetics and timing of sperm capacitation in vitro as determined by an ionophore-induced acrosome reaction; 2) the incidence of spontaneous acrosomal loss; 3) the ability of capacitated, swim-up processed sperm to acrosome-react in response to chemical (calcium ionophore) or physiological (solubilized zonae pellucidae) inducers; and 4) differences in acrosomal ultrastructure by use of transmission electron microscopy (TEM). Acrosomal status was determined with the fluorescent probe *Arachis hypogaea* (peanut) agglutinin. The timing of in vitro capacitation differed ($p < 0.05$) between cat populations. Normospermic samples were capacitated at 2.0 h postcentrifugation, whereas teratospermic samples required 2.5 h to become capacitated. At 2.5 h, sperm from teratospermic males were less capable ($p < 0.05$) of completing the acrosome reaction after ionophore exposure ($49.3 \pm 8.0\%$) than sperm from normospermic males ($73.3 \pm 3.8\%$). Levels of spontaneous acrosomal loss/reaction over time were similar ($p > 0.05$) between cat groups (range, 7.6–17.8%). In swim-up separated sperm from normospermic cats, ionophore A23187 was a more potent inducer ($p < 0.05$) of the acrosome reaction ($70.1 \pm 6.5\%$) than solubilized zonae pellucidae ($31.1 \pm 1.2\%$). Swim-up separated sperm from teratospermic cats, however, were compromised in the ability to acrosome react, regardless of inducer (ionophore, $23.9 \pm 3.3\%$; solubilized zonae pellucidae, $23.9 \pm 4.7\%$; $p > 0.05$). Sperm motility patterns over time indicated that differences in acrosomal status were not influenced by cell death. The frequency of abnormal acrosomes detected by TEM was higher ($p < 0.05$) in teratospermic ($30.0 \pm 3.9\%$) than in normospermic ($3.1 \pm 1.3\%$) samples. Swim-up separation failed to reduce ($p > 0.05$) the proportion of sperm cells with malformed acrosomes (swim-up, $33.5 \pm 3.5\%$; washed, $26.6 \pm 4.6\%$). These results indicate that sperm from teratospermic cats exhibit a high incidence of malformed acrosomes detectable only at the ultrastructural level. Nevertheless, acrosomal dysfunction is not related exclusively to structural defects because $> 40.0\%$ of swim-up separated sperm with structurally normal acrosomes still are incapable of completing the acrosome reaction. This suggests that compromised capacitation and acrosomal dysfunction may be responsible for low fertilization success in the teratospermic domestic cat.

INTRODUCTION

Teratospermia has a detrimental impact on in vitro gamete interaction in the domestic cat [1, 2]. Teratospermic cats produce poor quality ejaculates, characterized by a high incidence of pleiomorphic sperm ($> 60\%$ abnormal sperm/ejaculate) and low circulating testosterone concentrations [3]. Although swim-up separation increases the number of morphologically normal sperm recovered from teratospermic ejaculates, oocyte penetration and fertilization in vitro are compromised [1, 2, 4]. It appears that even morphologically normal sperm from teratospermic males have a specific dysfunction that prevents normal sperm-oocyte interaction.

The diminished ability of normal sperm from teratospermic cats to penetrate and fertilize conspecific oocytes

may originate at the level of the acrosome. Capacitated sperm from infertile men with teratospermia are less capable of completing the acrosome reaction in response to calcium ionophore [5] or progesterone [6] than are sperm from fertile men. Sperm capacitation is a prerequisite for the acrosome reaction in most eutherian mammals [7]; however, the timing of capacitation in the domestic cat is uncertain. In previous studies, in vitro fertilization [8–10] or zona penetration [11, 12] of homologous oocytes and zona penetration of salt-stored homologous oocytes [13] have been used as determinants of cat sperm capacitation. These studies reported that in vitro capacitation of epididymal sperm occurs within 30 min [10, 12] but may be maximal after 4 h of incubation with oocytes [12]. In vitro capacitation of ejaculated sperm, however, has been reported to occur from 1.5 h [11] to 7 h [13] after removing seminal plasma.

We hypothesized that both capacitation and the acrosome reaction in the domestic cat were influenced by teratospermia. Using normospermic and teratospermic males, we determined the timing of in vitro capacitation and assessed the level of spontaneous acrosomal loss during in vitro capacitation. After establishing the time course for in

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vitro capacitation, we compared the ability of capacitated sperm from both cat populations to acrosome-react in response to either chemical or physiological inducers. Swim-up separation was used to assess the ability of morphologically normal sperm to capacitate and acrosome react. *Arachis hypogaea* (peanut) agglutinin (PNA) was utilized as the determinant of acrosomal status and was validated in felid spermatozoa. Finally, transmission electron microscopy (TEM) was used to evaluate the acrosomal ultrastructure of both washed and swim-up processed sperm.

MATERIALS AND METHODS

Animals

Six adult males from two populations of domestic cats consistently producing either normospermic (> 60% normal sperm/ejaculate, $n = 3$ males) or teratospermic (< 40% normal sperm/ejaculate, $n = 3$ males) ejaculates were used as sperm sources [3]. Similar cats from this colony have been used in a series of studies to examine the influence of teratospermia on zona penetration [1, 4] and in vitro fertilization [2]. Male cats were housed as previously described [3]. Briefly, individually caged males were maintained at the National Institutes of Health Animal Center (Poolesville, MD) and exposed to 12 h of natural daylight per day. Males were provided dry, commercial cat food (Purina Cat Chow; Ralston Purina Co., St. Louis, MO) and water ad libitum.

Semen Collection, Analysis, and Processing

Semen was collected from normospermic and teratospermic cats according to a standardized electroejaculation procedure [3]. Males were anesthetized with an intramuscular injection of tiletamine hydrochloride-zolazepam (Telazol; A.H. Robbins, Richmond, VA; 4.5 mg/kg). A total of 80 electrical stimuli were delivered in three series using a rectal probe (1-cm diameter, 13-cm length, 3 longitudinal electrodes; P.T. Electronics, Boring, OR) and an AC, 60-Hz sine wave stimulator (P.T. Electronics).

Fresh semen was evaluated for ejaculate volume, sperm concentration, percentage of motile sperm, and progressive forward motility (scale of 0–5; 0 = no forward movement and 5 = rapid, linear forward movement) as previously described [3]. Semen was diluted 1:1 with Ham's F-10 medium (Sigma Chemical Company, St. Louis, MO) supplemented with 5% heat-treated fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). Seminal plasma was removed from diluted semen after centrifugation ($300 \times g$, 10 min). Pellets were either 1) resuspended with 100 μ l Ham's F-10 containing FBS (washed) or 2) overlaid with 100 μ l Ham's F-10 with FBS, and sperm were allowed to migrate from the pellet into fresh medium for 1 h at room temperature (swim-up). The upper layer (80 μ l) was removed after swim-up incubation. Sperm morphology was determined from fixed aliquots (0.3% glutaraldehyde) of raw semen and swim-up

processed sperm by means of phase-contrast microscopy ($\times 1000$) [3].

Evaluation of Acrosomal Ultrastructure

Acrosomal ultrastructure of sperm from normospermic (3 males; 2 ejaculates/male) and teratospermic (3 males; 2 ejaculates/male) cats was evaluated by TEM. Washed and swim-up processed sperm were fixed in 2.5% glutaraldehyde for 2 h and then postfixed in 1% osmium tetroxide for 1 h. Fixed sperm were stained en bloc for 1 h with 2% uranyl acetate and dehydrated with a series of ethyl alcohols (70%, 80%, 90%, and 100%). Dehydrated sperm were infiltrated with plastic resin. Sections were cut on an ultramicrotome, stained with lead citrate, and then examined under a Zeiss EM10 CA transmission electron microscope (Carl Zeiss, Inc., Oberkochen, Germany).

A total of 360 sperm heads from normospermic and teratospermic cats (180 sperm heads/population; a minimum of 20 sperm heads/ejaculate) were examined for acrosomal integrity by TEM. Acrosomal dimensions (apical and equatorial regions) and entire sperm head (length and width) were measured. Acrosomes were classified as structurally normal if the sperm nucleus was encompassed by a continuous, symmetrical, moderately electron-dense acrosomal matrix that was uniformly thickened at the apical region of the sperm head [14]. Acrosomes were considered to be abnormal only when distinct structural anomalies were present. These anomalies consisted primarily of 1) knob-like protrusions of extraneous acrosomal material or 2) vesicular protrusions of acrosomal material containing a lumen or with an apparent lumen partially occupied by redundant acrosomal material. These malformations generally were located near the apex of the acrosomal cap and had a uniformly granular quality of electron density consistent with the acrosomal matrix. Luminal structures generally appeared to be lined with acrosomal membrane but not plasma membrane.

Validation of PNA Acrosome Stain

As we prepared to conduct the present study, we compared PNA (Sigma) as an acrosomal marker to other common acrosomal stains, including Pope stain [15], Coomassie blue stain [16], Triple stain [17] and PSA (*Pisum sativum agglutinin*) [18]. Both the Pope and Coomassie blue stains were useful acrosomal markers, but the status of individual acrosomes was not always clearly defined. Triple stain did not elicit the characteristic staining patterns in cat sperm previously reported for human sperm [17]. PSA detected similar numbers of acrosome-reacted sperm to those detected by PNA, but the ethanol permeabilization required for PSA was more cumbersome. Because PNA had not previously been used as an acrosomal marker in cat sperm, we needed to verify that the lectin binding sites were similar to those reported for other species [19].

Ultrastructural localization of PNA binding sites in cat sperm was determined by TEM. Swim-up processed sperm from normospermic males (2 males; 1 ejaculate/male) were permeabilized with 0.1% (w/v) saponin in PBS for 5 min at room temperature [19]. Permeabilized sperm were labeled in suspension with a preparation of colloidal gold-conjugated PNA (140 $\mu\text{g}/\text{ml}$ PBS; 10-nm particle size; Sigma) for 15 min at room temperature. Labeled sperm were diluted with 1.5 ml of PBS and centrifuged ($300 \times g$; 10 min) to remove excess gold-conjugated PNA. Washed, labeled sperm were processed for TEM as previously described.

Assessment of Timing of In Vitro Capacitation

Fresh ejaculates from normospermic (3 males; 2 ejaculates/male) and teratospermic (3 males; 2 ejaculates/male) cats were diluted 1:1 with Ham's F-10 containing FBS and processed as washed samples as described above. Washed sperm were maintained at room temperature (25°C) for up to 3 h postcentrifugation. Ten-microliter aliquots were removed at 30, 60, 90, 120, 150, and 180 min postcentrifugation (time 0 = time of pellet resuspension) and were either processed as controls or induced to undergo the acrosome reaction with the calcium ionophore A23187 (Sigma). Ionophore was dissolved in dimethyl sulfoxide (DMSO) to yield an 8- μM stock and stored at -20°C until needed. The acrosome reaction was induced by incubating the 10- μl sperm aliquots with an equal volume of 8 μM ionophore to yield final concentrations of 4 μM ionophore and 0.1% DMSO. Sperm were incubated with ionophore for 30 min at 38°C in a humidified atmosphere of 5% CO_2 . Simultaneously, the 10- μl control sperm aliquots were incubated with 0.1% DMSO under identical temperature and gas conditions. Percentages of motile sperm and progressive forward motility were evaluated immediately before and after the 30-min exposure to ionophore or DMSO.

After incubation with ionophore or DMSO, sperm aliquots (5–10 μl) were air-dried onto slides at room temperature, stained with fluorescein isothiocyanate (FITC)-conjugated PNA (100 $\mu\text{g}/\text{ml}$) at 4°C for 10–15 min, rinsed with PBS, and allowed to dry. Slides were mounted with a permanent, nonfluorescing medium (Mowiol; Calbiochem, San Diego, CA) containing an antibleaching agent (*n*-propyl galate). PNA-stained sperm (200 sperm/slide) were evaluated with epifluorescence and scored as 1) acrosome-intact (AI) if medium-to-bright fluorescence was observed over the entire acrosomal region of the sperm head or 2) acrosome-reacted (AR) if fluorescence was not observed or was present only along the equatorial segment. Sperm that completed an ionophore-induced acrosome reaction were considered to be fully capacitated. The percentages of AR sperm in control and ionophore-treated samples were compared, and significant ($p < 0.05$) differences between these percentages were interpreted as the completion of capacitation by a subset of the sperm population.

Assessment of Spontaneous Acrosomal Loss

Fresh ejaculates from normospermic (3 males; 2 ejaculates/male) and teratospermic (3 males; 2 ejaculates/male) cats were processed as washed sperm (described above), except that samples were not exposed to either ionophore or DMSO. A 10- μl aliquot was removed at 30, 60, 90, 120, 150, and 180 min postcentrifugation (time 0 = time of pellet resuspension) and air-dried onto a slide. Air-dried slides were stained with PNA and scored for acrosomal status as outlined above.

Chemical Versus Physiological Induction of the Acrosome Reaction

Fresh ejaculates from normospermic (3 males; 2 ejaculates/male) and teratospermic (3 males; 2 ejaculates/male) cats were split into 2 aliquots, diluted 1:1 with Ham's F-10 containing FBS, and processed as either washed or swim-up samples as previously described. Both washed and swim-up sperm were capacitated in Ham's F-10 with FBS for 3 h at room temperature.

Sperm from washed and swim-up processed samples were induced to undergo the acrosome reaction with 4 μM ionophore A23187 (as described above) or solubilized zonae pellucidae (ZP). For the latter, intact ZP were prepared from immature, cumulus-free domestic cat oocytes, as described by Dunbar et al. [20]. In brief, ovaries were homogenized in PBS and filtered sequentially through a series of nylon mesh grids. Isolated ZP were heat-solubilized at 60°C for 1 h and then stored at -20°C until used. Washed and swim-up sperm samples (2×10^5 sperm/ml) were incubated with either Ham's F-10 (control) or solubilized ZP (2 ZP/ μl) for 1 h in a humidified, 5% CO_2 atmosphere at 38°C . After incubation with either ionophore, solubilized ZP, or the respective control medium (DMSO or Ham's F-10), sperm were air-dried onto slides, stained with PNA, and scored for acrosomal status, as described earlier. Sperm motility was evaluated immediately before and after incubation of sperm with inducers.

Statistical Analysis

Values are presented as means \pm standard error of the mean (SEM). Statistical analyses were performed by use of the Statistical Analysis System (analysis of variance) [21] or StatView (Student's *t*-test, chi-square analysis) [22]. Semen characteristics (ejaculate volume, sperm concentration, percent motile sperm, progressive forward motility, sperm morphology) for each cat population were analyzed by paired Student's *t*-tests. Chi-square analysis was performed on the frequencies of normal and malformed acrosomes detected by TEM. Acrosome reaction and motility data were analyzed by least-squares analysis of variance. For in vitro capacitation and spontaneous acrosomal loss trials, percentage of AI sperm, percentage of AR sperm, and per-

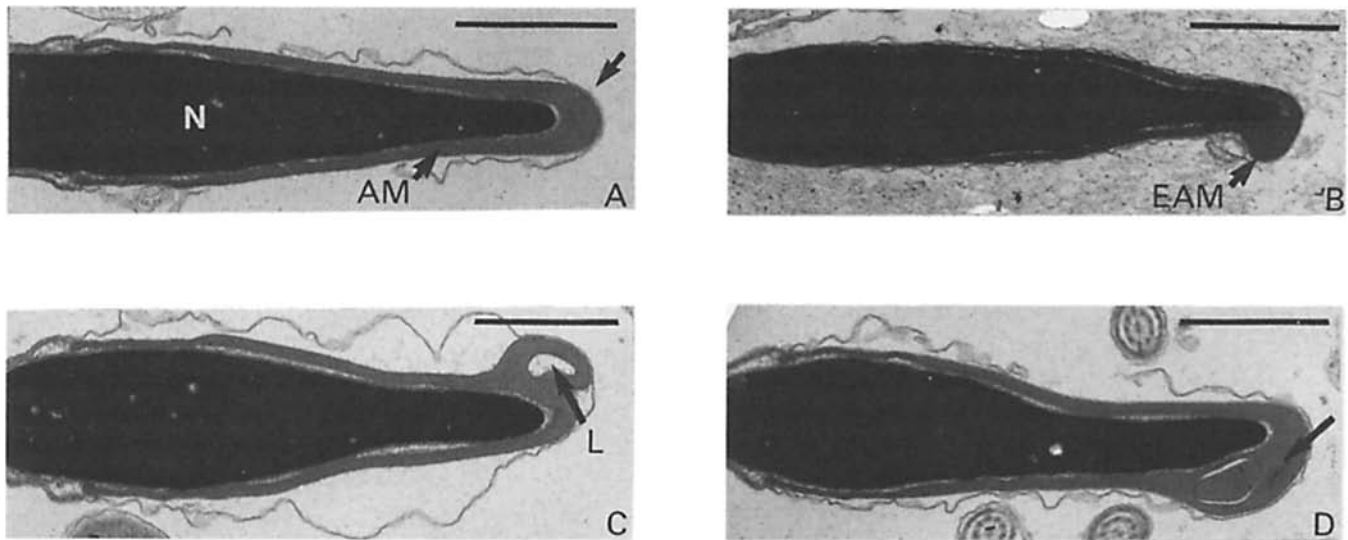


FIG. 1. Acrosomal ultrastructure of spermatozoa. Transmission electron micrographs of sperm heads from teratospermic males. **A)** Structurally normal acrosome consisting of symmetrical, moderately electron-dense, acrosomal matrix (AM) that surrounds the nucleus (N) and is uniformly thickened at apical region (arrow) of the sperm head. **B)** Structurally abnormal acrosome exhibiting knob-like fold of extraneous acrosomal material (EAM) near sperm head apex. **C)** Structurally abnormal acrosome with vesicular protrusion of acrosomal material containing large lumen (L) lined with acrosomal membrane. **D)** Structurally abnormal acrosome consisting of vesicular protrusion (arrow) with lumen partially occupied by redundant acrosomal material. Bar = 1 μ m.

centage of motile sperm for each cat population were analyzed as repeated measures. For trials using chemical and physiological inducers, percentage of AI sperm, percentage of AR sperm, and percentage of motile sperm for each cat population were analyzed by a split-split plot design, where cat population (normospermic, teratospermic) was designated as the whole plot, sperm processing group (washed, swim-up) as the sub-plot, and sperm treatment (ionophore, solubilized ZP, control) as the sub-sub plot.

RESULTS

Reproductive Characteristics of Normospermic and Teratospermic Populations

The ejaculate traits of normospermic and teratospermic males, shown in Table 1, were consistent with those reported previously in a study using similar analysis procedures [3]. Ejaculate volume, sperm concentration, percentage of motile sperm, and progressive forward motility of raw ejaculates were similar ($p > 0.05$) in normospermic and teratospermic populations. Both raw ejaculate and swim-up separated samples from normospermic males contained a similar ($p > 0.05$) high incidence of morphologically normal sperm. In contrast, the low proportion of morphologically normal sperm in raw ejaculates from teratospermic males was improved ($p < 0.05$) by swim-up processing.

Acrosomal Ultrastructure

Overall, higher ($p < 0.01$) proportions of malformed acrosomes were detected by TEM in teratospermic than in normospermic samples, regardless of sperm processing

method. The frequency of abnormal acrosomes was similar ($p > 0.05$) in washed ($26.6 \pm 4.6\%$) and swim-up separated ($33.3 \pm 3.5\%$) sperm from teratospermic males, as well as in washed ($1.1 \pm 1.0\%$) and swim-up separated ($4.5 \pm 2.7\%$) sperm from normospermic cats. Normal domestic cat sperm heads, including the acrosome, averaged $4.5 \pm 0.1 \mu$ m in length, $1.1 \pm 0.1 \mu$ m in cross-sectional width (Fig. 1, A-D), and $2.9 \pm 0.07 \mu$ m in sagittal width (Fig. 2A). Ultrastructurally normal acrosomes exhibited a symmetrically thickened apical region ($0.2 \pm 0.01 \mu$ m) of acrosomal matrix that narrowed ($0.04 \pm 0.002 \mu$ m) near the equatorial segment (Fig. 1A). Prevalent acrosomal abnormalities, consisting of extraneous protrusions of acrosomal material, are demonstrated in Figure 1, B-D.

Ultrastructural Localization of PNA Binding Sites

TEM analysis revealed that PNA lectin binding sites were localized on the outer acrosomal membrane of cat sperm.

TABLE 1. Ejaculate characteristics of normospermic and teratospermic domestic cats.

	Normospermic ejaculates (n = 6)	Teratospermic ejaculates (n = 6)
Ejaculate volume (μ l)	246.9 ± 10.1	256.7 ± 7.1
Sperm concentration ($\times 10^6$ /ml)	222.3 ± 13.1	199.0 ± 50.3
Sperm motility (%)	82.1 ± 1.1	69.6 ± 8.9
Progressive forward motility*	4.1 ± 0.1	3.7 ± 0.1
Structurally normal sperm (%)		
Raw ejaculate	77.7 ± 1.6	24.0 ± 5.0^a
Swim-up separated	80.0 ± 2.2	56.4 ± 2.3^b

* Scale of 0 to 5; 0 = no forward movement and 5 = rapid, linear movement.

^{a,b} Within columns, means (\pm SEM) with different superscripts differ ($p < 0.05$).

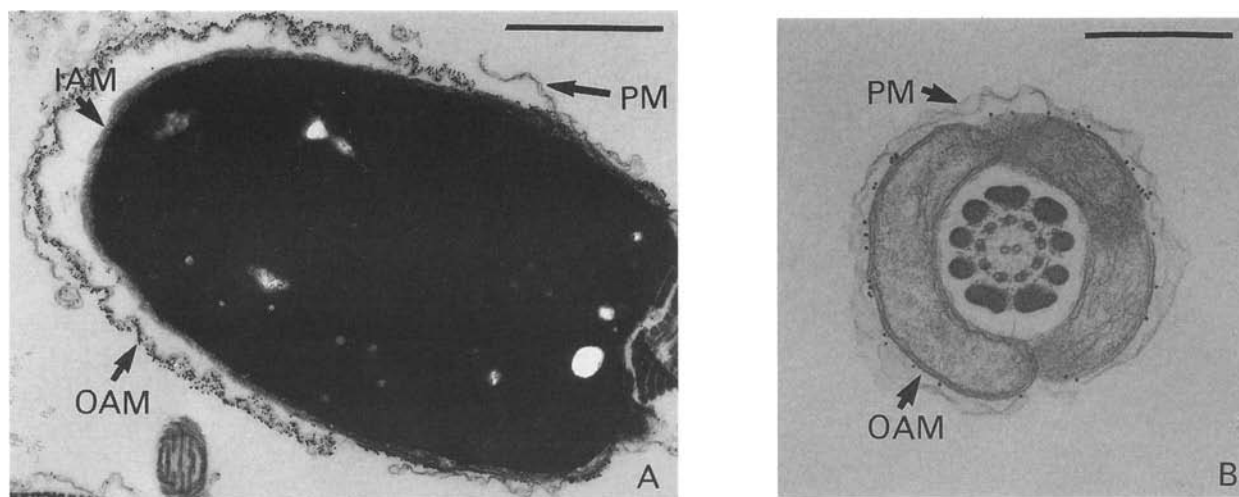


FIG. 2. Ultrastructural localization of PNA binding sites in cat spermatozoa. Swim-up processed, saponin-treated cat sperm were labeled in suspension with colloidal gold-conjugated PNA. **A)** Electron micrograph of spermatozoon from normospermic cat. Gold particles are aligned along outer acrosomal membrane, while neither residual plasma membrane or inner acrosomal membrane are labeled. **B)** Electron micrograph of midpiece of spermatozoon from normospermic cat. Gold particles are localized on outer acrosomal membrane, while intact plasma membrane is unlabeled. OAM, outer acrosomal membrane; PM, plasma membrane; IAM, inner acrosomal membrane. Bar = 1 μ m.

Figure 2A depicts the prevalence of PNA-conjugated, colloidal gold particles along the outer acrosomal membrane of a spermatozoon from a normospermic cat. Although a few gold particles were present on the remnants of the plasma membrane, the low frequency indicated minimal, nonspecific binding. Minimal, nonspecific labeling of the plasma membrane was further demonstrated by the lack of colloidal gold particles on the intact plasma membrane of the midpiece (Fig. 2B).

On the basis of the specificity of the PNA lectin for the outer acrosomal membrane, AI sperm exhibited medium-to-bright fluorescence over the entire acrosomal region of the head (Fig. 3A). AR sperm exhibited either fluorescence only along the equatorial segment (Fig. 3B) or no fluorescence in the head region.

Timing of In Vitro Capacitation

Incubating sperm from normospermic and teratospermic cats under capacitating conditions (in the presence of a serum protein source) revealed different kinetic patterns with respect to the onset of capacitation (Fig. 4A). No differences ($p > 0.05$) were detected in acrosome reactivity between control and ionophore-treated sperm for either cat population at 0, 0.5, 1, or 1.5 h. Sperm from normospermic males were first induced to acrosome-react by ionophore exposure at 2 h postcentrifugation (ionophore, $56.8 \pm 8.5\%$ AR sperm; control, $19.5 \pm 3.0\%$ AR sperm; $p < 0.05$). Maximal proportions of AR sperm in this group were achieved by 2.5 h (ionophore, $73.3 \pm 3.8\%$; control, $26.3 \pm 4.5\%$; $p < 0.05$). In contrast, sperm from teratospermic males did not

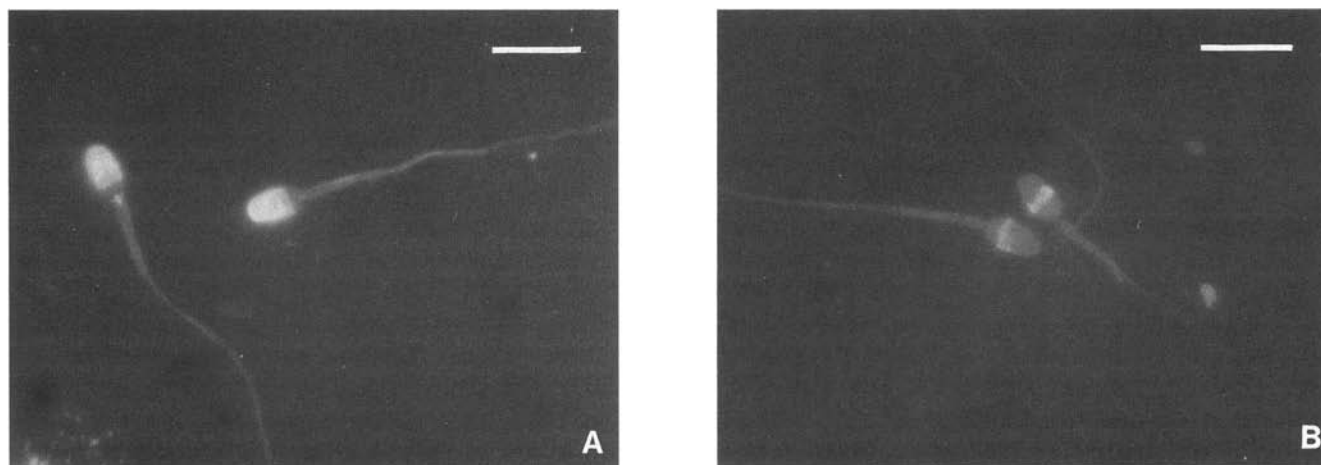


FIG. 3. Patterns of fluorescence of PNA-labeled cat spermatozoa. Sperm were air-dried onto slides and stained with FITC-conjugated PNA. **A)** AI sperm cells exhibited bright fluorescence over head region. **B)** AR sperm cells expressed fluorescence only at equatorial segment. Bar = 9 μ m.

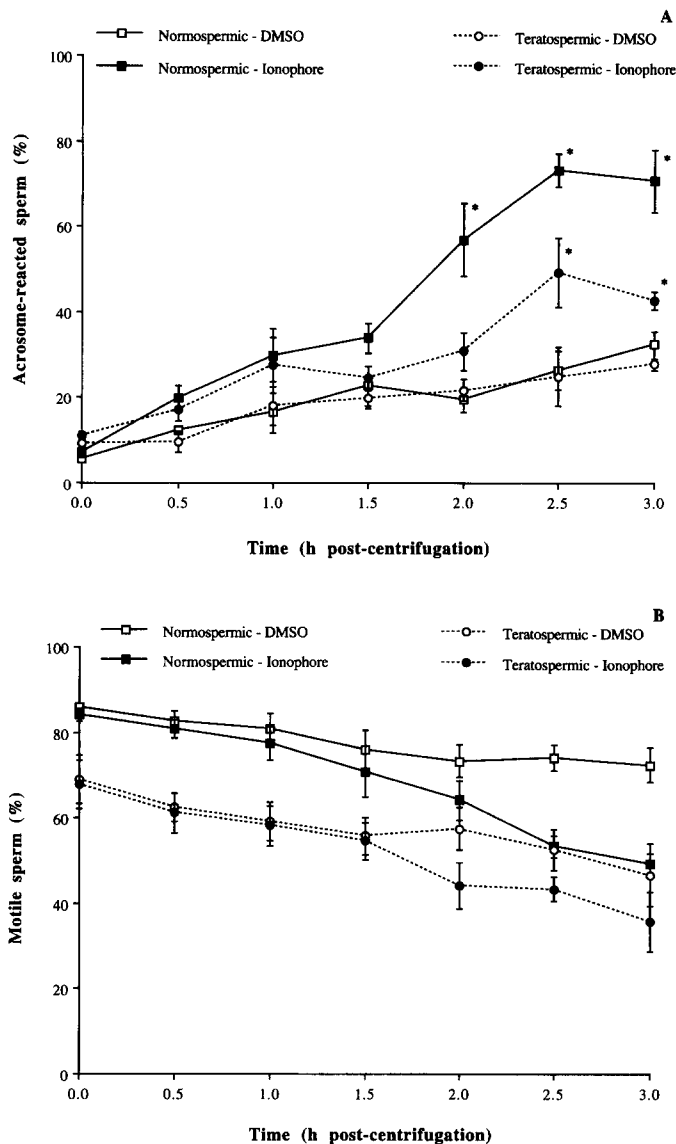


FIG. 4. Timing of in vitro capacitation in cat spermatozoa. **A)** Washed sperm from normospermic and teratospermic males were challenged with ionophore A23187 or DMSO (control) at 30-min intervals and evaluated for percentage of AR sperm. Time 0 = time of pellet resuspension after centrifugation. Acrosomal status was determined with FITC-PNA. Asterisks indicate significant differences ($p < 0.05$) in percentages of AR sperm between ionophore and DMSO treatments within cat population. Sperm from normospermic cats acquired ability to acrosome-react within 2 h of incubation and were fully capacitated by 2.5 h ($p < 0.05$). In contrast, sperm from teratospermic cats were unable to acrosome-react until after 2.5-h incubation ($p < 0.05$), and maximal AR was consistently lower for teratospermic than for normospermic samples ($p < 0.05$). **B)** Sperm motility was assessed before and after incubation with ionophore or DMSO. Data presented are motility ratings after 30 min of incubation with ionophore or DMSO. Sperm from both cat populations exhibited loss ($p < 0.05$) of motility at 2 h postcentrifugation after ionophore challenge.

acquire the ability to acrosome-react in response to ionophore challenge until 2.5 h (ionophore, $49.3 \pm 8.0\%$ AR sperm; control, $24.9 \pm 7.0\%$ AR sperm; $p < 0.05$). Overall, fewer ($p < 0.05$) sperm from teratospermic males underwent the acrosome reaction by 3 h ($42.6 \pm 2.1\%$) than did

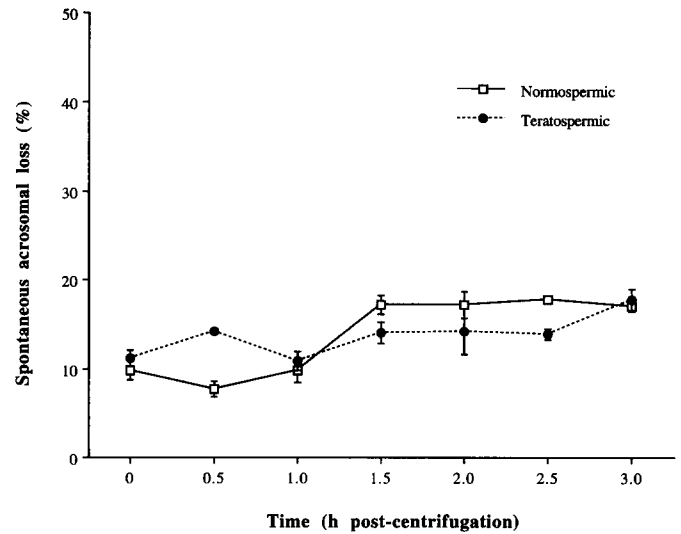


FIG. 5. Spontaneous acrosomal loss during in vitro capacitation of sperm (without exposure to ionophore or DMSO) from normospermic and teratospermic cats was similar ($p > 0.05$) between cat populations over time.

their normospermic counterparts ($70.8 \pm 7.4\%$).

Sperm motility declined gradually during the 3-h incubation (Fig. 4B). Although the rate of decline was similar for both cat populations, the percentages of motile sperm were lower ($p < 0.05$) for teratospermic samples than normospermic counterparts at each time point. Motility ratings did not differ ($p > 0.05$) between control and ionophore-treated sperm from either normospermic or teratospermic males during the first 1.5 h of incubation; however, after 2 h, the percentage of motility for each cat population was lower in ionophore-treated sperm than in controls ($p < 0.05$).

Spontaneous Acrosomal Loss during In Vitro Capacitation

Low levels of spontaneous acrosomal loss occurred in both normospermic and teratospermic samples that were incubated under capacitating conditions but not challenged with ionophore (Fig. 5). The percentages of sperm exhibiting spontaneous acrosomal loss were similar ($p > 0.05$) between normospermic and teratospermic males and linear with respect to time (normospermic, $y = 6.76 + 1.76x$, $R^2 = 0.73$; teratospermic, $y = 10.48 + 0.08x$, $R^2 = 0.571$). Spontaneous acrosomal loss increased from 9.8% to 17.0% in normospermic males and from 11.1% to 17.8% in teratospermic cats.

Chemical Versus Physiological Induction of the Acrosome Reaction

The incidence of the acrosome reaction in capacitated sperm after an ionophore A23187 or a solubilized ZP challenge differed between normospermic and teratospermic males, regardless of sperm processing method (Table 2). Fewer ($p < 0.05$) washed sperm from teratospermic males

TABLE 2. Percentages of acrosome-reacted sperm in washed or swim-up separated normospermic and teratospermic cat ejaculates capacitated for 3 h and challenged with calcium ionophore A23187 or solubilized ZP.

Sperm treatment	Normospermic ejaculates (n = 6)		Teratospermic ejaculates (n = 6)	
	Washed	Swim-up	Washed	Swim-up
Capacitated only	12.1 ± 1.9 ^{xya}	10.1 ± 1.9 ^{xa}	18.3 ± 1.9 ^{yzb}	19.5 ± 1.6 ^{zba}
DMSO (control)	24.1 ± 3.6 ^{xyb}	19.8 ± 3.1 ^{xb}	28.1 ± 1.7 ^{ybc}	20.7 ± 1.2 ^{xb}
Ionophore A23187	71.1 ± 6.2 ^{xc}	70.1 ± 6.5 ^{xc}	38.3 ± 3.7 ^{yd}	23.9 ± 3.3 ^{zb}
Ham's F-10 (control)	15.9 ± 2.5 ^{xya}	8.8 ± 1.9 ^{xa}	30.9 ± 4.6 ^{zc}	19.8 ± 1.4 ^{yba}
Solubilized ZP	40.7 ± 2.4 ^{xd}	31.1 ± 1.2 ^{yd}	24.4 ± 1.0 ^{yzb}	23.9 ± 4.7 ^{zb}

^{x,y,z} Within rows, means ± SEM with different superscripts differ ($p < 0.05$).

^{a,b,c,d} Within columns, means ± SEM with different superscripts differ ($p < 0.05$).

acrosome-reacted in response to ionophore or solubilized ZP than did sperm from corresponding normospermic samples. Similarly, a lower ($p < 0.05$) proportion of swim-up separated sperm from teratospermic males acrosome-reacted in response to ionophore or solubilized ZP than of swim-up separated sperm from normospermic males.

Swim-up separation did not alter the ability of sperm from normospermic males to undergo the acrosome reaction, except in response to solubilized ZP (Table 2). In this case, fewer ($p < 0.05$) swim-up separated sperm from normospermic males completed the acrosome reaction than did washed sperm. In teratospermic males, swim-up separation failed to enhance the ability of sperm to complete the acrosome reaction in response to ionophore A23187 or solubilized ZP. After ionophore challenge, the percentage of AR sperm was 1.6-fold higher ($p < 0.05$) for washed than for swim-up separated sperm from teratospermic samples. Similar ($p > 0.05$) percentages of AR sperm occurred after solubilized ZP challenge in both swim-up separated and washed teratospermic samples, and neither had higher than basal acrosome reactivity levels measured in corresponding Ham's F-10 controls.

Overall, ionophore A23187 clearly was a more ($p < 0.05$) potent inducer of the acrosome reaction than solubilized ZP for both washed and swim-up separated normospermic and washed teratospermic samples. For both cat populations, sperm motility ratings were similar ($p > 0.05$) for both Ham's F-10 control sperm (normospermic, $76.9 \pm 3.7\%$ motile; teratospermic, $43.8 \pm 2.4\%$ motile) and solubilized ZP-challenged sperm (normospermic, $72.8 \pm 2.3\%$ motile; teratospermic, $40.9 \pm 2.3\%$ motile), but were lower ($p < 0.05$) for ionophore-challenged sperm (normospermic, $61.7 \pm 4.4\%$ motile; teratospermic, $33.3 \pm 2.2\%$ motile) than for DMSO control sperm (normospermic, $76.3 \pm 2.5\%$ motile; teratospermic, $46.7 \pm 1.6\%$ motile), regardless of sperm processing.

DISCUSSION

Sperm capacitation has long been recognized as a time-dependent, species-dependent phenomenon [23]. Similar to human [24] sperm, domestic cat sperm appear to require a shorter time (2–2.5 h) to capacitate than ejaculated sperm

of pigs (4–5 h) [25], bulls (4 h) [26], or dogs (4–5 h) [27]. Additionally, the conditions for in vitro capacitation of domestic cat sperm are less complex than for other species that require exposure to a hypertonic medium [28], to heparin [26], or to a high calcium concentration [25]. On the contrary, removing seminal plasma is unnecessary for cat oocyte penetration; simple semen dilution is sufficient for gamete interaction in the domestic cat [1] and tiger (*Panthera tigris*) [29]. The discovery that domestic cat sperm exhibit a relatively low frequency of spontaneous acrosomal loss in vitro also differs from that observed in sheep [30] and dog [31] sperm. Additionally, cat sperm undergo capacitation at room temperature, whereas many other species studied require 37–39°C temperatures [7]. Therefore, the domestic cat spermatozoon is unique with respect to that of other species in terms of the timing and relative ease of sperm capacitation and the absence of spontaneous acrosomal loss in vitro.

Hamner et al. [8], the first to study capacitation requirements in the domestic cat, reported that washed, ejaculated sperm require 0.5–24 h of incubation in utero before acquiring fertilization capacity in vitro. Bowen [9] demonstrated that ductus deferens cat sperm could fertilize domestic cat oviductal oocytes in vitro without incubation in the female reproductive tract. Some earlier published work suggested an almost remarkable onset of sperm capacitation in vitro. For example, Niwa et al. [10] achieved in vitro penetration of oocytes within 30 min of coincubation, but these sperm were of epididymal origin. Goodrowe et al. [11] and Goodrowe and Hay [12] also used oocyte penetration as an indicator of capacitation and observed sperm-zona interaction within 30 min of coincubation. In these studies, however, no distinction was made between sperm attachment and zona penetration. In our systematic approach using the ionophore-induced acrosome reaction as a measure of capacitation, we determined that domestic cat sperm require at least a 2-h incubation in vitro after seminal plasma removal to become capacitated. Further, we found that the kinetics of in vitro sperm capacitation were affected by male reproductive status. Almost half the sperm from normospermic males were fully capacitated 2 h after seminal plasma removal, as demonstrated by the ability to respond

to ionophore challenge. In contrast, sperm from teratospermic males were unable to respond to ionophore challenge until 2.5 h after separation from seminal plasma. Sperm from teratospermic males not only experienced delayed capacitation *in vitro* but also were less efficient in completing the acrosome reaction than normospermic counterparts. Sperm motility patterns over time confirmed that differences in acrosomal status between cat populations were not influenced by cell death.

The ability of sperm to respond to a stimulus like ionophore A23187 is indicative of two important sperm functions, capacitation and the acrosome reaction. The fact that capacitated sperm from subfertile men are less capable of responding to ionophore challenge than sperm from fertile men [32] suggests that there is a direct relationship between acrosomal dysfunction and infertility. Further affirmation of this theory is illustrated by a correlation between the ionophore response of human sperm and sperm penetration rate of zona-free hamster oocytes [33]. We have reported that sperm from teratospermic domestic cats (i.e., those males consistently producing < 40% structurally normal sperm per ejaculate) are compromised in the ability to bind, penetrate, and fertilize conspecific oocytes *in vitro* [1, 2]. New data generated here suggest that diminished capacitation and resulting acrosomal dysfunction may be one mechanism by which teratospermia exerts its influences on the fertilization process in the domestic cat.

To reduce the impact of gross sperm malformations on the acrosome reaction, swim-up processing was used to recover a high proportion of morphologically normal sperm from teratospermic ejaculates, as detected by light microscopy. However, the TEM examination of acrosomal ultrastructure demonstrated the limitation of light microscopy because sperm with structurally malformed acrosomes still were present after swim-up separation. Although this type of processing failed to eliminate all sperm with structurally defective acrosomes, most (> 60%) sperm had normally shaped acrosomes. Even so, swim-up separated sperm from teratospermic cats remained compromised in the ability to undergo the acrosome reaction. This indicated that acrosomal dysfunction associated with teratospermia was not related exclusively to morphology. In turn, this finding may explain why swim-up separation of ejaculates collected from teratospermic cats fails to enhance zona penetration or fertilization of homologous oocytes [1, 2].

We were interested in the ability of chemical and physiological stimuli to induce the acrosome reaction in cat sperm, which we investigated by simultaneously comparing these stimuli. Overall, the biological activity of domestic cat ZP membranes was similar to that reported for other species including the mouse [34], rabbit [35], dog [31], and human [36]. For most species, a fairly low concentration of solubilized ZP (1–2 ZP/ μ l) induces the acrosome reaction in approximately 15–29% of homologous sperm after a 30-min

to 1-h incubation. Physiological induction of the acrosome reaction reinforced the differences in sperm function between the normospermic and teratospermic cat populations. Although solubilized ZP induced the acrosome reaction in normospermic males, this stimulus failed to elicit the acrosome reaction in teratospermic males. Likewise, the chemical ionophore stimulus induced a response in swim-up separated sperm no greater than the basal acrosome reaction measured in teratospermic controls. Together, these observations further demonstrated the inability of morphologically normal sperm from teratospermic males to undergo one of the sperm functions critical to fertilization.

The mechanism by which teratospermia causes acrosomal dysfunction in the cat may involve one or more regulating factors at the cellular level. For example, in mouse sperm, phosphorylation is one regulatory component of the acrosome reaction; using a tyrosine kinase inhibitor reduces the level of solubilized ZP-induced acrosome reaction [37]. A similar mechanism may be in place in the cat. Recently, we demonstrated that tyrosine phosphorylation of a 95-kDa sperm receptor is compromised by teratospermia in the cat [38]. The inability of solubilized ZP to induce the acrosome reaction in capacitated sperm from teratospermic males in the present study is most likely a result of diminished phosphorylation efficiency associated with this cat population. Detailed studies are in progress to determine the role of tyrosine kinase in regulation of the acrosome reaction by using specific tyrosine kinase inhibitors.

An important prerequisite event for membrane fusion during the acrosome reaction is the translocation of extracellular calcium ions. Calcium ionophore A23187 facilitates the passage of calcium ions across the plasma membrane [39] and thus artificially initiates the acrosome reaction. The inability of sperm from teratospermic cats to respond to the ionophore A23187 may reflect abnormalities in membrane permeability or intracellular mechanisms regulating calcium transport. A recent report indicated that sperm from teratospermic men exhibit abnormal basal calcium levels and little or no calcium influx in response to progesterone-stimulated acrosome reactions [6]. One of our planned studies is to examine the role of calcium ions in regulating the acrosome reaction in domestic cat sperm to further define the impact of teratospermia on sperm function.

All evidence to date suggests that, as the incidence of teratospermia increases, reproductive efficiency decreases. There are two taxonomic groups that benefit from this expanding database. First, because of a similarly high incidence of pleiomorphic sperm ejaculated in the teratospermic cat and most human males, we have asserted that the teratospermic cat is an excellent model for studying functional fertility in the human male [40]. Secondly, the majority of endangered felids (28 of the 36 species) routinely ejaculate high proportions (> 30%) of pleiomorphic sperm [41]. Therefore, the teratospermic domestic cat also

is an important model for studying the etiology of interspecies similarities and differences associated with sperm function mechanisms. For the wild felid species, in particular, it is likely that the physiology of teratospermia will be useful to improving assisted reproductive techniques that have both conservation and management potential. From an applied perspective, our new data here indicate that selection of functionally competent sperm from teratospermic ejaculates requires more definitive separation techniques than swim-up processing alone. Elucidating the underlying biochemical and molecular events that are influenced by teratospermia may ultimately lead to identification and perhaps recovery of the subset of functionally competent sperm most useful for enhancing fertility.

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