BIOLOGY OF REPRODUCTION 49, 131–139 (1993)

Zona Pellucida Filtration of Structurally Abnormal Spermatozoa and Reduced Fertilization in Teratospermic Cats[1]

J.G. HOWARD,[2] A.M. DONOGHUE, L.A. JOHNSTON, and D.E. WILDT

National Zoological Park, Smithsonian Institution, Washington, District of Columbia 20008

ABSTRACT

Zona pellucida (ZP) penetration, in vitro fertilization, embryo development, and the morphology of fertilizing sperm were examined through use of normospermic (> 60% structurally normal sperm/ejaculate) versus teratospermic (< 40% structurally normal sperm/ejaculate) ejaculates from domestic cats. In addition, the effect of swim-up processing on sperm-oocyte interaction was compared with that of simple sperm washing. Normospermic and teratospermic ejaculates were evaluated for sperm motility and morphology. Sperm were preincubated for 1 h, then coincubated with in vivo-matured follicular cat oocytes (n = 401) for 20 h and with ZP-intact, salt-stored oocytes (n = 202) for 6 h. In vivo-matured oocytes were assessed for percent cleavage and stage of embryo development over time. Salt-stored oocytes were assessed for percent ZP penetration (proportion of oocytes containing sperm within or through the inner ZP), mean (± SEM) number of inner ZP-penetrated sperm, and the morphology of all bound and penetrated sperm. The incidence of pleiomorphic sperm in raw ejaculates averaged 29% in normospermic versus 67% in teratospermic males, but all ejaculates contained high sperm motility ratings (> 60%). Swim-up processing increased ($p < 0.05$) the number of normal sperm recovered/teratospermic inseminate (66.5 ± 2.3%) compared to recovery after simple washing (28.6 ± 2.2%). Percent sperm motility also increased ($p < 0.05$) in teratospermic males after swim-up (90.0 ± 1.3%) as compared to sperm washing (64.2 ± 3.7%). Cleavage rate in vitro was higher ($p < 0.05$) using sperm from normospermic (86.3%) compared to teratospermic (50.3%) males, but rates of embryo development to the morula/blastocyst stage were similar ($p > 0.05$). ZP penetration of salt-stored oocytes by normospermic ejaculates (73.7%) was superior ($p < 0.01$) to that of teratospermic ejaculates (24.1%); the number of ZP-penetrated sperm/oocyte was 5-fold higher ($p < 0.05$) in the normospermic than in the teratospermic group. The proportion of structurally normal bound and ZP-penetrated sperm was similar ($p > 0.05$) between cat populations regardless of the morphologic forms in the inseminate. Structurally abnormal sperm from teratospermic males were capable of ZP binding (29%) and initial penetration into the outer ZP (17%). However, only 3% of the inner ZP sperm were pleiomorphic, and every sperm within the perivitelline space was morphologically normal. Although swim-up processing increased sperm motility and the number of structurally normal sperm in the teratospermic inseminates, no difference ($p > 0.05$) was observed in cleavage or ZP penetration. These results indicate that the ZP is an efficient filter for structurally abnormal sperm, but even normal sperm from teratospermic cats appear impaired in fertilizing ability. Although teratospermia compromises ZP penetration and fertilization, the few embryos produced via teratospermic ejaculates develop normally in vitro.

INTRODUCTION

In vitro fertilization (IVF) is a valuable tool for assessing sperm functionality and for studying the success or failure of gamete interaction in the domestic cat and its wild relatives [1, 2]. Viable-appearing IVF embryos have been generated in the cat (*Felis catus*) [3–6], leopard cat (*Felis bengalensis*) [7], Indian desert cat (*F. silvestris*) [8], puma (*F. concolor*) [9], tiger (*Panthera tigris*) [10, 11], and cheetah (*Acinonyx jubatus*) [12]. Furthermore, IVF embryos from the domestic cat [3], Indian desert cat [8], and tiger [10] have proven biologically competent as demonstrated by the production of live offspring after embryo transfer.

Although relatively consistent within species, IVF efficiency varies notoriously among felid taxa. At least two male factors appear to contribute to species-specific differences in IVF success. Donoghue et al. [12] recently reported that the inability of sperm from certain cheetah males to sustain motility for 6 h in vitro was highly correlated to IVF failure. We also have speculated that species differences in the proportions of structurally abnormal sperm in the inseminate influence sperm-oocyte interaction in vitro and the formation of cleaved embryos [1, 2, 13–15]. Certain felid taxa ejaculate extraordinarily high proportions of structurally deformed sperm [16, 17]. One survey revealed that 22 of 28 nondomesticated species commonly produce more than 40% abnormal sperm/ejaculate [18], a finding perhaps related to diminished genetic variation within specific populations or entire species [15, 16]. The cheetah, which experiences poor sperm motility, also routinely produces more than 70% structurally abnormal sperm/ejaculate and exhibits IVF embryo cleavage rates of ~17% [12]. Likewise, pumas produce an extremely high proportion of pleiomorphic sperm (> 90%) and show a cleavage rate of < 10% [9]. This is in marked contrast to most domestic cats, which routinely produce more than 70% normal sperm and exhibit IVF cleavage rates of ~60–80% [4–6, 14, 15]. Likewise, tigers produce few pleiomorphic sperm/ejaculate (< 20%), while IVF rates usually exceed 60% from either fresh or frozen-thawed spermatozoa [10, 11].

Accepted February 24, 1993.

Received August 12, 1992.

[1]This research was supported, in part, by grants from the National Institutes of Health (RR00045 and HD23853), NOAHS (New Opportunities of Animal Health Sciences) Center, the Friends of the National Zoo (FONZ), and the Ralston Purina Company/American Association of Zoological Parks and Aquariums Conservation Endowment Fund.

[2]Correspondence. FAX: (202) 673–4733.

In testing the hypothesis that pleiomorphic sperm contribute to IVF inefficiency in felids, one problem is that sperm quality is fairly uniform among individuals within species. However, recently we determined that certain adult domestic cats consistently ejaculate relatively high proportions (> 60%) of deformed spermatozoa [19]. Compared with normospermic cats, teratospermic males produce similar serum FSH and LH concentrations but lower testosterone levels [19]. We have demonstrated that compared to sperm from normospermic males, sperm from these androgen-deficient, teratospermic cats are less capable of penetrating ZP-free hamster ova and of binding and penetrating the ZP of immature, conspecific oocytes [13]. Interestingly, swim-up separation enhances sperm motility and the number of structurally normal cells recovered, but fails to improve the ability of these sperm to interact with immature oocytes in vitro [13].

The present study was designed to determine whether sperm from teratospermic domestic cats are compromised in their ability to bind, penetrate, and fertilize mature, conspecific oocytes in vitro. The use of swim-up processing allowed comparison of the functionality of structurally normal as well as abnormal sperm from teratospermic males. A second objective was to assess the fate of pleiomorphic sperm, especially to determine whether these cells are capable of binding the ZP, penetrating the ZP bilayers [20], and entering the perivitelline space. Two populations of cats (normospermic and teratospermic), two sperm processing techniques (washing and swim-up), and two types of ZP-intact oocytes (in vivo-matured follicular oocytes and in vitro-matured, salt-stored oocytes) were used to achieve these objectives.

MATERIALS AND METHODS

Animals

Adult males from two domestic cat populations producing either consistently high (> 60%/ejaculate; normospermic) or low (< 40%/ejaculate; teratospermic) proportions of structurally normal spermatozoa were studied simultaneously. These same males had been used in earlier studies to assess the impact of teratospermia upon sperm interaction in vitro with ZP-free hamster oocytes and immature, conspecific oocytes [13, 19]. Sixteen adult female cats with unknown reproductive histories were used as donors of in vivo-matured oocytes. Details on animal housing and maintenance have been described previously [19]. Briefly, cats were exposed to ~12 h of natural daylight/day, 10 h of which were supplemented with artificial lighting. Males were maintained individually, females housed in pairs, and all animals provided dry cat food (Purina Cat Chow; Ralston-Purina Co., St. Louis, MO) and water ad libitum.

Induction of Ovarian Activity and Laparoscopic Aspiration of Follicular Oocytes

Females were treated with a single i.m. injection of 150 IU eCG (Sigma Chemical Company, St. Louis, MO) to stimulate ovarian follicular development. Final oocyte maturation was induced with 100 IU hCG (Sigma) given i.m. 84 h after eCG. Twenty-four to 26 h after the hCG injection, oocytes were recovered laparoscopically from ovarian follicles (≥ 2 mm in diameter) via a standard laparoscopic procedure for domestic cats [3]. In brief, a surgical plane of anesthesia was induced with a ketamine hydrochloride (Vetalar; Parke-Davis, Morris Plains, NJ) and acepromazine maleate (Ayerst Labs., Rouses Pt., NY) mixture (10:1 ratio; 18.0–20.0 mg/kg b.w. and 0.2 mg/kg b.w., respectively, i.m.). To sustain this anesthetic plane, each female was maintained on isoflurane gas/oxygen through use of a face mask. Follicular contents were aspirated transabdominally under laparoscopic observation into collection tubes containing Ham's F-10 medium plus 5% fetal calf serum (FCS) and heparin (40 IU/ml). The contents of each tube were emptied into plastic culture dishes and examined by stereomicroscopy. Only oocytes designated as mature (on the basis of expansion of the corona radiata and cumulus cell mass) were used [14, 21]. Mature oocytes were washed three times in Ham's F-10/FCS under oil and maintained at 38°C in a humidified environment of 5% CO_2 in air.

Semen Collection, Analysis, and Processing

Semen was collected from normospermic (6 ejaculates; 3 males) and teratospermic (6 ejaculates; 3 males) adult cats through use of a standardized electroejaculation procedure [16, 19]. Each male was anesthetized for semen collection with an i.m. injection of tiletamine hydrochloride-zolazepam (Telazol; 4.5 mg/kg, A.H. Robins, Richmond, VA). A 60-Hz sine wave stimulator (P.T. Electronics, Boring, OR) and a rectal probe (1 cm in diameter × 13 cm in length) with three longitudinal electrodes (P.T. Electronics, Boring, OR) were used to deliver the electrical stimuli. Fresh semen was evaluated as described previously [19] for ejaculate volume, sperm concentration, sperm percent motility, and forward progressive motility (scale of 0 to 5: 0 = no forward movement and 5 = rapid, linear forward progression) [19]. A sperm motility index (SMI) was calculated to provide an overall estimate of sperm motility characteristics: SMI = (sperm % motility + [forward progressive motility × 20]) ÷ 2. Sperm morphology was evaluated by fixing an ejaculate aliquot (10 μl) in 0.3% glutaraldehyde followed by phase-contrast microscopic examination (1000×) of 200 sperm/aliquot [13, 16, 19].

Each ejaculate was diluted (1:1) in Ham's F-10 medium containing 5% FCS and divided into two aliquots for comparison of washed sperm with sperm processed by swim-up separation. Both aliquots were centrifuged (300 × *g*, 10 min); the supernatant was discarded and 100 μl of fresh

Ham's F-10/FCS layered gently onto the sperm pellet. The washed-only aliquots were resuspended in the layered medium, whereas the swim-up sperm were allowed to migrate into fresh medium. After a 1 h swim-up preincubation at room temperature (25°C), an aliquot of each sperm suspension was evaluated for sperm percent motility, progressive motility, morphology, and concentration. Each sample was diluted to a final concentration of 4×10^6 motile sperm/ml and maintained at 38°C in 5% CO_2 in air. To determine the longevity of sperm motility in vitro, sperm percent motility and progressive motility were evaluated hourly for 7 h (i.e., after 1 h of preincubation and 6 h of sperm-oocyte coincubation), and an SMI rating was calculated for each time period.

Insemination of In Vivo-Matured Oocytes and Assessment of Fertilization and Embryo Development

To standardize sperm concentration during the gamete coincubation period, 5 μl of each sperm suspension (at 4×10^6 motile sperm/ml) was added to a 95-μl drop of medium to generate a final concentration of 2×10^5 motile sperm/ml. Mature oocytes (n = 401) were transferred into 100-μl drops of processed sperm suspension and cocultured (15–18 oocytes/drop) under oil at 38°C in 5% CO_2 in air. After 20 h of culture, oocytes were removed from fertilization dishes and washed in a 0.2% hyaluronidase solution (type 1-S from bovine testis, Sigma Chemical Co.) in Ham's F-10 to remove cumulus cells and loosely attached spermatozoa. Oocytes were returned to culture in 100-μl drops of fresh medium and cultured for an additional 10 h before fertilization was assessed. Fertilization was defined as having occurred on the basis of the presence of two polar bodies, two pronuclei, or cleavage to at least the two-cell stage [3]. Cleaved embryos were assessed for symmetry, blastomere shape, and uniform darkness [3]. Embryos of good-to-excellent quality were perfectly symmetrical (or only slightly asymmetrical), spherical, and uniformly dark. Embryos were classified as fair or poor in quality if they were partially or severely degenerating, were pale in color, or contained lysed blastomeres. To assess fertilization in oocytes failing to cleave within 30 h of culture, oocytes were incubated with Hoechst #33342 (bisbenzamide, a DNA-specific fluorescent stain; H342, Sigma), counterstained with 0.1% Trypan blue (Sigma; 0.09 mg/ml, 15 min, 25°C) for 1–2 min, and then examined via differential interference contrast (DIC) and fluorescence optics (250× and 400×) [3]. After initial assessment at 30 h postinsemination, cleaved embryos were examined at 48, 72, 96, and 120 h for stage of development and quality grade.

Salt-Stored Cat Oocyte Preparation, Insemination, and Assessment of ZP Penetration

Fresh domestic cat ovaries were obtained from local veterinary clinics following elective surgeries and maintained in PBS at 4°C for 1–4 h before processing. For oocyte recovery, ovaries were placed into Eagle's Minimum Essential Medium (Eagle's MEM; Sigma) supplemented with 5% FCS and were punctured repeatedly with a 22-gauge needle to release cumulus-oocyte complexes [20]. Oocytes with homogeneously dark vitelli, tightly compacted corona radiata, and cumulus cell masses were washed three times in fresh medium and matured (38°C, 5% CO_2 in air) for 48 h in Eagle's MEM containing 0.23 mM pyruvate, 5% FCS, 1 μg/ml ovine FSH (NIADDK-oFSH-17 AFP-6446C), 1 μg/ml ovine LH (NIADDK-oLH-25 AFP-5551B), and 25 μg/ml estradiol-17β (Sigma). To remove cumulus cells after maturation, oocytes were transferred to Eagle's MEM containing 0.2% hyaluronidase for 15 min (38°C) and then mechanically pipetted. Oocytes were rinsed and transferred to a salt storage solution [20] consisting of 0.5 M $(NH_4)_2SO_4$, 0.75 M $MgCl_2$, 0.2 mM $ZnCl_2$, 0.1 mg/ml polyvinylalcohol, and 40 mM HEPES buffer (pH 7.4). Salt-stored oocytes were maintained at 4°C until the day of insemination, when they were removed from the salt solution and rinsed twice (1.0 h/rinse) in equilibrated (38°C, 5% CO_2 in air) Ham's F-10 medium supplemented with 5% FCS.

Salt-stored oocytes (n = 202) were placed in 100-μl drops (8–10 oocytes/drop) of fresh Ham's F-10/FCS medium containing either washed or swim-up processed sperm at a final concentration of 2×10^5 motile cells/ml. In vivo-matured follicular oocytes and salt-stored oocytes were cultured simultaneously using the same sperm suspensions as for insemination. Following a 6-h coincubation, salt-stored oocytes were fixed in a 2% glutaraldehyde/2% formaldehyde solution and maintained at 4°C until evaluated for sperm-ZP interaction. Using DIC microscopy (320×) and a micromanipulator-maneuvering pipette [13], oocytes were examined and scored for 1) percent ZP penetration, 2) numbers of ZP-bound and ZP-penetrated sperm per ovum, and 3) morphological structure of bound and ZP-penetrated sperm. To define ZP penetration, oocytes were categorized into four groups: 1) those with sperm heads in the outer half of the ZP (< 1/2 ZP); 2) those with sperm heads in the inner half of the ZP (> 1/2 ZP); 3) those in which more than one sperm head had reached at least the inner half of the ZP (poly > 1/2 ZP); and 4) those with sperm heads in the perivitelline space (PVS). Percent ZP penetration was defined as the number of oocytes with sperm penetrating the specific layer divided by the total number of oocytes inseminated times 100. The morphological structure of each bound and ZP-penetrated sperm was classified as normal or as having a specific structural defect.

Statistical Analysis

Mean values are presented as ± SEM. Differences in sperm traits between the normospermic and teratospermic groups were compared by Student's *t*-test [22]. Analysis of variance was used in the oocyte studies to determine differences in fertilization and ZP penetration between sperm morphol-

ogy populations (normospermic versus teratospermic) and between washed sperm and swim-up treatments [22]. The sperm morphology population was used as the whole plot, and sperm treatment was the split plot. Probabilities of < 0.05 were considered to be significantly different.

RESULTS

Ejaculate and Inseminate Characteristics

Seminal traits including ejaculate volume and sperm concentration were similar ($p > 0.05$) between normospermic (136.6 ± 21.8 μl and 148.8 ± 49.2 × 10^6/ml, respectively) and teratospermic (146.8 ± 9.4 μl and 242.7 ± 47.7 × 10^6/ml, respectively) cats. Percent sperm motility, progressive motility, and SMI in raw ejaculates also were not different ($p > 0.05$) between populations (normospermic: 81.0 ± 1.9%, 4.1 ± 0.3, and 81.5 ± 4.1, respectively; teratospermic: 71.7 ± 5.1%, 3.5 ± 0.2, and 71.8 ± 3.9, respectively). Consistent differences ($p < 0.01$) were detected in sperm morphology between the two groups, with teratospermic males producing ejaculates with fewer ($p < 0.01$) structurally normal spermatozoa (33.3 ± 4.1%) than normospermic males (70.7 ± 6.1%). Abnormalities in normospermic samples consisted of spermatozoa with head defects (macro- or microcephaly and bicephaly; 3.0%), midpiece aplasia (0.1%), a coiled flagellum (3.0%), a bent midpiece (10.2%), a bent flagellum (5.0%), and a proximal or distal cytoplasmic droplet (8.0%). In general, teratospermic ejaculates contained a higher ($p < 0.05$) incidence of each type of sperm abnormality. Spermatozoa in the teratospermic samples contained either head defects (1.2%), midpiece aplasia (0.2%), a coiled flagellum (8.3%), a bent midpiece (35.5%), a bent flagellum (10.9%), or a proximal or distal cytoplasmic droplet (10.6%).

Seminal traits including sperm motility ratings and structural morphology were influenced ($p < 0.05$) by processing. In teratospermic cats, swim-up separation improved ($p < 0.05$) percent sperm motility, progressive motility, and motility index above values observed after sperm washing (Table 1). Following swim-up, sperm motility ratings in the teratospermic aliquots were similar ($p > 0.05$) to those in both the washed and swim-up aliquots from normospermic males (Table 1). In teratospermic ejaculates, swim-up processing increased ($p < 0.01$) the percentage of normal spermatozoa by 2.3-fold over the proportion in the washed sperm aliquots (Table 1). Reductions ($p < 0.05$) were observed in the percentage of sperm that had a coiled flagellum, a bent midpiece, a bent flagellum, and a cytoplasmic droplet (Table 2). Swim-up treatment also increased ($p < 0.05$) the number of structurally normal spermatozoa in the normospermic cats by ~10% (Table 1).

SMI profiles were sustained at > 50 throughout the 7-h coincubation interval in both cat populations. In normospermic males, the mean SMI at 7 h was similar ($p > 0.05$) between the washed (70.0 ± 2.8) and swim-up (75.0 ± 2.5) sperm suspensions. For the teratospermic group, swim-up separation increased ($p < 0.05$) the height of the entire SMI profile. The mean SMI at 7 h in the swim-up aliquots was 77.5 ± 1.8, which was superior ($p < 0.05$) to that of the washed sperm (55.7 ± 2.6).

TABLE 1. Mean ± SEM sperm motility and morphology traits of washed vs. swim-up processed ejaculates from normospermic and teratospermic domestic cats.[a]

	Normospermic males (n = 6 ejaculates)	Teratospermic males (n = 6 ejaculates)
Sperm motility (%)		
Washed sperm	83.3 ± 3.3[d]	64.2 ± 3.7[e]
Swim-up sperm	91.7 ± 1.7[d]	90.0 ± 1.3[d]
Sperm progressive motility[b]		
Washed sperm	4.0 ± 0.2[de]	3.6 ± 0.2[d]
Swim-up sperm	4.0 ± 0.2[de]	4.3 ± 0.2[e]
Sperm motility index[c]		
Washed sperm	81.7 ± 3.3[d]	67.9 ± 4.1[e]
Swim-up sperm	85.8 ± 5.8[d]	87.5 ± 2.6[d]
Structurally normal sperm (%)		
Washed sperm	71.8 ± 4.2[d]	28.6 ± 2.2[f]
Swim-up sperm	82.0 ± 1.5[e]	66.5 ± 2.3[d]

[a]Normospermic cats produced >60% structurally normal sperm/ejaculate. Teratospermic cats produced <40% structurally normal sperm/ejaculate.
[b]Sperm progressive motility was based on a scale of 0 to 5; 5 = most rapid forward progression.
[c]Sperm motility index = [(% motility) + (progressive motility × 20)] ÷ 2.
[def]Within rows and columns for each sperm trait, means with different superscripts differ ($p < 0.05$).

Fertilization of In Vivo-Matured Oocytes

Following eCG/hCG treatment, the ovaries of all 16 females contained distinct preovulatory follicles (≥ 2 mm diameter; range, 19–64 follicles/female; mean, 26.1 ± 3.5 follicles/female). A high proportion (94.7%) of the oocytes from these follicles were recovered by laparoscopic aspiration (mean, 24.7 ± 2.9 oocytes/female). To assess sperm fertilizing ability between cat populations, 401 mature oocytes were inseminated in vitro (Table 3). Following the 20-h sperm-oocyte coincubation, sperm from normospermic and teratospermic cats were capable of fertilizing in vivo-matured oocytes; however, overall fertilization rate was more than 1.6-fold greater for the normospermic group (Table 3). There was at least a 1.7-fold increase ($p < 0.05$) in the

TABLE 2. Influence of swim-up processing upon sperm structural morphology (mean ± SEM) in teratospermic cat ejaculates (n = 6).

	Washed sperm	Swim-up sperm
Structurally-normal sperm (%)	28.6 ± 2.2[a]	66.5 ± 2.3[b]
Structurally-abnormal sperm (%)		
Microcephalic	1.6 ± 0.8[a]	1.7 ± 0.8[a]
Bicephalic	0.3 ± 0.2[a]	0.0[a]
Midpiece aplasia	0.1 ± 0.1[a]	0.0[a]
Coiled flagellum	8.6 ± 4.9[a]	0.8 ± 0.4[b]
Bent midpiece	31.2 ± 2.7[a]	14.5 ± 2.2[b]
Bent flagellum	22.9 ± 2.2[a]	12.9 ± 1.2[b]
Cytoplasmic droplet	6.7 ± 1.3[a]	3.6 ± 0.5[b]

[ab]Within rows, means with different superscripts differ ($p < 0.05$).

TABLE 3. Fertilization, cleavage, and embryonic development in vitro of in vivo-matured follicular oocytes via washed or swim-up aliquots from normospermic vs. teratospermic ejaculates.[a]

	Normospermic males (n = 6 ejaculates)	Teratospermic males (n = 6 ejaculates)
Number of oocytes	204	197
Fertilization rate (%)		
Washed sperm	81.3 ± 2.0[b]	49.7 ± 3.0[c]
Swim-up sperm	94.6 ± 5.4[b]	53.2 ± 6.1[c]
Overall	86.9 ± 3.1[b]	51.6 ± 4.2[c]
Cleavage rate (%)		
Washed sperm	81.3 ± 2.0[b]	46.6 ± 3.3[c]
Swim-up sperm	93.5 ± 6.5[b]	53.2 ± 6.1[c]
Overall	86.3 ± 3.7[b]	50.3 ± 3.6[c]
Percent achieving morula or blastocyst stage by 120 h	82.6[b]	81.8[b]

[a]Mean values are ± SEM.
[bc]Within rows, means with different superscripts differ ($p < 0.05$).

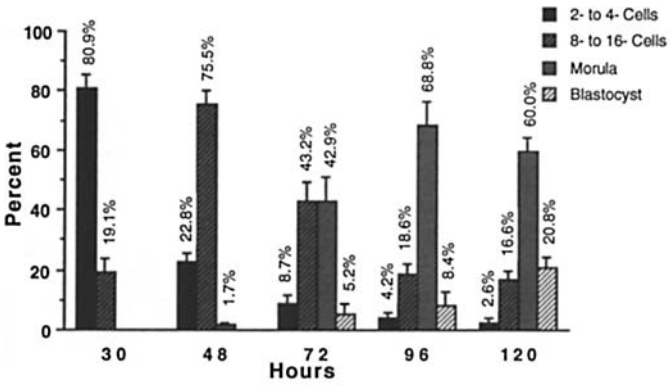

FIG. 1. Time sequence of cat embryonic development in vitro following IVF using teratospermic ejaculates (0 h = time of insemination). Sperm processing did not influence embryonic development; therefore, washed and swim-up aliquots were combined. Rate of embryo cleavage was similar between normospermic and teratospermic populations.

number of cleaved oocytes produced by normospermic cats compared to teratospermic males (Table 3). Hoechst staining revealed that a low proportion (< 2%) of uncleaved oocytes contained 2 polar bodies or 2 pronuclei.

Swim-up separation had no effect ($p > 0.05$) on the ability of sperm from teratospermic males to fertilize oocytes (Table 3) even though sperm motility, progressive motility, and normal sperm morphology were improved ($p < 0.05$) in these inseminates (Table 1). Cleavage rates also did not differ ($p > 0.05$) between the washed and swim-up sperm (Table 3).

Embryo Development

The ability of the embryos to develop in vitro was similar ($p > 0.05$) between cat groups, with more than 80% of all embryos reaching the morula or blastocyst stage by 120 h of culture (Table 3). The rate of embryo development by 30, 48, 72, 96, or 120 h of coculture was not different ($p > 0.05$) between the normospermic and teratospermic populations. Sperm processing treatment also did not influence ($p > 0.05$) the rate of embryo development in either cat group (data not shown). Therefore, embryo cleavage data from the washed and swim-up inseminates were combined (Fig. 1). Of the cleaved embryos detected at 30 h in the teratospermic group, 28.5% contained 2 blastomeres, 52.4% had 4 cells, and 19.1% had 8 cells. Of these 2–4-cell or 8-cell embryos, 91.0% and 100.0% were classified as of good-to-excellent quality, respectively. At 48 h, 3.3% and 19.5% of the embryos remained at 2 or 4 cells, but only 60.0% of these were good to excellent. The majority of embryos at 48 h contained 8 (49.4%) or 16 (26.1%) cells, and > 93% were good to excellent. A few (1.7%) embryos at 48 h were morulae, all of which were good to excellent. By 72 h, 8.7% of embryos had failed to develop beyond 4 cells (66.7% fair to poor); most of these embryos were 8 to 16 cells (43.2%; 80.5% good to excellent) or morulae (42.9%; 100% good to excellent), and only a few were blastocysts (5.2%; 100% good to excellent). By 96 h, 68.8% of the embryos were morulae (92% good to excellent); the remainder were at the 4-cell (4.2%; 75% fair to poor), 8- to 16-cell (18.6%; > 50% fair to poor), or blastocyst (8.4%; > 88% good to excellent) stage. At 120 h, the majority of embryos (60.0%) remained as morulae with minimum development to blastocysts (20.8%), but more than 79% of these were good to excellent.

Penetration of Salt-Stored ZP-Intact Oocytes

Spermatozoa from both normospermic and teratospermic males were capable of binding to and penetrating the ZP of salt-stored cat oocytes (Fig. 2); however, overall sperm-ZP interaction was greater for the normospermic group (Table 4). Sperm from both populations penetrated the outer ZP of a high proportion (> 79%) of oocytes, but ZP penetration was superior ($p < 0.05$) for normospermic cats (Table 4). There was a 3-fold increase ($p < 0.05$) in mean percent inner ZP penetration and a greater than 5-fold increase ($p < 0.05$) in poly-inner ZP and PVS penetration for normospermic compared to teratospermic males (Table 4). The number of sperm bound to the ZP or penetrating into the outer ZP and inner ZP was 3- to 5-fold higher ($p < 0.05$) for normospermic than for teratospermic cats (Table 4). Only a very few spermatozoa reached the PVS in the teratospermic group after the 6-h coincubation (Table 4).

A high proportion of the spermatozoa bound to salt-stored, ZP-intact oocytes was structurally normal in the teratospermic (72.9%) and normospermic (79.7%) aliquots, regardless of sperm treatment. In aliquots of washed and swim-up sperm, the proportion of structurally normal sperm bound to the ZP from teratospermic ejaculates was comparable ($p > 0.05$) to that for similarly processed aliquots in the normospermic group. To illustrate the difference in morphology between inseminated sperm and ZP-interacted sperm in the teratospermic cat population, Table 5 compares the

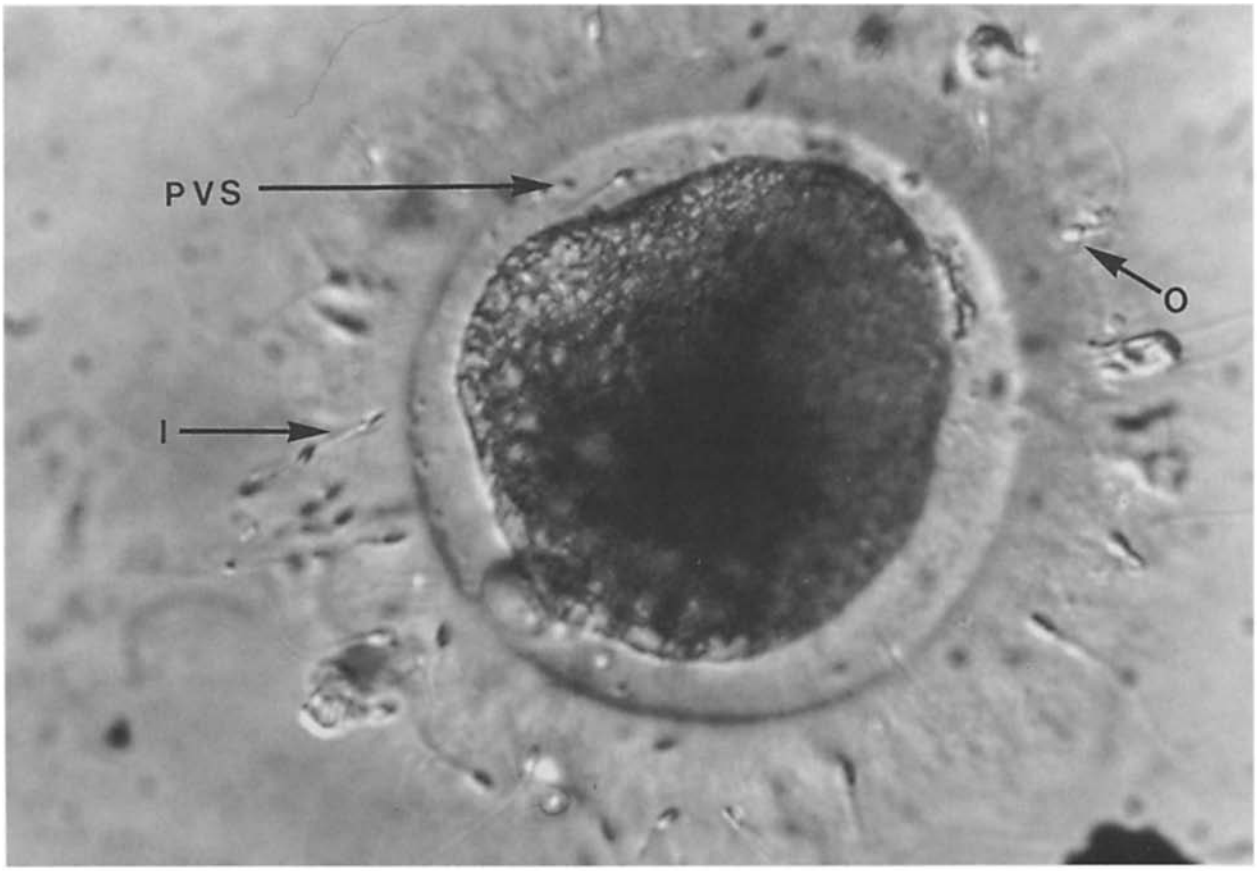

FIG. 2. A salt-stored, ZP-intact domestic cat oocyte. Three sperm are indicated with arrows, one in the outer (O) ZP layer, one in the inner (I) ZP layer, and one in the perivitelline space (PVS).

TABLE 4. Penetration of salt-stored ZP-intact cat oocytes via washed or swim-up aliquots from normospermic vs. teratospermic ejaculates.[a]

	Normospermic males (n = 6 ejaculates)	Teratospermic males (n = 6 ejaculates)
Number of oocytes	96	106
Zona penetration (%)[b]		
<1/2 ZP	98.8 ± 1.3[c]	79.7 ± 6.3[d]
>1/2 ZP	73.7 ± 7.7[c]	24.1 ± 4.9[d]
Poly >1/2 ZP	54.4 ± 8.3[c]	9.6 ± 3.2[d]
PVS	14.9 ± 3.9[c]	2.7 ± 1.9[d]
Number of sperm		
Bound	26.2 ± 0.8[c]	8.3 ± 0.4[d]
<1/2 ZP	26.4 ± 4.4[c]	8.9 ± 2.6[d]
>1/2 ZP	2.1 ± 0.3[c]	0.4 ± 0.1[d]
PVS	0.3 ± 0.1[c]	0.02 ± 0.01[d]

[a]Values are means ± SEM. Within each cat group, no difference ($p > 0.05$) existed between washed and swim-up aliquots; therefore, data were combined.

[b]Categories of ZP penetration include: 1) <1/2 ZP = % oocytes with sperm in the outer one half of the ZP; 2) >1/2 ZP = % oocytes with sperm in the inner one half of the ZP; 3) poly >1/2 ZP = % oocytes with >1 sperm in the inner ZP; and 4) PVS = % oocytes with sperm in the perivitelline space.

[cd]Within rows, means with different superscripts differ ($p < 0.05$).

morphological characteristics of the washed sperm with those of the ZP-bound or penetrating sperm. The percentage of normal sperm bound to ova and penetrating each zona layer was greater ($p < 0.05$) than the proportion of normal sperm in the washed sperm inseminates. Structurally abnormal sperm were capable of ZP binding (29.2%) and penetration into the outer ZP (17.0%). However, only 3.3% of the inner ZP sperm were abnormal and every sperm within the PVS was morphologically normal, even though inseminates contained an average of 71.4% pleiomorphic forms (Table 5). The specific types of structurally abnormal spermatozoa bound to and penetrating ZP-intact oocytes are presented in Table 5.

DISCUSSION

This study provides compelling evidence that teratospermia in domestic cats has a detrimental impact upon fertilization in vitro. Compared with spermatozoa from normospermic males, those from teratospermic cats were less

TABLE 5. Morphological characteristics of washed sperm in the inseminate compared to sperm bound to the ZP and within or through the ZP in teratospermic cats.[a]

	Inseminate	Bound	Outer ZP	Inner ZP	PVS
Structurally normal sperm (%)	28.6 ± 2.2[b]	70.8 ± 8.3[c]	83.0 ± 2.3[c]	96.7 ± 3.3[d]	100.0 ± 0.0[d]
Structurally abnormal sperm (%)					
Head defects	1.9 ± 0.4	0.0	0.0	0.0	0.0
Midpiece defects	31.3 ± 1.6[b]	14.9 ± 3.7[c]	12.6 ± 2.4[c]	0.0[d]	0.0[d]
Coiled or bent flagellum	31.5 ± 3.8[b]	13.2 ± 6.4[c]	3.8 ± 1.7[d]	0.0[d]	0.0[d]
Cytoplasmic droplets	6.7 ± 1.3[b]	1.1 ± 0.1[c]	0.6 ± 0.4[c]	3.3 ± 1.2[c]	0.0[c]

[a]Means ± SEM, based upon 6 ejaculates collected from 3 teratospermic males.
[b–d]Within rows, means with different superscripts differ ($p < 0.05$).

capable of ZP penetration and oocyte fertilization. The proportion of all ZP-intact cat oocytes with sperm in the inner ZP was markedly lower for the teratospermic (24.1%) than for the normospermic (73.7%) group. This compromised sperm function appeared inherent to the teratospermic ejaculate and did not depend upon the actual number of structurally abnormal sperm in the inseminate. This was evident from comparison of the results on the basis of semen handling. Swim-up processing increased the percentage of normal sperm forms in teratospermic ejaculates from an average of 28.6 to 66.5%, a value similar to that observed in the washed sperm aliquots from normospermic ejaculates (71.8%). However, swim-up sperm from teratospermic males still were physiologically incapable of binding to and penetrating the ZP at the higher rates detected in normospermic samples. This resulted in a consistently higher number of ZP-penetrated sperm/oocyte in the normospermic group, regardless of sperm treatment.

The finding of compromised fertilizing capacity of sperm from teratospermic cats was similar to more general observations made in several other species. For example, structurally abnormal spermatozoa from certain mouse strains are compromised in ability to bind the ZP of conspecific ova [23]. There also is decreased ovum penetration during human IVF when the inseminates contain an increased incidence of abnormal spermatozoa [24], a problem that apparently is related to decreased sperm-ZP interaction [25–28]. The ZP binding ability of sperm and the number of sperm within the ZP are diminished in samples from subfertile men who fail to achieve fertilization during IVF as compared to proven fertile sperm samples [25, 28]. Our previous studies have focused upon identifying those factors most important in influencing the ability of felid follicular oocytes to fertilize in vitro and to develop into biologically competent embryos [14]. Among the most influential include exogenous gonadotropin dose, interval between the FSH-like and LH-like stimulus, and sperm motility-longevity [3–5, 7, 10]. It now is apparent that not only the absolute presence of sperm pleiomorphisms, but also unknown factors associated with structurally normal sperm from teratospermic donors can have a marked impact upon IVF success in the cat.

There appears to be a fundamental functional deficit in sperm from teratospermic cats. Compromised sperm function in these androgen-deficient cats may be associated with defects in spermatogenesis and epididymal transport/maturation [13, 19]. Teratospermic cats produced certain midpiece abnormalities similar to anomalies that have been observed in bulls with defective spermatogenesis [29]. Morphological abnormalities also are known to occur during sperm transport through the epididymis. In boars, few pleiomorphic sperm are found in the caput epididymis; however, cells recovered distal to the caput can exhibit a bent midpiece containing a cytoplasmic droplet within the bend [30]. This specific type of defect was the most prevalent abnormality observed in the teratospermic cat spermatozoon. In all species studied to date, spermatozoa also undergo critical androgen-dependent changes during epididymal maturation that ultimately result in the capacity to fertilize ova [31]. Functional modifications include alteration or accumulation of epididymal proteins on the sperm surface during transit and the acquisition of ZP binding capacity [31]. Our studies suggest that the diminished ability of spermatozoa from teratospermic cats to interact with and penetrate the ZP may be related to epididymal dysfunction and to a deficiency in the appropriate sperm surface proteins or receptor sites involved in ZP binding. To study this further, we are screening specific factors that might help explain why "normal" and abnormal sperm from teratospermic males are compromised in fertilization ability. These include sperm ultrastructure, motility/metabolism, ability to achieve the acrosome reaction, sperm binding receptors, and ability to penetrate the ZP barrier and interact with the vitellus after ZP micromanipulation (ZP piercing and sperm injection). In a preliminary study assessing sperm ultrastructure by transmission electron microscopy, the acrosomes of sperm from teratospermic cat ejaculates appeared intact and normal in shape (Howard and Wildt, unpublished).

Salt-stored ZP appear to be a useful alternative for testing sperm function when fresh oocytes are unavailable. We recently have modified the traditional ZP penetration assay, developed in the hamster [32, 33], rabbit [34], and human [35–37], by using salt-stored oocytes collected from cats [20]. This material has been valuable for studying sperm capacitation in the domestic cat [20] and for determining the ability of heterologous felid gametes to interact in vitro

[2, 12, 14, 20, 38]. This is an attractive approach for assessing a sperm population, because salt storage destroys the block to polyspermy and allows many sperm to reach the perivitelline space. Salt-stored ZP also retain the ability to distinguish between capacitated and noncapacitated sperm [33]. For example, protein-free culture medium containing polyvinylalcohol sustain sperm motility in vitro in the leopard cat [20] and cheetah [38]; however, protein supplementation (albumin or serum) is necessary to facilitate sperm capacitation and inner ZP penetration. The leopard cat, a species that produces high proportions of structurally normal sperm, has the same number of spermatozoa reaching the inner ZP or the PVS of salt-stored domestic cat oocytes as have normospermic domestic cats [20]. The present results were important because they demonstrated the potent "filtering" capacity of the domestic cat ZP to keep the inner ZP or PVS free of pleiomorphic sperm. A similar observation has been made for cheetah sperm cocultured with domestic cat oocytes [38]. Although 58.5% of cheetah sperm that are bound to the outer ZP are morphologically abnormal, only structurally normal sperm reach the inner sanctum of the ZP or PVS [38].

In a recent cheetah IVF study, a low IVF rate obtained with conspecific oocytes (26.2%) was highly correlated to the ability of cheetah sperm to reach the inner ZP of salt-stored, domestic cat oocytes [12]. This predictive utility also was demonstrated in the present study, because sperm penetration results from the salt-stored ZP assay mimicked the IVF cleavage rates obtained with in vivo-matured oocytes. When the incidence of IVF ("true" fertilization) was low, few sperm reached the inner half of the ZP or the PVS of the salted oocytes. Similar to the situation in humans [25–27], the salt-stored ZP penetration assay accurately reflected IVF outcome in the cat. These results confirmed the benefits of the assay as an effective tool for predicting fertilizing capacity in felids and discriminating where sperm dysfunction occurs at a specific level of ZP penetration.

The present results were important because they conclusively demonstrated that pleiomorphic sperm in the domestic cat did not participate in fertilization and that even structurally normal sperm cells from teratospermic males appeared partially compromised. It was evident that once oocytes were fertilized and cleavage occurred, the origin of the sperm donor had no influence upon embryonic development in vitro. Embryos resulting from sperm collected from teratospermic males grew at rates and to stages comparable to those from normospermic males [39]. This finding alone has interesting implications for the field of conservation biology, because the degree of teratospermia is serious in certain rare felid species and has the potential for influencing reproductive performance after both natural and artificial breeding. We have contended in previous reports that assisted reproductive technologies, like IVF, have considerable conservation potential [1, 2, 14]. Many of the common problems (including sexual incompatibility, physical handicaps, and the need for infusing new germ plasm into isolated populations) could be overcome through use of these approaches. Although the present data suggested that ejaculates containing many pleiomorphic sperm could be used to produce IVF embryos, the findings also indicated that teratospermia is one of the most important factors influencing IVF efficiency in this taxon.

ACKNOWLEDGMENTS

We thank Teresa Cummings for her technical assistance during laparoscopic oocyte aspiration and Cathy Just for conducting the sperm morphology evaluations. We are grateful to Kathleen James, Bobby Fry, Dr. Martin Kriete, and the National Institutes of Health Animal Center Section for animal management and veterinary care.

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