Journal of Andrology, Vol. 12, No. 1, January/February 1991

Teratospermia in Domestic Cats Compromises Penetration of Zona-Free Hamster Ova and Cat Zonae Pellucidae

JOGAYLE HOWARD, MITCHELL BUSH, AND DAVID E. WILDT

From the National Zoological Park, Smithsonian Institution, Washington, District of Columbia.

ABSTRACT: The ability of spermatozoa to bind and penetrate zona-free hamster ova and the zonae pellucidae of domestic cat oocytes *in vitro* was compared between normospermic (>60% structurally normal spermatozoa per ejaculate) and teratospermic (<40% normal spermatozoa per ejaculate) domestic cats. The effects of culture media (Biggers, Whitten, Whittingham [BWW] versus modified Krebs Ringer bicarbonate [mKRB]) and simple dilution (DR), ejaculate centrifugation, and either resuspension (NS) or swim-up processing (SU) on penetration also were examined. High percentages of structurally normal spermatozoa were bound to zona-pellucida-free hamster ova regardless of the morphological forms in the inseminant. Mean percent normal spermatozoa bound to ova in DR, NS, and SU sperm aliquots from teratospermic male cats were not different ($P > 0.05$) from similarly treated normospermic aliquots. However, the percent penetration of hamster ova by normospermic ejaculates (10.5%) was superior ($P < 0.05$) to that of teratospermic ejaculates (2.8%). Although swim-up processing improved percent sperm motility, progressive motility, and normal morphology in teratospermic ejaculates ($P < 0.05$), no difference was observed in ovum penetration among the DR-treated, NS-treated, and SU-treated spermatozoa ($P > 0.05$). Culture medium had no effect on sperm binding in the hamster assay, but ovum penetration rate by spermatozoa in the normospermic ejaculates was enhanced ($P < 0.05$) using mKRB (13.5%) when compared with BWW (7.6%) medium. Spermatozoa from teratospermic cats were capable of binding and penetrating cat zonae; however, sperm–zona interaction (defined as percent of oocytes with spermatozoa binding to or penetrating into the zona) was different ($P < 0.05$) between normospermic (65.3%) and teratospermic (24.2%) cats. The number of bound spermatozoa per ovum was five-fold less in males producing high proportions of pleiomorphic spermatozoa ($P < 0.05$). Zona penetration was also different between normospermic (58.1%) and teratospermic (16.9%) males. Within cat populations, the DR, NS, and SU treatments had no influence on sperm binding or zona penetration. These results indicate that teratospermia in domestic cats has a detrimental impact on gamete interaction and penetration of zona-free hamster ova and cat zonae pellucidae. The *in vitro* effect is not entirely dependent on absolute numbers of structurally normal spermatozoa but appears to be related to an unidentified characteristic of teratospermic ejaculates.

Key words: Sperm morphology, teratospermia, sperm function, zona-free hamster ova, zona pellucida.

J Androl 1991;12:36–45.

As the incidence of teratospermia increases, reproductive potential decreases in humans (Bostofte et al, 1982), cattle (Saacke et al, 1968), sheep (Hulet et al, 1965), horses (Bader et al, 1988), pigs (Larsson et al, 1980), and laboratory rodents (Burkhart and Malling, 1981). Compared with structurally normal spermatozoa, morphologically abnormal forms are disadvantaged in transport through the female reproductive tract (Krzanowska, 1974; Nestor and Handel, 1984; Saacke et al, 1988), suggesting that there are natural barriers preventing these cells from reaching the site of fertilization. We know less about the ability of abnormal spermatozoa to actually participate in the fertilization event; however, *in vitro* fertilization (IVF) offers one powerful approach for assessing the functional capabilities of these gametes.

The sperm penetration assay, introduced by Yanagimachi (1972), measures the capability of a spermatozoon to undergo nuclear decondensation within the ooplasm of zona-pellucida-free hamster ova. This assay has been used extensively to study the function of human spermatozoa (Yanagimachi, 1984; Rogers, 1985, 1986). Hamster ova also can be penetrated *in vitro* by rodent (Hanada and Chang, 1976), rabbit (Hanada and Chang, 1978), boar (Imai et al, 1980), bull (Brackett et al, 1982), horse (Brackett et al, 1982), goat (Kim et al, 1980), ram (Pavlok et al, 1983), macaque (Boatman and Bavister, 1984), and tiger (Byers et al, 1989) spermatozoa.

The hamster bioassay does not assess the ability of the spermatozoon to bind and penetrate the zona pellucida, and that is a serious limitation since defective spermatozoa–zona interaction is one cause of infertility in humans (Mahadevan et al, 1987) and mice (Kot and Handel, 1987). Inclusion of the zona barrier is therefore important for as-

This research was supported by NIH grants RR00045 and HD23853, and a grant from Friends of the National Zoo (FONZ).

Correspondence to: JoGayle Howard, DVM, PhD, Department of Animal Health, National Zoological Park, Smithsonian Institution, Washington, District of Columbia 20008.

Received for publication February 27, 1990; accepted for publication August 20, 1990.

sessing certain sperm functions, including zona binding and penetration. Ovarian antral follicles are one source of zona-intact ova, and homologous immature oocytes are useful for assessing sperm function in humans (Overstreet et al, 1980) and rodents (Bleil and Wassarman, 1980).

The domestic cat is a valuable animal model in biomedical research and in comparative studies of rare *Felidae* species (Wildt et al, 1986). Many nondomestic felid species ejaculate high proportions (range, 36% to 84%) of pleiomorphic spermatozoa (Howard et al, 1984), which is possibly related to low circulating concentrations of testosterone, but not pituitary, gonadotropin secretion (Wildt et al, 1983, 1987a,b, 1988). In general, the domestic cat ejaculates a low proportion (2% to 30%) of structurally abnormal spermatozoa (Sojka et al, 1970; Wildt et al, 1983); however, certain domestic cats consistently ejaculate high proportions (>60%) of pleiomorphic spermatozoa (Howard et al, 1990). Compared with normospermic male cats, teratospermic males produce comparable serum follicle-stimulating hormone and luteinizing hormone concentrations but lower levels of testosterone.

The overall goal of this study was to develop methods to assess sperm function in felids and, specifically, the impact of teratospermia on *in vitro* sperm–ovum interaction in domestic cats. Two approaches were compared: penetration of heterologous, zona-free hamster ova and homologous, cat zonae pellucidae.

Materials and Methods

Animals and Semen Collection

Adult males from two domestic cat populations producing either high (>60% per ejaculate; normospermic) or low (<40% per ejaculate; teratospermic) proportions of structurally normal spermatozoa were studied. Details on laboratory maintenance of these animals have been described (Howard et al, 1990). In brief, cats were maintained individually in stainless-steel cages and exposed to approximately 12 hours of natural daylight per day; 8 of these hours were supplemented with artificial fluorescent illumination. Animals were provided with dry, commercial cat food (Purina Cat Chow, Ralston Purina Co, St. Louis, MO) and water *ad libitum*.

A total of 48 ejaculates (24 normospermic and 24 teratospermic) was collected by electroejaculation from six male cats (3 normospermic, 3 teratospermic males; 8 ejaculates/individual). Each male was anesthetized for semen collection with an intramuscular injection of ketamine-hydrochloride (Vetalar®, Parke-Davis, Morris Plains, NJ; 25 mg/kg). After induction of a surgical plane of anesthesia, a Teflon rectal probe (1 cm in diameter) and an alternating current electroejaculator (PT Electronics, Boring, OR) were used to deliver the electrical stimuli (Howard et al, 1990). The electroejaculation regimen, consisting of a total of 80 electrical stimuli divided into three series, was described previously (Howard et al, 1990).

Seminal Analysis and Processing

Fresh semen from each of the three electroejaculation series was combined and evaluated immediately (Howard et al, 1986, 1990). In brief, this involved measuring ejaculate volume and sperm concentration (Howard et al, 1986), and subjectively assessing percent sperm motility and progressive motility at 37°C by phase-contrast microscopy (250x). Sperm progressive motility was based on a scale of 0 to 5, with 0 being no motility and 5 being rapid, steady, forward progression. Sperm morphology was evaluated by fixing ejaculate aliquots (20 μl) in 1% glutaraldehyde followed by phase-contrast microscopic examination (1000x) of 200 spermatozoa/aliquot (Howard et al, 1990). Each cell was classified as normal or having one of the following anomalies: abnormal head (macrocephalic, microcephalic, bicephalic, or tricephalic), mitochondrial sheath aplasia (including partial or complete aplasia of the mitochondrial sheath), tightly coiled flagellum, bent midpiece with or without a cytoplasmic droplet, bent flagellum with or without a cytoplasmic droplet, or a proximal or distal cytoplasmic droplet.

Ejaculates containing spermatozoa with at least a 70% motility rating were transferred to 1.5-ml conical tubes (Sarstedt Inc, Princeton, NJ) and processed for coincubation with either hamster ova or domestic cat oocytes. Semen was diluted slowly in 200 μl of either Biggers, Whitten, and Whittingham (BWW) medium containing 3 mg/ml bovine serum albumin (BSA; Biggers et al, 1971) or modified Krebs Ringer bicarbonate (mKRB) medium containing 4 mg/ml BSA (Niwa et al, 1985). BWW medium has been used traditionally for the hamster ovum assay (Rogers, 1985). Comparison of the mKRB medium was based on previous use in our laboratory for processing domestic cat ejaculates for *in vitro* fertilization (Goodrowe et al, 1988b).

To assess the influence of centrifugation, seminal plasma removal, and swim-up separation on sperm traits and subsequent ovum penetration, diluted semen was split into three aliquots: diluted raw (DR), non-swim-up (NS), and swim-up (SU). NS and SU aliquots were centrifuged (300g, 8 minutes), supernatant was discarded, and 50 μl of fresh medium was layered gently onto each sperm pellet. The SU spermatozoa were allowed to migrate into fresh medium, whereas NS spermatozoa were resuspended immediately in supernatant by gentle aspiration-pipetting action. Following a 1-hour preincubation period at room temperature (25°C) for spermatozoal capacitation (Goodrowe et al, 1988a), an aliquot of each of the three sperm suspensions was evaluated for sperm motility, progressive motility, morphology, and concentration. To standardize sperm concentration during the sperm–ovum coincubation period, each sample was diluted to a final concentration of 10×10^6 motile spermatozoa/ml.

Zona-Free Hamster Ovum Assay

A total of 36 electroejaculates (18 normospermic and 18 teratospermic; 6 ejaculates/male) was used to study gamete interaction and penetration of zona-pellucida-free hamster ova by domestic cat spermatozoa. In addition to determining if seminal processing influenced heterologous ovum penetration, the effect of culture medium was assessed by comparing BWW and mKRB media.

To obtain ova, immature hamsters were administered an intraperitoneal injection of 40 IU pregnant mares' serum gonadotropin (PMSG; Sigma Chemical Co, St. Louis, MO) and 25 IU human chorionic gonadotropin (hCG; Sigma Chemical Co) 56 hours later. Fifteen hours after hCG, oviducts were retrograde-flushed with either BWW or mKRB medium. Hamster ova were recovered and treated with 0.1% hyaluronidase (Type I-S, Sigma Chemical Co) and 0.1% trypsin (Sigma Chemical Co) to remove cumulus cells and zonae pellucidae, respectively (Rogers, 1985). Following preincubation of spermatozoa, zona-free hamster ova (20 ova/drop) were added to a 20-μl drop of diluted spermatozoal suspension (2×10^5 motile sperm cells/drop). After a 3-hour coincubation period in a 5% carbon dioxide in air, humidified environment (37°C), hamster ova were washed three times and mounted on a microscopic slide as described previously (Rogers, 1985). Using phase-contrast microscopy (400x), hamster ova were examined and scored for the following: 1) sperm–ovum interaction; 2) numbers of bound spermatozoa per ovum; 3) morphological structure of bound spermatozoa; and 4) ovum penetration. Sperm–ovum interaction was defined as the percentage of ova with spermatozoa either binding to the vitelline membrane or penetrating the vitellus and having at least one swollen sperm head. Spermatozoa remaining on the vitelline membrane following three washes were recorded as bound. Morphological structure of bound spermatozoa was classified as normal or having one of the structural abnormalities described earlier. Ovum penetration was defined by the presence of a swollen or decondensed sperm head with a corresponding flagellum in the vitellus. Penetration results were expressed as: 1) percent penetration rate, which was calculated as the number of ova penetrated divided by the total number of ova inseminated times 100; and 2) penetration index, which was calculated as the total number of decondensed sperm heads divided by the total number of ova inseminated.

Zona-Intact Domestic Cat Oocyte Assay

Sperm function and zona penetration of cat oocytes were assessed in 12 electroejaculates (6 normospermic and 6 teratospermic; 2 ejaculates/male). Semen was diluted in mKRB and processed as described for the hamster assay. Each diluted ejaculate was split into three aliquots (DR, NS, and SU), incubated at 25°C for 1 hour, and diluted to a final concentration of 10×10^6 motile spermatozoa/ml.

Domestic cat ovaries, obtained by ovariectomy, were placed immediately in physiological saline and maintained at 5°C for 1 to 3 hours before processing. Ovaries were punctured repeatedly with a 22-gauge needle to release cumulus-oocyte complexes into the surrounding mKRB medium. Oocytes of various stages of maturity were released. Only oocytes with homogeneously dark vitelli and tightly compacted corona radiata and cumulus cell masses, previously described as immature (Goodrowe et al, 1988b), were selected for insemination. Approximately 10 to 20 viable oocytes at the desired maturational status were recovered from each ovary. To allow loosening of the cumulus oophorus cells, oocytes were transferred into fresh mKRB and cultured under lightweight paraffin oil (Fisher Scientific Co, Pittsburgh, PA) in a 5% carbon dioxide in air, humidified environment (37°C) for 16 hours. To visualize zona penetration more readily, cumulus cells were removed by transferring oocytes to mKRB containing 0.2% bovine testicular hyaluronidase (Type I-S, Sigma Chemical Co) for 15 minutes (37°C). Oocytes were washed three times in mKRB and manipulated individually using a siliconized micropipette with an opening of similar diameter to the zona pellucida (~162 μm). This was essential for removal of all residual cumulus cells attached to the zona pellucida.

For insemination, 20 cat oocytes were placed in a 35 × 10 mm culture dish containing a 20-μl drop of the processed sperm suspension (2×10^5 motile sperm/drop). After a 3-hour coincubation period in a 5% carbon dioxide in air, humidified environment (37°C), ova were washed three times in fresh mKRB medium using a fine bore, siliconized micropipette for removing loosely attached spermatozoa. Ten oocytes were transferred to a concave, single-well microscopic slide containing 40 μl mKRB medium. Each oocyte was examined individually by differential interference-contrast microscopy (320x) using a micromanipulator consisting of a holding pipette for stabilizing and manipulating the oocyte during examination. Spermatozoa remaining on the zona pellucida were recorded as bound, whereas spermatozoa within the zona pellucida were classified as penetrating. Percent zona penetration was determined as the number of ova with spermatozoa penetrating into the zona pellucida divided by the total number of ova inseminated times 100. Zona penetration index was calculated as the total number of spermatozoa penetrating into the zona pellucida divided by the number of ova inseminated. Sperm–zona interaction (defined as the percentage of ova with spermatozoa binding to or penetrating into the zona pellucida) was used as an index of the functional ability of spermatozoa to bind and penetrate the zonae pellucidae of homologous oocytes.

Statistical Analysis

The hamster ovum penetration study was a split-split plot design in which the whole plot was the sperm morphology population (normospermic vs. teratospermic groups), the sub-plot was medium (BWW vs. mKRB), and the sub-sub plot was sperm treatment (DR vs. NS vs. SU treatments). The design for the cat zona penetration study was similar, with the whole plot being the sperm morphology population (normospermic and teratospermic) and the sub-plot being sperm treatment (DR, NS, and SU treatments). Data were analyzed using a general linear models program (SAS, 1986). Dependent variables presented as percentages (eg, the percentage of structurally normal spermatozoa) were analyzed using raw and arc sine transformed data. Because analysis of variance revealed no interpretive difference between the raw and transformed data, results were presented as least squares means ± SEM of raw values. When a significant variance value was calculated ($P < 0.05$), differences among sperm treatment means were determined by the least significant difference (LSD) multiple-comparison procedure. Comparisons within each sperm morphology population included differences among sperm treatments within each medium and among both media. Each SEM was based on pooled variances.

Results

Zona-Free Hamster Ovum Assay

Ejaculate and Inseminant Characteristics Between Populations—Data concerning seminal traits and the influence of seminal processing on sperm motility, progressive motility, concentration, and normal morphology in normospermic and teratospermic cats are presented in a parallel report (Howard et al, 1990). To facilitate interpretation of the sperm–ovum interaction data, relevant information on ejaculate and inseminant characteristics from that article is presented here and in Tables 1 and 3. Seminal traits, including ejaculate volume and the total number of motile spermatozoa per ejaculate, were similar ($P > 0.05$) between the normospermic (124.1 ± 9.5 μl, 17.0 ± 3.8 × 10^6, respectively) and teratospermic (97.4 ± 9.5 μl, 24.6 ± 3.8 × 10^6, respectively) cats. Percent sperm motility and progressive motility also were similar ($P > 0.05$) between populations; however, teratospermic males consistently produced ejaculates with fewer ($P < 0.05$) structurally normal spermatozoa than normospermic males. In both normospermic and teratospermic males, the addition of either BWW or mKRB to raw ejaculates increased ($P < 0.05$) the number of structurally abnormal spermatozoa (Table 1), primarily by increasing the number of cells with a bent flagellum (Howard et al, 1990). Compared with the DR and NS treatments, the swim-up (SU) technique improved ($P < 0.05$) motility ratings and recovery of structurally normal, motile spermatozoa for both normospermic and teratospermic males (Table 1).

Sperm-Ovum Interaction and Penetration of Hamster Ova by Cat Spermatozoa—Spermatozoa from both cat populations were capable of interacting similarly ($P > 0.05$) with zona-free hamster ova as demonstrated by high sperm–ovum interaction values in normospermic (93.8 ± 0.1%) and teratospermic (96.2 ± 0.1%) cats. Neither culture medium nor the DR, NS, and SU treatments influenced ($P > 0.05$) sperm–ovum interaction ratings in either cat population.

Spermatozoa from normospermic and teratospermic cats penetrated hamster ova as demonstrated by the presence of a decondensed sperm head within the cytoplasm (Fig 1). The penetration index and penetration rate for normospermic males were superior ($P < 0.05$) to that of teratospermic males (Table 2). These findings were consistent within medium and sperm processing treatment groups. Although

TABLE 1. *Influence of sperm treatment on sperm motility and structural morphology**

	Sperm treatment			
	Undiluted raw	Diluted raw	Non-swim-up	Swim-up
Sperm motility (%)†	78.7 ± 1.4§	70.9 ± 1.4¶	69.4 ± 1.4¶	91.2 ± 1.4‖
Sperm progressive motility†,‡	4.0 ± 0.1§	3.6 ± 0.1¶	3.7 ± 0.1¶	4.3 ± 0.1‖
Normal spermatozoa (%)				
Normospermic ejaculates	74.2 ± 2.1§	44.6 ± 2.1¶	43.5 ± 2.1¶	69.1 ± 2.1§
Teratospermic ejaculates	34.0 ± 2.1§	25.4 ± 2.1¶	27.7 ± 2.1¶	59.4 ± 2.1‖

* Results from normospermic and teratospermic domestic cat ejaculates used for co-incubation with zona-free hamster ova and zona-intact domestic cat oocytes. Values are means ± pooled SEM of combined oocyte assays. No difference in sperm traits was detected between the zona-free hamster ovum assay and the zona-intact cat oocyte assay.

† Values are means ± pooled SEM of normospermic (n = 24) and teratospermic (n = 24) ejaculates. No difference in sperm percent motility and progressive motility was detected between the cat populations.

‡ Sperm progressive motility was based on a scale of 0 to 5, 5 = most rapid forward progression.

§,‖,¶ Within rows, means with different superscipts differ ($P < 0.05$).

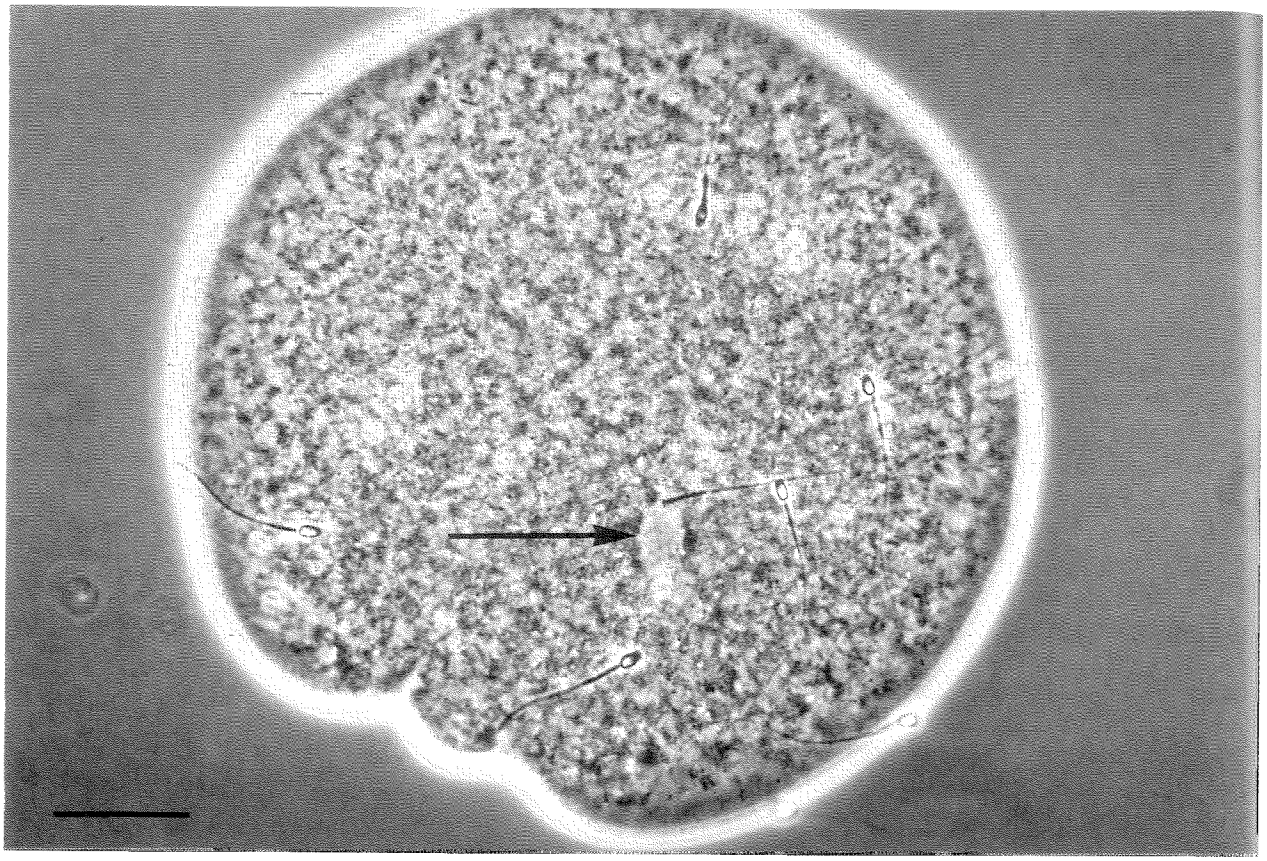

FIG. 1. Penetration of a zona-free hamster ovum by a domestic cat spermatozoon. One swollen sperm head (arrow) with attached flagellum is visible in the vitellus of the ovum. Scale bar = 30 μm.

ovum penetration rates were similar between the BWW and mKRB treatments in the teratospermic group, the latter medium enhanced ($P < 0.05$) the ovum penetration rate in the normospermic population (Table 2). The DR, NS, and SU treatments had no effect on penetration rate or index, even though the latter process improved ($P < 0.05$) sperm motility, progressive motility, and normal cellular morphology of the inseminants.

Morphological Characteristics of Spermatozoa Bound to Hamster Ova—A high proportion of spermatozoa bound to the vitelline membranes of hamster ova was structurally normal in the teratospermic (73.3%) and normospermic (78.4%) aliquots. In teratospermic males, the percentage of normal spermatozoa bound to ova was greater ($P < 0.05$) than the proportion of normal spermatozoa in the inseminants, regardless of sperm treatment (Table 3). Similar contrasts were observed in the DR and NS aliquots from normospermic ejaculates (data not shown). The proportion

TABLE 2. *Influence of sperm morphology and culture media on zona-free hamster ovum penetration*

	Ova (total number)	Bound sperm/ovum* (number)	Penetration index†	Penetration rate‡
Normospermic ejaculates (n = 18)				
BWW medium	540	29.9 ± 5.7	0.08§	7.6%§
mKRB medium	525	24.2 ± 5.8	0.15¶	13.5%¶
Teratospermic ejaculates (n = 18)				
BWW medium	536	22.2 ± 5.7	0.04‖	3.2%‖
mKRB medium	540	31.0 ± 5.7	0.02‖	2.4%‖

* Values are means ± pooled SEM.
† Penetration index = the total number of decondensed sperm heads divided by the number of ova inseminated.
‡ Penetration rate = the number of ova with decondensed sperm heads divided by the total number of ova inseminated multiplied by 100.
§,¶,‖ Within columns, values with different superscripts differ ($P < 0.05$).

TABLE 3. *Morphological characteristics of inseminated spermatozoa compared to spermatozoa bound to zona-free hamster ova in teratospermic domestic cats**

Teratospermic ejaculates	Inseminated spermatozoa			Bound spermatozoa†
	Diluted raw	Non-swim-up	Swim-up	
Normal spermatozoa (%)	22.7 ± 2.3‡	25.8 ± 2.3‡	56.4 ± 2.3§	73.3 ± 3.0¶
Abnormal spermatozoa (%)				
Abnormal sperm head	2.1 ± 0.1	2.3 ± 0.2	0.3 ± 0.1	2.1 ± 0.8
Mitochondrial sheath aplasia	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.0
Coiled flagellum	10.0 ± 2.0	8.7 ± 1.8	2.3 ± 1.0	5.2 ± 1.7
Bent midpiece with droplet	14.6 ± 1.2	14.3 ± 1.6	11.5 ± 2.4	4.9 ± 1.5
Bent midpiece, no droplet	4.8 ± 0.7	4.1 ± 0.5	2.6 ± 0.6	0.8 ± 0.2
Bent flagellum with droplet	19.9 ± 3.2	18.9 ± 3.4	7.4 ± 2.0	1.0 ± 0.5
Bent flagellum, no droplet	21.7 ± 2.3	21.5 ± 2.3	15.9 ± 2.4	5.1 ± 1.4
Proximal droplet	2.3 ± 0.5	2.5 ± 0.5	2.0 ± 0.6	6.8 ± 1.7
Distal droplet	1.8 ± 0.4	1.7 ± 0.3	1.5 ± 0.5	0.8 ± 0.4

* Values are means ± SEM of combined media groups (BWW and mKRB).
† Values are means ± SEM of combined sperm treatment groups. The proportion of normal sperm bound to ova was similar among the diluted raw, non-swim-up, and swim-up treatments.
‡,§,¶ Within-row means with different superscripts differ ($P < 0.05$).

of normal spermatozoa bound in the DR, NS, and SU aliquots from normospermic ejaculates incubated in BWW and mKRB were comparable ($P > 0.05$) to similarly processed aliquots in the teratospermic group. The specific morphologic types of spermatozoa bound to hamster ova are depicted in Table 3. With one exception, there were at least a few spermatozoa exhibiting each type of abnormality found attached to ova. Only those cells with partial or complete mitochondrial sheath aplasia appeared incapable of binding (Table 3).

Zona-Intact Domestic Cat Oocyte Assay

Ejaculate and Inseminant Characteristics Between Populations—Raw ejaculate traits for each cat population were similar to the values reported for these same males in the characterization study of Howard et al (1990) and used for heterologous fertilization of hamster ova. The number of motile spermatozoa per ejaculate was similar ($P > 0.05$) between groups, and the mean number of normal cells per ejaculate was greater ($P < 0.05$) in normospermic than teratospermic males (Table 1). Mean percent sperm motility and progressive motility were similar ($P > 0.05$) between normospermic and teratospermic cats. As observed in the hamster ovum study, diluting raw ejaculates in mKRB medium reduced ($P < 0.05$) the number of structurally normal spermatozoa. The SU treatment, however, improved the proportion of structurally normal spermatozoa within each cat population compared with the DR and NS aliquots and enhanced percent sperm motility and progressive motility (Table 1).

Oocyte Penetration—Spermatozoa from normospermic and teratospermic cats were capable of binding to and penetrating into the zona pellucida of homologous oocytes (Fig 2); however, overall sperm–zona interaction was more than two-fold greater for the normospermic group (Table 4). There was at least a five-fold increase ($P < 0.05$) in the number of bound spermatozoa per ovum and zona penetration index, and more than a three-fold increase ($P < 0.05$) in zona penetration rate for normospermic compared to teratospermic males (Table 4). The various sperm treatments (DR, NS, and SU) had no influence on any of the interactions, or binding or penetration characteristics examined (Table 4).

Discussion

This study provides the first evidence that teratospermia in domestic cats has a detrimental impact on gamete interaction *in vitro*. Compared with those of normospermic males, the spermatozoa from teratospermic cats were less capable of penetrating heterologous ova and binding and penetrating homologous zonae pellucidae. The most striking observation was that this compromised ability appeared inherent to the teratospermic ejaculate and was not dependent on the absolute number of structurally defective spermatozoa. Teratospermic ejaculates subjected to swim-up processing (to increase the number of normal sperm forms) were still three to six times less likely to penetrate zona-free hamster ova and almost four times less likely to penetrate zona-intact cat oocytes than similarly treated spermatozoa from normospermic cats. The differences between cat populations were similar to those observed in humans, in which the number of hamster ovum penetrations correlate positively to the number of structurally normal spermatozoa, more so than to other seminal characteristics (Rogers et al, 1983; Marsh et al, 1987; Kruger et al, 1988). In oligospermic men (Aitken

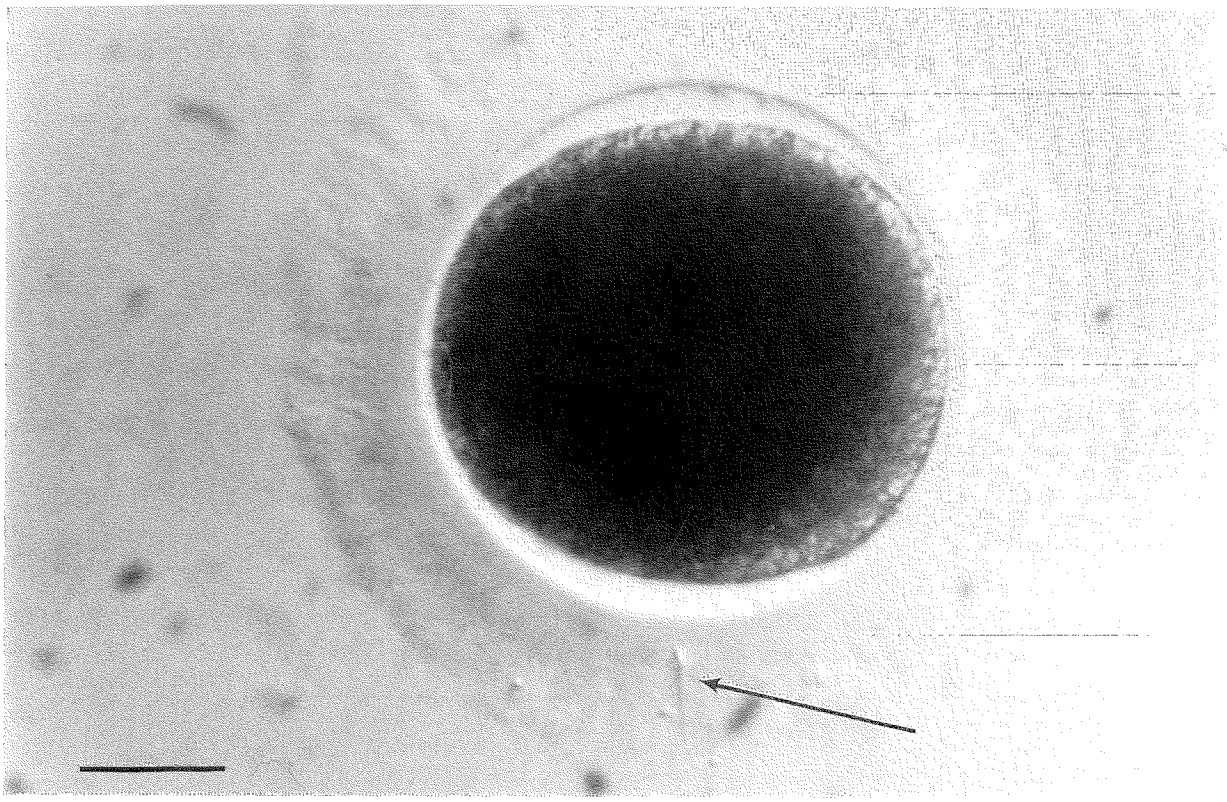

FIG. 2. Homologous penetration of a zona-intact domestic cat oocyte. Cat spermatozoon is visible within the zona pellucida (arrow). Scale bar = 30 μm.

et al, 1982) and in cases of unexplained infertility (Aitken et al, 1983), spermatozoal morphology and fertilizing capacity appear to be closely related. Kot and Handel (1987) reported that structurally abnormal spermatozoa from certain strains of mice bind to the zonae pellucidae of conspecific ova at a much lower frequency than might be predicted. Studies also have related decreased human ovum penetration rates to an increased incidence of abnormal spermatozoa in the inseminant (Mahadevan and Trounson, 1984). Studies in our laboratory have demonstrated that many factors influence the ability of cat follicular oocytes to fertilize *in vitro* and develop into biologically competent embryos *in vivo* (Goodrowe et al, 1988b; Miller et al, 1988; Johnston et al, 1989, 1991). It now is apparent that abnormally high numbers of pleiomorphic spermatozoa also would decrease IVF success rate in this species.

In the context of sperm penetration, both the zona-free hamster ovum and cat zonae pellucidae assays produced similar results. The concordance of the bioassays in demonstrating functional differences between spermatozoa from normospermic and teratospermic ejaculates confirmed the results and also indicated that these approaches were complementary. Numerous studies have demonstrated that the zona-free hamster ovum assay correlates positively with the penetration of zona-intact human oocytes (Overstreet et al, 1980; Wolf et al, 1983; Margalioth et al, 1986; Kruger et al, 1988). In the current study, however, the hamster assay was ineffective in distinguishing the binding dysfunction in the teratospermic cats, which was readily apparent when homologous, zona-intact oocytes were used. There appears to be a fundamental functional defect in spermatozoa from teratospermic cats, which is expressed as a diminished ability of the spermatozoa to interact with, bind to, and penetrate the zona pellucida.

The various sperm treatments, including swim-up processing, had no effect on sperm–ovum binding or penetration in either bioassay. The swim up method, however, is known to enhance cat sperm motility and morphology *in vitro* (Howard et al, 1990). Reducing the opportunity for abnormal spermatozoa to participate in fertilization may reduce the risk of producing defective or chromosomally abnormal embryos. The penetration rate and index were increased in the normospermic ejaculates exposed to hamster ova in the presence of mKRB medium. Fertilization *in vitro* is facilitated by albumin (Miyamoto and Chang, 1973; Hoppe and Whitten, 1974), and this protein component dif-

TABLE 4. *Penetration of zona-pellucida-intact, domestic cat oocytes by normospermic and teratospermic domestic cat spermatozoa*

	Normospermic ejaculates (n = 6)	Teratospermic ejaculates (n = 6)
Total number of ova inseminated	360	360
Sperm–zona interaction (%)*	65.3 ± 0.1§	24.2 ± 0.1¶
Number of bound sperm/ovum*	1.0 ± 0.1§	0.2 ± 0.1¶
Zona penetration index†	1.7§	0.3¶
Zona penetration rate‡	58.1%§	16.9%¶

* Values are means ± SEM. Sperm–zona interaction was the percentage of ova with spermatozoa binding to or penetrating into the zonae pellucidae.

† Zona penetration index = the total number of spermatozoa penetrating into the zonae pellucidae divided by the number of ova inseminated.

‡ Zona penetration rate = the number of ova with spermatozoa penetrating into the zonae pellucidae divided by the total number of ova inseminated times 100.

§,¶ Within rows, values with different superscripts differ ($P < 0.05$).

fers slightly in the mKRB (0.4%) and BWW (0.3%) medium. Also, in comparison to BWW, mKRB medium contains a higher concentration of sodium pyruvate, an important energy source for sperm respiration (Bavister and Yanagimachi, 1977). It is possible that cat spermatozoa are highly sensitive to the slightly higher albumin concentration and energy substrate level in the mKRB, which improved sperm fusion and penetration of the hamster ova.

Spermatozoa from a variety of species bind to and penetrate zona-free hamster ova *in vitro*; however, this is the first study demonstrating this ability for domestic carnivore spermatozoa. Spermatozoa from one species of nondomestic felid, the Siberian tiger (*Panthera tigris altaica*), have been shown to penetrate zona-free hamster ova (Byers et al, 1989). In this species, the bioassay was effective for assessing sperm function and various preincubation and coculture conditions. Optimum ovum penetration by tiger spermatozoa was observed after a 2-hour preincubation period at 37°C and a 3-hour co-incubation period. Since BWW medium was used in the tiger study, it is possible that tiger spermatozoa are similar to domestic cat spermatozoa and penetration rates would be enhanced if mKRB were used for gamete incubation. Although cat spermatozoa require capacitation before fertilization (Hamner et al, 1970; Goodrowe et al, 1988a), seminal plasma is not detrimental to ovum penetration in either the domestic cat or tiger. As detected in this study, removal of seminal plasma from domestic cat spermatozoa is not a prerequisite for sperm–oocyte interaction; neither hamster ovum nor cat oocyte penetration *in vitro* was enhanced by centrifuging the semen and decanting the seminal plasma. Likewise, the presence of seminal plasma does not affect the ability of tiger spermatozoa to penetrate zona-free hamster ova (Byers et al, 1989).

Because increasing the proportion of structurally normal spermatozoa or enhancing sperm motility had no affect on the number of penetrations of hamster ova or binding and penetration of cat zonae, some other factor inherent to the teratospermic ejaculate may be compromising gamete function. Ultrastructural or biochemical defects warrant further study, and attention also should be focused on the etiology of teratospermia. The increased numbers of pleiomorphic spermatozoa observed in certain populations of androgen-deficient domestic or nondomestic felids may be the result of defective spermatogenesis or epididymal dysfunction (Howard et al, 1990). In all mammals studied at this time, critical androgen-dependent changes occur during epididymal maturation and sperm storage (Orgebin-Crist and Fournier-Delpech, 1982). Because this critical interval is accompanied by significant changes in nuclear chromatin and DNA stabilization (Huret, 1986), defective epididymal function may be a cause of the diminished ability of spermatozoa to interact with ova and undergo nuclear decondensation. In the human, an apparent correlation exists between the progressive accumulation of epididymal proteins on the sperm surface during transit and the simultaneous ability to penetrate zona-free hamster oocytes (Moore et al, 1983). Deposition of these proteins on the sperm surface also has been correlated with the acquisition of zona pellucida binding capacity and fertilizing ability (Orgebin-Crist and Fournier-Delpech, 1982; Cuasnicu et al, 1984). These studies suggest that the compromised binding and penetrating ability of spermatozoa from the androgen-deficient, teratospermic cats may be related to epididymal dysfunction and a deficiency or alteration in sperm surface proteins.

In summary, this investigation demonstrated that cat spermatozoa from teratospermic ejaculates were compromised in three initial stages of sperm–ovum interaction: binding to the zona, penetration into the zona pellucida, and fusion with the oolemma. It is encouraging, however, that spermatozoa recovered from cats producing such high percentages of pleiomorphisms were still capable of gamete interaction, albeit at a compromised rate. Assuming that such a finding is not species specific, this observation is relevant to conservation biology since certain species, such as the cheetah (Wildt et al, 1987a), leopard (Wildt et al, 1988), and Asiatic lion (Wildt et al, 1987b), consistently produce a high proportion (>70%) of abnormal spermatozoa per ejaculate. Our studies of the Florida panther (*Felis concolor coryii*), a highly endangered puma subspecies, reveal that males routinely produce 94% sperm pleiomor-

phisms per ejaculate, with an average of 47% structurally defective acrosomes. Spermatozoa from individual males producing as many as 99% total pleiomorphisms and 63% defective acrosomes are still capable of fertilizing homologous oocytes *in vitro* (Miller et al, 1990). The zona-free hamster oocyte and zona-intact cat oocyte bioassays described here will be important tools for assessing numerous factors controlling fertilization and will improve our overall understanding of the impact and, perhaps, etiology of teratospermia.

Acknowledgments

The authors thank Dr. Mark A. Barone, Karen J. Clingerman, and Sandy L. Hurlbut for their valuable expertise and technical assistance. We also are grateful to the following veterinary clinics for their cooperation and for providing cat ovaries: Maple Springs Veterinary Office, Gaithersburg, MD; Montgomery Animal Hospital, Rockville, MD; Peachtree Veterinary Clinic, Beallsville, MD; and Seneca Valley Veterinary Clinic, Gaithersburg, MD.

References

Aitken RJ, Best FSM, Richardson DW, Djahanbakkch D, Mortimer D, Templeton AA, Lees MM. An analysis of sperm function in cases of unexplained infertility: conventional criteria, movement characteristics and fertilizing capacity. *Fertil Steril.* 1982;38:212–221.

Aitken RJ, Warner P, Best FSM, Templeton AA, Djahanbakhch O, Mortimer D, Lees MM. The predictability of subnormal penetrating capacity of sperm in cases of unexplained infertility. *Int J Androl.* 1983;6:212–280.

Bader H, Hoppen HO, Wockener A, Merkt H. Case studies in stallions with fertility problems: endocrine and spermatological aspects. *Proc Int Congr Anim Reprod Artif Insemin.* 1988;3:367.

Bavister BD, Yanagimachi R. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa *in vitro*. *Biol Reprod.* 1977;16:228–237.

Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos *in vitro*. In: Daniel JC, ed. *Methods of Mammalian Embryology.* New York: Raven Press, Ltd; 1971:86–116.

Bleil JD, Wassarman PM. Mammalian sperm–egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell.* 1980;20:873–882.

Boatman DE, Bavister BD. Stimulation of rhesus monkey sperm capacitation by cyclic nucleotide mediators. *J Reprod Fertil.* 1984;71:357–366.

Bostofte E, Serup J, Rebbe H. Relation between morphologically abnormal spermatozoa and pregnancies obtained during a twenty-year follow-up period. *Int J Androl.* 1982;5:379–386.

Brackett BG, Cofone MA, Boice ML, Bousquet D. Use of zona-free hamster ova to assess fertilizing ability of bull and stallion. *Gamete Res.* 1982;5:217–227.

Burkhart JG, Malling HV. Sperm abnormalities in the PL/J mouse strain: a description and proposed mechanism for malformation. *Gamete Res.* 1981;4:171–183.

Byers AP, Hunter AG, Seal US, Binczik GA, Graham EF, Reindl NJ, Tilson RL. In-vitro induction of capacitation of fresh and frozen spermatozoa of the Siberian tiger (*Panthera tigris*). *J Reprod Fertil.* 1989;86:599–607.

Cuasnicu PS, Gonzalez Echeverria F, Piazza A, Pineiro L, Blaquier JA. Epididymal proteins mimic the androgenic effect on zona pellucida recognition by immature hamster spermatozoa. *J Reprod Fertil.* 1984;71:427–431.

Goodrowe KL, Miller AM, Wildt DE. Capacitation of domestic cat spermatozoa as determined by homologous zona pellucida penetration. *Proc Int Congr Anim Reprod Artif Insemin.* 1988a;3:245–247.

Goodrowe KL, Wall RL, O'Brien SJ, Schmidt PM, Wildt DE. Developmental competence of domestic cat follicular oocytes after fertilization *in vitro*. *Biol Reprod.* 1988b;39:355–372.

Hamner CE, Jennings L, Sojka NJ. Cat (*Felis catus L.*) spermatozoa require capacitation. *J Reprod Fertil.* 1970;23:477–480.

Hanada A, Chang MC. Penetration of hamster and rabbit zona-free eggs by rat and mouse spermatozoa with special reference to sperm capacitation. *J Reprod Fertil.* 1976;46:239–241.

Hanada A, Chang MC. Penetration of the zona-free or intact eggs by foreign spermatozoa and the fertilization of deer mouse eggs in vitro. *J Exp Zool.* 1978;203:277–285.

Hoppe PC, Whitten WK. An albumin requirement for fertilization of mouse eggs *in vitro*. *J Reprod Fertil.* 1974;39:433–436.

Howard JG, Bush M, Hall LL, Wildt DE. Morphological abnormalities in spermatozoa of 28 species of non-domestic felids. *Proc Int Congr Anim Reprod Artif Insemin.* 1984;2:57–59.

Howard JG, Bush M, Wildt DE. Semen collection, analysis and cryopreservation in nondomestic mammals. In: Morrow DA, ed. *Current Therapy in Theriogenology.* Philadelphia: WB Saunders Co; 1986:1047–1053.

Howard JG, Brown JL, Bush M, Wildt DE. Teratospermic and normospermic domestic cats: ejaculate traits, pituitary-gonadal hormones and improvement of spermatozoal viability and morphology after swim-up processing. *J Androl.* 1990;11:204–215.

Hulet CV, Foote WC, Blackwell RL. Relationship of semen quality and fertility in the ram to fecundity in the ewe. *J Reprod Fertil.* 1965;9:311–341.

Huret JL. Nuclear chromatin decondensation of human sperm: a review. *Arch Androl.* 1986;6:97–109.

Imai H, Niwa K, Iritani A. Ultrastructural observations of boar spermatozoa penetrating zona-free hamster eggs. *Biol Reprod.* 1980;23:481–486.

Johnston LJ, O'Brien SJ, Wildt DE. *In vitro* maturation and fertilization of domestic cat follicular oocytes. *Gamete Res.* 1989;24:343–356.

Johnston LJ, Donoghue AM, O'Brien SJ, Wildt DE. Culture medium and protein supplementation influence *in vitro* fertilization and embryo development in the domestic cat. *J Exp Zool.* 1991 (in press).

Kim CI, Niwa K, Imai H, Iritani A. Penetration of zona-free hamster eggs *in vitro* by goat spermatozoa preincubated in the reproductive tract isolated from a maturing gilt. *J Exp Zool.* 1980;213:181–183.

Kot MC, Handel MA. Binding of morphologically abnormal sperm to mouse egg zonae pellucidae *in vitro*. *Gamete Res.* 1987;18:57–66.

Kruger TF, Swanson RJ, Hamilton M, Simmons KF, Acosta AA, Matta JF, Oehninger S, Morshedi M. Abnormal sperm morphology and other semen parameters related to the outcome of the hamster oocyte–human sperm penetration assay. *Int J Androl.* 1988;11:107–113.

Krzanowska H. The passage of abnormal spermatozoa through the uterotubule junction of the mouse. *J* Reprod Fertil. 1974;38:81–90.

Larrson K, Darenius K, Johansson K. Sperm morphology and *in vitro* viability in diluted semen in relation to fertility of AI boars. *Nord Veterinaermed.* 1980;32:533–535.

Mahadevan MM, Trounson AO. The influence of seminal characteristics on the success rate of human *in vitro* fertilization. *Fertil Steril.* 1984;42:400–405.

Mahadevan MM, Trounson AO, Wood C, Leeton JF. Effect of oocyte quality and sperm characteristics on the number of spermatozoa bound to the zona pellucida of human oocytes inseminated *in vitro*. *J In Vitro Fertil Embryo Transfer.* 1987;4:223–227.

Margalioth EJ, Navot D, Laufer N, Lewin A, Rabinowitz R, Schenker JG. Correlation between the zona-free hamster egg sperm penetration assay and human *in vitro* fertilization. *Fertil Steril.* 1986;45:665–670.

Marsh SK, Bolton VN, Braude PR. The effect of morphology on the ability of human spermatozoa to penetrate zona-free hamster oocytes. *Hum Reprod.* 1987;2:499–503.

Miller AM, Goodrowe KL, Wildt DE. Influence of gonadotropin source and treatment interval on *in vitro* fertilization of domestic cat oocytes. *Biol Reprod.* 1988;38(Suppl):73.

Miller AM, Roelke ME, Goodrowe KL, Howard JG, Wildt DE. Oocyte recovery, maturation and fertilization *in vitro* in the puma (*Felis concolor*). *J Reprod Fertil.* 1990;88:249–258.

Miyamoto H, Chang MC. The importance of serum albumin and metabolic intermediates for capacitation of spermatozoa and fertilization of mouse eggs *in vitro*. *J Reprod Fertil.* 1973;32:193–205.

Moore HDM, Hartman TD, Pryor JP. Development of the oocyte-penetrating capacity of spermatozoa in the human epididymis. *Int J Androl.* 1983;6:310–318.

Nestor A, Handel MA. The transport of morphologically abnormal sperm in the female reproductive tract of mice. *Gamete Res.* 1984;10:119–125.

Niwa K, Ohara K, Hosoi Y, Iritani A. Early events of *in vitro* fertilization of cat eggs by epididymal spermatozoa. *J Reprod Fertil.* 1985;74:657–660.

Orgebin-Crist MC, Fournier-Delpech S. Sperm–egg interaction. Evidence for maturational changes during epididymal transit. *J Androl.* 1982;3:429–433.

Overstreet JW, Yanagimachi R, Katz DF, Hayashi K, Hanson FW. Penetration of human spermatozoa into the human zona pellucida and the zona-free hamster egg: a study of fertile donors and infertile patients. *Fertil Steril.* 1980;33:534–542.

Pavlok A, Petelikova J, Flechon JE. Interaction of zona-free hamster eggs with fresh and frozen ram spermatozoa *in vitro*. In: Andre J, ed. *The Sperm Cell.* The Hague, Netherlands: Martinus Nijhoff; 1983:51–54.

Rogers BJ. The sperm penetration assay: its usefulness reevaluated. *Fertil Steril.* 1985;43:821–840.

Rogers BJ. The usefulness of the sperm penetration assay in predicting in vitro fertilization (IVF) success. *J In Vitro Fertil Embryo Transfer.* 1986;3:209–211.

Rogers BJ, Bentwood BJH, Van Campen H, Helmbrecht G, Soderdahl D, Hale RW. Sperm morphology assessment as an indicator of human fertilizing capacity. *J Androl.* 1983;4:119–125.

Saacke RG, Amann RR, Marshall CE. Acrosomal cap abnormalities of sperm from subfertile bulls. *J Anim Sci.* 1968;27:1391–1400.

Saacke RG, Barne JH, Karabinus DS, Mullins KJ, Whitman S. Transport of abnormal spermatozoa in the artificially inseminated cow based upon accessory spermatozoa in the zona pellucida. *Proc Int Congr Anim Reprod Artif Insemin.* 1988;3:292.

SAS. In: SAS User's Guide. Cary, NC: Statistical Analysis System Institute, Inc; 1986.

Sojka NJ, Jennings LL, Hamner CE. Artificial insemination in the cat (*Felis catus L.*). *Lab Anim Care.* 1970;20:198–204.

Wildt DE, Bush M, Howard JG, O'Brien SJ, Meltzer D, van Dyk A, Ebedes H, Brand DJ. Unique seminal quality in the South African cheetah and a comparative evaluation in the domestic cat. *Biol Reprod.* 1983;29:1019–1025.

Wildt DE, Schiewe MC, Schmidt PM, Goodrowe KL, Howard JG, Phillips LG, O'Brien SJ, Bush M. Developing animal model systems for embryo technologies in rare and endangered wildlife. *Theriogenology.* 1986;25:33–51.

Wildt DE, O'Brien SJ, Howard JG, Caro TM, Roelke ME, Brown JL, Bush M. Similarity in ejaculate-endocrine characteristics in captive versus free-ranging cheetahs of two subspecies. *Biol Reprod.* 1987a;36:351–360.

Wildt DE, Bush M, Goodrowe KL, Packer C, Pusey AC, Brown JL, Joslin P, O'Brien SJ. Reproductive and genetic consequences of founding isolated lion populations. *Nature.* 1987b;329:328–331.

Wildt DE, Phillips LG, Simmons LG, Chakraborty PK, Brown JL, Howard JG, Teare A, Bush M. A comparative analysis of ejaculate and hormonal characteristics of the captive male cheetah, tiger, leopard, and puma. *Biol Reprod.* 1988;38:245–255.

Wolf DP, Sokoloski JE, Quigley MM. Correlation of human *in vitro* fertilization with the hamster egg bioassay. *Fertil Steril.* 1983;40:53–59.

Yanagimachi R. Penetration of guinea-pig spermatozoa into hamster eggs *in vitro*. *J Reprod Fertil.* 1972;28:477–480.

Yanagimachi R. Zona-free hamster eggs: their use in assessing fertilizing capacity and examining chromosomes of human spermatozoa. *Gamete Res.* 1984;10:187–232.