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Abstract: Cheetahs (*Acinonyx jubatus*) produce poor quality ejaculates that can limit the efficiency of standard assisted reproduction including artificial insemination (AI) and in vitro fertilization (IVF). The purpose of this study was to: (1) further study sperm-oocyte interaction in this teratospermic species by examining the ability of malformed sperm to interact with various oocyte barriers; and (2) assess the potential of zona piercing for assisting IVF in a teratospermic felid. Zonae of salt-stored (SS), domestic tat oocytes were mechanically pierced (ZnPd) three times each. Semen was collected by electroejaculation from six male cheetahs and ejaculates were processed for IVF. Sperm aliquots from each ejaculate were assessed for a sperm motility index (SMI) over time. Zona-intact (ZnIn-SS) oocytes (n = 78) and ZnPd-SS oocytes (n = 74) were coincubated with spermatozoa in vitro for 6 h. The proportion of morphologically abnormal spermatozoa per ejaculate was high for all males (range 81.5% to 95.9%). SMI values at 0 and 6 h were variable, ranging from 50 to 75 and 0 to 40, respectively. Spermatozoa from an ejaculates bound to and penetrated the outer zona pellucida of ZnIn-SS and ZnPd-SS oocytes similarly (P > 0.05), The proportion of oocytes containing spermatozoa within the inner zona layer and the average numbe of spermatozoa per oocyte in this region were greater (P < 0.05) for the ZnPd-SS than ZnIn-SS oocytes (39.2% and 1.0 versus 12.8% and 0.2, respectively). Although zona piercing enhanced sperm penetration, there was no increase (P > 0.05) in pleiomorphic spormatozoa penetrating the inner zona pellucida or PVS. Penetration of both ZnIn-SS and ZnPd-SS oocytes was positively correlated (P < 0.05; r = 0.91) with SMI at 6 h but not to the proportion of pleiomorphic spermatozoa ejaculated (P > 0.05). In summary, altering the integrity of the zona pellucida by creating artificial channels increases the number of cheetah spermatozoa entering the inner zona region, but not the PVS. This phenomenon occurs without increasing the number of pleiomorphic sperm entering the zona/oocyte interface, reinforcing the role of the zona pellucida, especially the inner region, as a powerful filter for malformed sperm.

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Enhancing Zona Penetration by Spermatozoa From a Teratospermic Species, the Cheetah (*Acinonyx jubatus*)

TERRI L. ROTH, WILLIAM F. SWANSON, EVAN BLUMER, AND DAVID E. WILDT
Conservation and Research Center, National Zoological Park, Smithsonian Institution, Front Royal, VA 22630 (T.L.R., W.F.S., D.E.W.), and Fossil Rim Wildlife Center, Glen Rose, Texas 76043 (E.B.)

ABSTRACT Cheetahs (*Acinonyx jubatus*) produce poor quality ejaculates that can limit the efficiency of standard assisted reproduction including artificial insemination (AI) and in vitro fertilization (IVF). The purpose of this study was to: (1) further study sperm-oocyte interaction in this teratospermic species by examining the ability of malformed sperm to interact with various oocyte barriers; and (2) assess the potential of zona piercing for assisting IVF in a teratospermic felid. Zonae of salt-stored (SS), domestic cat oocytes were mechanically pierced (ZnPd) three times each. Semen was collected by electroejaculation from six male cheetahs and ejaculates were processed for IVF. Sperm aliquots from each ejaculate were assessed for a sperm motility index (SMI) over time. Zona-intact (ZnIn-SS) oocytes ($n = 78$) and ZnPd-SS oocytes ($n = 74$) were coincubated with spermatozoa in vitro for 6 h. The proportion of morphologically abnormal spermatozoa per ejaculate was high for all males (range 81.5% to 95.9%). SMI values at 0 and 6 h were variable, ranging from 50 to 75 and 0 to 40, respectively. Spermatozoa from all ejaculates bound to and penetrated the outer zona pellucida of ZnIn-SS and ZnPd-SS oocytes similarly ($P > 0.05$). The proportion of oocytes containing spermatozoa within the inner zona layer and the average number of spermatozoa per oocyte in this region were greater ($P < 0.05$) for the ZnPd-SS than ZnIn-SS oocytes (39.2% and 1.0 versus 12.8% and 0.2, respectively). Although zona piercing enhanced sperm penetration, there was no increase ($P > 0.05$) in pleiomorphic spermatozoa penetrating the inner zona pellucida or PVS. Penetration of both ZnIn-SS and ZnPd-SS oocytes was positively correlated ($P < 0.05$; $r = 0.91$) with SMI at 6 h but not to the proportion of pleiomorphic spermatozoa ejaculated ($P > 0.05$). In summary, altering the integrity of the zona pellucida by creating artificial channels increases the number of cheetah spermatozoa entering the inner zona region, but not the PVS. This phenomenon occurs without increasing the number of pleiomorphic sperm entering the zona/oocyte interface, reinforcing the role of the zona pellucida, especially the inner region, as a powerful filter for malformed sperm. © 1995 Wiley-Liss, Inc.

The cheetah (*Acinonyx jubatus*) is the last surviving species of its genus and is threatened by extinction (CITES, '84). Research has revealed several biological characteristics that may be contributing to species decline. For example, the cheetah appears to have experienced a population bottleneck that resulted in a major loss of genetic diversity (O'Brien et al., '83, '85). In turn, this lack of genetic variability appears, in part, responsible for the cheetah's high susceptibility to disease (Munson, '93) and low reproductive efficiency (only 20% of cheetahs in captivity have reproduced; Wildt et al., '93). Cheetahs ejaculate unusually high proportions of structurally abnormal spermatozoa compared to most other felid species (Wildt et al., '83; Howard et al., '84), and this may contribute to poor reproductive performance. At least in mammals, there appear to be natural barriers preventing morphologically abnormal spermatozoa from fertilizing oocytes in vivo. For example, transport of abnormal spermatozoa through the female reproductive tract is compromised (Krzanowska, '74; Nestor and Handel, '84; Saacke et al., '88). More importantly, it now is known that the domestic cat zona pellucida plays a filtering role allowing only structurally normal

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Address reprint requests to Dr. Terri L. Roth, Department of Reproductive Physiology, Conservation and Research Center, 1500 Remount Road, Front Royal, VA 22630.
Evan Blumer's current address is The Wilds, 14000 International Road, Cumberland, OH 43732.

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spermatozoa to penetrate and participate in fertilization (Howard et al., '93; Roth et al., '94). Thus, there appears to be a fascinating and, as yet, unknown set of interactions at the sperm-oocyte level perhaps regulating reproductive success for felid species consistently producing teratospermic ejaculates.

It also is relevant that assisted reproduction is beginning to play an important role in the captive propagation of certain endangered species like the cheetah (Howard et al., '92). To date, laparascopic intrauterine artificial insemination (AI) has resulted in six pregnancies producing a total of 11 offspring. These findings are important in terms of managing the captive population because assisted reproduction could help overcome problems in propagating geographically disparate individuals or dealing with sexual incompatibility which is common in this species. Although early results are encouraging, the average litter size remains low (<2 cubs/litter) indicating that alternative "assisted" approaches need to be improved. We also know that sperm-oocyte interaction in vitro and the production of embryos by in vitro fertilization (IVF) are less efficient in the cheetah compared to more 'normospermic' felid species like the domestic cat (*Felis catus*) and tiger (*Panthera tigris*; Wildt et al., '92). It remains unclear whether this inefficiency in vitro is related more to number of malformed sperm or to impaired sperm motility (Donoghue et al., '92a).

Interestingly, the human serves as an excellent model for the Felidae family because ejaculate quality in men often parallels that of cats, especially in the tendency for ejaculates to contain high proportions of pleiomorphic spermatozoa and/or spermatozoa exhibiting poor motility longevity. In this context, gamete micromanipulation (including zona pellucida piercing) is being used to promote sperm-oocyte interaction and enhance IVF success when the human sperm donor consistently produces a poor quality ejaculate (Malter and Cohen, '89; Payne et al., '91; Tucker et al., '91). Recently, we have shown that zona piercing significantly enhances zona penetration by spermatozoa from both normospermic and teratospermic male domestic cats (Roth et al., '94). When the distinctly bilayered zona pellucida of cat oocytes is mechanically pierced with a finely pulled pipette to produce six artificial channels, sperm penetration into the inner zona layer and PVS is enhanced compared to penetration of zona-intact oocytes. Furthermore, there is no increase in the number of morphologically abnormal spermatozoa penetrating the inner zona layer or PVS, indicating that the selective filtering capacity of the zona is not disrupted by the piercing procedure.

In this study, we took advantage of our previous knowledge about the impact of zona piercing in the domestic cat and that sperm and oocytes from heterologous felid species interact in vitro (Andrews et al., '92; Donoghue et al., '92a,b). Specifically, we used salt-stored domestic cat oocytes (Andrews et al., '92) to further study sperm function in the teratospermic cheetah. Our primary objective was to understand more about the role of the zona pellucida by determining if zona piercing: (1) facilitated penetration by cheetah spermatozoa; and (2) allowed indiscriminate penetration by morphologically abnormal spermatozoa. Secondarily, to dissect the potential relative impact of sperm motility longevity versus percent pleiomorphisms, the influence of these two traits on sperm-oocyte interaction was examined.

MATERIALS AND METHODS

Sperm function was measured using domestic cat oocytes matured in vitro and then stored in a salt solution (Andrews et al., '92). Spermatozoa interact with these oocytes as they do with in vivo-matured counterparts, and salt-stored oocytes have proven useful for assessing penetrating ability of both homologous (domestic cat; Andrews et al., '92; Howard et al., '91, '93) and heterologous (leopard cat: *F. bengalensis;* Andrews et al., '92; cheetah: Donoghue et al., '92a; tiger: Donoghue et al., '92b) spermatozoa.

Oocyte preparation and storage

Immature oocytes, recovered from domestic cat ovaries following ovariohysterectomy, were processed according to previous procedures (Johnston et al., '89). Briefly, ovaries maintained in phosphate-buffered saline at 5°C for up to 48 h after ovariohysterectomy were placed in Eagle's Minimum Essential Medium (MEM; Sigma Chemical Company, St. Louis, MO) supplemented with 5% fetal calf serum (FCS; Irvine Scientific, Irvine, CA) and punctured repeatedly with a 22 gauge needle to release cumulus-egg complexes. Oocytes with a dark vitellus were rinsed three times and then cultured in maturation medium consisting of MEM containing 0.284 mg/ml L-glutamine, 0.011 mg/ml pyruvate, 5% FCS, 10.0 μg/ml LH (NIADDK-oLH-25 AFP-5551B), 1.0 μg/ml FSH (NIADDK-oFSH-17 AFP-6446C), 25.0 μg/ml estradiol-17β (Sigma), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). After 44 to 48 h

in culture, oocytes were treated with 0.2% hyaluronidase in maturation medium for 15 min (38°C) and mechanically pipetted to remove any cumulus cells still adhered to the zona pellucida. Oocytes then were rinsed through three drops of maturation medium and transferred into vials containing the salt storage solution 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; Andrews et al., '92) and then stored at 4°C.

Zona piercing

Microtools consisting of a holding pipette and a piercing pipette were used for micromanipulation and zona piercing of salt-stored oocytes (Roth et al., '94). The holding pipette was broken at an outer diameter of ~125 µm, and the opening was fire-polished to a smooth ~75 µm inner diameter. The piercing tool was pulled to form a fine, sharp tip. Salt-stored (SS) oocytes were retrieved from the salt solution and rinsed in a Petri dish containing 3 ml of Ham's F10 medium (Irvine Scientific) supplemented with 5% FCS, 0.011 mg/ml pyruvate, 0.284 mg/ml glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (HF10). Oocytes were transferred to 50 µl drops of HF10 medium (four oocytes/drop) overlaid with oil in a Petri dish (100 × 15 mm) that was placed on the stage of an inverted microscope. Two Leitz micromanipulators were used to perform all micromanipulations. A 1.0 ml syringe attached via tubing to the holding pipette provided a source of suction. Each oocyte was stabilized by gentle suction from the holding pipette and, with the piercing pipette, the zona was pierced in the area directly opposite and slightly above the holding pipette as described earlier (Malter and Cohen, '89; Roth et al., '94). The piercing needle was advanced slowly through the uppermost portion of the oocyte, being careful to avoid piercing the cytoplasmic membrane, until it passed through the zona on the opposite side. The needle then was withdrawn from the oocyte leaving two channels (~5–10 µm) in the zona. The suction from the holding pipette was relaxed and, with the piercing pipette, the oocyte was gently rolled to a new position on the end of the holding pipette. Results from a previous study in domestic cats indicated that piercing the zona three times increases zona penetration by spermatozoa from normospermic and teratospermic cats (Roth et al., '94). Therefore, oocytes were pierced a second time, rolled again and pierced a third time so that there were six holes in the zona of each zona-pierced (ZnPd) oocyte. Approximately 1 h was required to pierce 30 oocytes three times each.

All pierced oocytes were transferred back into vials containing salt-storage solution and stored at 4°C. Zona intact (ZnIn-) and ZnPd-SS oocytes were transported to the study site in salt-storage solution maintained at 4°C. On the day of use, ZnIn- and ZnPd-SS oocytes were retrieved from their respective vials, rinsed through a Petri dish containing 3 ml HF10 and then transferred into 100 µl drops of HF10 (12–15 oocytes/drop) overlaid with oil in a Petri dish. To equilibrate oocytes, dishes were placed on a slide warmer (37°C) protected from light for a 1 h incubation. Then ZnIn- and ZnPd-SS oocytes were transferred into fresh 100 µl drops of HF10 and allowed to equilibrate a minimum of 1 additional h.

Cheetahs, semen collection and processing

Semen was collected by electroejaculation (Wildt et al., '83; Howard et al., '86) from six adult cheetahs (2.5 to 6.5 years old). Animals were housed at the Fossil Rim Wildlife Center in Glen Rose, TX and fed a diet of 1 to 2 kg/day Nebraska Brand Canine Diet (North Platte, NE) 6 days/wk supplemented with fresh venison or beef on the bone 1 day/wk. Males initially were induced into a surgical plane of anesthesia with Telazol (A.H. Robins Company, Richmond, VA; ~4 mg/kg estimated body weight, i.m.). Animals were maintained under a surgical plane of anesthesia throughout the electroejaculation procedure (~30 min) by administering small supplemental dosages of ketamine hydrochloride (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA; 25–50 mg; i.v.) as needed. Total ejaculate volume, sperm percent motility and forward progressive motility (scale of 0 to 5; 0 = no forward motility, 5 = rapid, linear forward progression) were recorded using previous criteria (Wildt et al., '83; Howard et al., '86), and a 10 µl aliquot was fixed in 0.3% glutaraldehyde for assessing sperm morphology. For this analysis, 100 spermatozoa/sample were evaluated for gross acrosomal, head, neck, midpiece, and flagellar defects (Howard et al., '90). Ejaculates were diluted with an equal volume of HF10, centrifuged (150 × g, 8 min), and supernatants were discarded. Sperm pellets were resuspended in 40 or 50 µl of HF10, and sperm motility, progressive motility and concentration were recorded. Samples then were diluted with HF10 to a final concentration of 5 × 10^6 motile sperm/ml.

Oocyte insemination and evaluation

Fifteen oocytes from each treatment group (ZnIn-SS and ZnPd-SS), equilibrated in HF10, were transferred into 80 μl insemination drops of HF10 covered with oil. Twenty μl aliquots of diluted spermatozoa from each male were used to inseminate drops containing ZnIn-SS (n = 15) and ZnPd-SS (n = 15) oocytes (final IVF concentration, 1 × 10^6 motile sperm/ml). Petri dishes containing gametes were incubated on a slide warmer (37°C) protected from light for 6 h. Oocytes were fixed in 10% buffered formalin and stored at 4°C until evaluated. Remaining sperm samples that were not used for IVF were maintained in microcentrifuge tubes on the slide warmer protected from light. Aliquots (10 μl each) were removed and evaluated for sperm longevity at 0.5 h and then hourly for 6 h using a sperm motility index described by Howard et al., '86 (SMI = [sperm % motility + (forward progressive motility × 20)] ÷ 2).

Penetration of ZnIn-SS (n = 78) and ZnPd-SS (n = 74) oocytes was evaluated using differential interference contrast microscopy (320 ×; Andrews et al., '92). Oocytes were assessed for the number of: (1) spermatozoa bound to the outer zona; (2) sperm heads within the outer half of the zona; (3) sperm heads within the inner half of the zona; and (4) spermatozoa within the PVS. Number of sperm/oocyte in each category and the morphology of all penetrating spermatozoa were recorded. For the ZnPd-SS oocytes, the number of spermatozoa penetrating the inner half of the zona and clearly within the pierced channels was recorded.

Statistical analysis

Average values are means ± standard errors of the mean (S.E.M.). Oocyte penetration data were analyzed statistically by analysis of variance (ANOVA), and treatment means were compared using Fischer PLSD (SAS, '84). Chi square (X^2) analysis was used to determine differences in the proportion of bound and penetrated ZnIn-SS and ZnPd-SS oocytes. Relationships between sperm motility and pleiomorphisms with oocyte penetration were evaluated using correlation coefficients (Steele and Torrie, '60).

RESULTS

All six cheetahs produced spermic ejaculates. On average, seminal characteristics of cheetahs in this study (Table 1) were similar to those previously reported for the species at large (Wildt et al., '83, '87, '93; Donoghue et al., '92a). However, among individuals, there was considerable variation in ejaculate volume (range, 0.16 to 1.15 ml) and total number of ejaculated spermatozoa (range, 1.9 to 71.0 × 10^6). The proportion of abnormally shaped spermatozoa ejaculated was high for all males (range, 81.5% to 95.9%). The most common morphological defects were a bent and/or coiled flagellum, together comprising more than 50% of all sperm shapes.

TABLE 1. Cheetah ejaculate traits following a standard electroejaculation regimen (n = 6 males)

	Mean ± S.E.M.
Ejaculate volume (μl)	827.0 ± 0.3
Total spermatozoa per ejaculate (×10^6)	25.2 ± 11.1
Sperm percent motility	66.7 ± 4.2
Sperm forward progressive motility (0–5)[1]	3.0 ± 0.2
Sperm motility index (SMI)[2]	63.3 ± 3.3
Sperm morphology (%)[3]	
Normal	11.9 ± 2.4
Abnormal	
Abnormal head[4]	1.5 ± 0.8
Abnormal acrosome	11.3 ± 2.5
Midpiece hypoplasia	1.6 ± 0.7
Bent midpiece	9.9 ± 2.2
Coiled flagellum	20.8 ± 3.6
Bent flagellum	33.8 ± 4.8
Cytoplasmic droplet	7.8 ± 1.3
Spermatid	1.1 ± 0.4

[1]Subjective assessment of forward progressive motility on a graded scale: 0, no movement to 5, rapid, steady forward progression.
[2]SMI = [% motility + (progressive motility × 20)] ÷ 2 (Howard et al., '86).
[3]Values based upon evaluations of 100 spermatozoa/sample. Fewer than 3.0% of spermatozoa exhibited malformations not fitting into these classification categories. These were excluded from tabular data.
[4]Includes microcephalic, macrocephalic and polycephalic.

Spermatozoa from all six cheetahs bound to and penetrated the zona pellucida of ZnIn-SS and ZnPd-SS oocytes (Fig. 1; Table 2). Spermatozoa bound to a high proportion (> 97%) of oocyte regardless of treatment. The proportion of ZnIn-SS and ZnPd-SS oocytes with spermatozoa penetrating the outer zona was high (> 79%) and not different ($P > 0.05$). In contrast, > 25% more ($P < 0.05$) ZnPd-SS oocytes had spermatozoa in the inner zona compared to ZnIn-SS oocytes. Furthermore, two of the ZnPd, but none of the ZnIn, oocytes contained spermatozoa within the PVS.

Oocyte type (ZnIn-SS versus ZnPd-SS) did not influence ($P > 0.05$) average number of spermatozoa bound to the zona (Table 2). Frequently, numerous (> 100) spermatozoa were bound to the zona making exact sperm counts impossible. For these oocytes, the arbitrary value of 100 bound

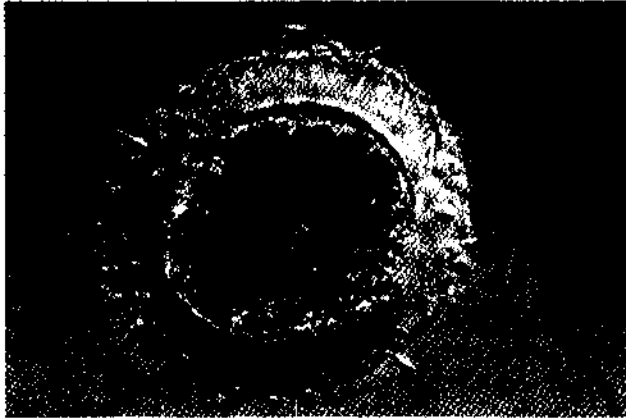

Fig. 1. Salt-stored, zona-pierced domestic cat oocyte with cheetah spermatozoa penetrating the outer layer of the zona pellucida (O) and penetrating the inner zona layer within an artificial channel (I).

spermatozoa was assigned. Number of spermatozoa penetrating the outer zona layer also did not differ ($P > 0.05$) with oocyte type. However, there was a fivefold increase in the number of spermatozoa penetrating the inner zona layer of ZnPd-SS compared to ZnIn-SS oocytes. Nevertheless, the number of morphologically defective spermatozoa (n = 3) penetrating ZnPd oocytes remained negligible ($P > 0.05$). Furthermore, no malformed spermatozoa were observed within the PVS of the 152 oocytes evaluated. Most spermatozoa (> 50%) penetrating the inner zona layer of ZnPd oocytes were within the artificially created channels (Table 2; Fig. 1).

The longevity of sperm motility (defined by SMI profiles over time) differed ($P < 0.05$) among individuals, but for all males, SMI decreased markedly ($P < 0.05$) over time (Fig. 2). At 3 h and 6 h, respectively, the SMI for two males was zero, but for the remaining four males, it ranged from 17.5 to 40. The SMI at 6 h was correlated positively ($P < 0.05$) with the number of spermatozoa penetrating the inner zona of ZnPd-SS oocytes and outer zona of ZnIn-SS oocytes ($r = 0.91$ and $r = 0.90$, respectively). Additionally, there was a positive correlation ($P < 0.05$) between the SMI at 0 h and the number of spermatozoa penetrating the inner zona of ZnIn oocytes ($r = 0.95$; $P < 0.01$). However, there was no significance ($P > 0.05$) to the correlation between the proportion of normal spermatozoa per ejaculate and the number of spermatozoa penetrating the zona pellucida regardless of oocyte type.

DISCUSSION

Assisting reproduction in endangered species requires understanding unique, physiological characteristics and overcoming obstacles not generally encountered in domestic animals. For example, the cheetah produces poor quality ejaculates comprised of high proportions of structurally abnormal spermatozoa that demonstrate poor longevity in vitro. IVF success is poor in this species, and we are challenged with identifying the factors re-

TABLE 2. Penetration of zona-intact and zona-pierced salt-stored domestic cat oocytes by cheetah spermatozoa (n = 6 males)

	Zona-intact	Zona-pierced
No. oocytes	78	74
Penetration (%)[1]		
Bound	100.0 ± 0.00	97.3 ± 0.02
< 1/2 ZP	83.3 ± 0.04	79.7 ± 0.05
> 1/2 ZP	12.8 ± 0.04[2]	39.2 ± 0.06[3]
PVS	0.0 ± 0.00	2.7 ± 0.02
No. spermatozoa/oocyte		
Bound	56.3 ± 4.1	50.8 ± 4.4
< 1/2 ZP	19.0 ± 2.2	15.9 ± 2.3
> 1/2 ZP	0.2 ± 0.1[2]	1.0 ± 0.2[3]
PVS	0.0 ± 0.0	0.1 ± 0.1
No. abnormal spermatozoa/ total spermatozoa		
> 1/2 ZP	0/14 (0.0%)	3/78 (4.0%)
PVS	NA	0/5 (0.0%)
No. spermatozoa within channels		
> 1/2 ZP	NA	44/78 (56.4%)

[1]Categories of zona penetration include: (1) Bound = spermatozoa bound to the zona; (2) < 1/2 ZP = spermatozoa within the outer half of the zona; (3) > 1/2 ZP = spermatozoa within the inner half of the zona; and (4) PVS = spermatozoa within the perivitelline space. Values are means ± S.E.M.

[2,3]Within rows, means with different superscripts differ ($P < 0.01$).

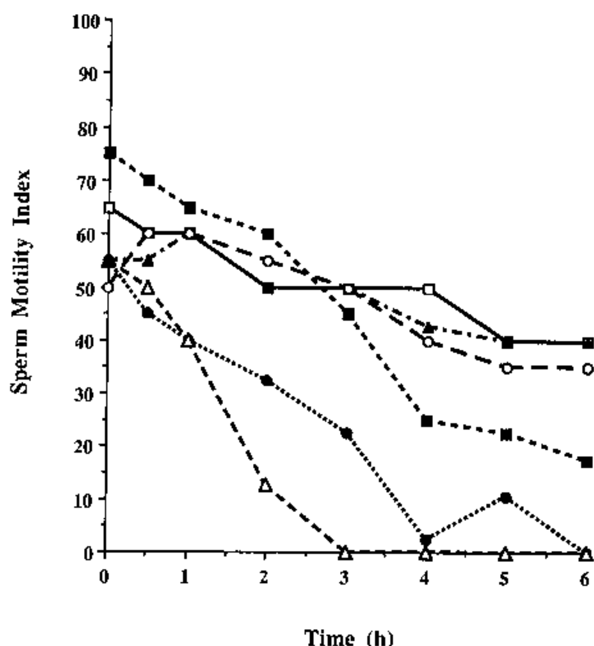

Fig. 2. Sperm motility index (SMI) over time for six male cheetahs. Despite dilution and washing, sperm from two males demonstrated no motility at 3 and 6 h, respectively. Zona penetration was positively correlated ($P < 0.05$) with SMI at 6 h.

sponsible for inefficient gamete interaction and for developing alternative methods for overcoming fertilization failure. In this study, piercing the zona pellucida increased heterologous zona penetration by cheetah spermatozoa. The zona pellucida plays several roles in the physiological events preceding fertilization by providing sperm receptors, inducing the acrosome reaction and regulating sperm penetration (reviewed by Wassarman, '87, '94). Therefore, studying sperm interaction with zona-manipulated oocytes may lead to identifying specific physiological deficiencies associated with spermatozoa from nondomestic, teratospermic felids. Furthermore, gamete manipulation may enhance fertilization success when genetically valuable males produce ejaculates too poor to fertilize oocytes in a standard IVF system.

It is becoming clear that the inner layer of the cat zona pellucida plays a crucial role in selecting the fertilizing spermatozoon by inhibiting penetration by most spermatozoa. Morphologically abnormal spermatozoa frequently are observed bound to or penetrating the outer zona layer, but only rarely appear in the inner zona (Howard et al., '93; Roth et al., '94). Furthermore, spermatozoa from one felid species, the snow leopard, readily enter the outer zona but appear incapable of penetrating the inner zona layer of the domestic cat oocyte, suggesting a potential species-specific function (Roth et al., '94). Evidence for the filtering role of the inner zona layer is apparent when total numbers of spermatozoa penetrating the two layers of the zona pellucida are compared. In this study, spermatozoa from all males were capable of binding and penetrating the outer half of the intact domestic cat zona pellucida, and the average number of spermatozoa penetrating the outer zona was high (19 sperm/oocyte). However, fewer than one sperm/oocyte was found within the inner zona, observations generally consistent with results from previous work (Howard et al., '93; Roth et al., '94). Therefore, the inner zona layer deserves special attention when studying felid sperm-oocyte interaction.

Zona piercing introduces artificial channels that could compromise these strict selection processes to allow more spermatozoa access to the vitellus. Our data indicated that this did occur because the proportion of ZnPd-SS oocytes with spermatozoa penetrating the inner zona layer increased > threefold, and the average number of spermatozoa within the inner zona increased ~fivefold compared to ZnIn-SS oocytes. Additionally, a high proportion of spermatozoa penetrating the inner zona of ZnPd oocytes was clearly within the artificial channels, providing strong evidence that these channels were responsible for increasing sperm penetration. Similar results were reported when zona piercing was tested in domestic cats (Roth et al., '94). Interestingly, enhanced penetration is not resulting from the unrestricted passage of morphologically abnormal spermatozoa through the zona. Similar to reports in the teratospermic domestic cat (Roth et al., '94), in this study, only three malformed sperm cells were observed within the inner zona layer, and no abnormal spermatozoa were within the PVS. Thus, it was evident that zona piercing did not disrupt the ability of the cat zona to filter out structurally abnormal cheetah spermatozoa, even when ~88% of the spermatozoa inseminated exhibit structural anomalies. One explanation for this result is that the zona of the pierced oocyte immediately resumed its original shape after the piercing pipette was withdrawn. Likely this immediate "reannealing" of the zona at least partially allowed oocytes to retain a filtering capability. Although there frequently were multiple sperm lodged within the artificial channels, some progressive penetrating ability still was required by sperm to achieve inner zona

or PVS penetration, a function potentially deficient in malformed spermatozoa.

Studies in the domestic cat have demonstrated that IVF and penetration of salt-stored oocytes are compromised when oocytes are inseminated with spermatozoa from teratospermic ejaculates, even after swim-up processing to select for structurally normal sperm (Howard et al., '93). However, in the cheetah, IVF success also is related to sperm motility over time (i.e., SMI profiles; Donoghue et al., '92a). In the present study, there was a positive correlation between sperm motility at 6 h and outer and inner zona penetration for ZnIn-SS and ZnPd-SS oocytes, respectively. These data also support the theory of the inner zona barrier because, although enhanced motility increased outer zona penetration of intact oocytes, inner zona penetration only increased if artificial channels had been drilled through the zona. Therefore, although enhanced sperm motility improved penetration, there still appeared to be a functional deficiency that rendered most cheetah spermatozoa incapable of penetrating the inner zona pellucida unless assisted by artificial channels. We speculate that spermatozoa cannot penetrate this second zona layer because they are deficient either in ability to bind with zona receptors, acrosome react or become hyperactivated (studies currently in progress).

Our earlier assertions that the teratospermic domestic cat is an excellent model for teratospermic, nondomestic felids (Howard et al., '91, '93) is clearly supported by the present results. Both the proportion of intact cat oocytes with cheetah spermatozoa penetrating the inner zona (~13%) and the average number of spermatozoa found within the inner zona (~0.2) were very similar to those reported for conspecific inseminations using teratospermic domestic cat sperm donors (~17% and 0.3 sperm/oocyte, respectively; Roth et al., '94). Even more interesting, zona piercing enhanced penetration similarly in both teratospermic populations with more oocytes containing spermatozoa in the inner zona (~39% for the cheetah and 32% for the cat), more sperm/oocyte within the inner zona (1.0 for the cheetah and 0.9 for the cat) and very few sperm/oocyte in the PVS (0.1 for the cheetah and 0.2 for the cat). However, within channels of ZnPd oocytes, spermatozoa have been noted traveling from the PVS back out into the surrounding medium (Roth et al., '94). Therefore, it is possible that there are more spermatozoa entering the PVS and exiting before oocytes are fixed. Together, these data suggested that sperm deficiencies inhibiting oocyte penetration in the cheetah may be similar to those affecting spermatozoa in the teratospermic domestic cat. Therefore, studying the domestic cat may lead to understanding problems associated with spermatozoa in the cheetah and other nondomestic, teratospermic felids.

In summary, zona piercing enhances heterologous sperm penetration in the cheetah without disrupting the ability of the zona pellucida to filter out morphologically abnormal spermatozoa. Sperm motility longevity, but not percent pleiomorphic spermatozoa, is positively associated with zona penetration. The inner zona layer appears to play a distinct, crucial role as a natural barrier to sperm penetration. By artificially introducing channels through the zona, this partial barrier can be circumvented without substantially increasing the chance of the oocyte being fertilized by a malformed spermatozoon. We now contend that one of the secrets to discovering the physiological deficiencies of spermatozoa in teratospermic ejaculates appears to lie in first determining the role(s) of the inner zona pellucida barrier. Although zona piercing offers one method for potentially assisting IVF by facilitating sperm penetration of the inner zona, the physiological competence of the penetrating spermatozoa will be determined only when the technique is tested in viable oocytes. Alternatively, considering the low number of oocytes containing spermatozoa in the PVS in both this study and the previous study with domestic cats (Roth et al., '94), it is possible that more invasive techniques, like sperm injection, will be necessary. Studies currently are in progress with the teratospermic domestic cat model to begin answering these questions.

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