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Normospermic Versus Teratospermic Domestic Cat Sperm Chromatin Integrity Evaluated by Flow Cytometry and Intracytoplasmic Sperm Injection[1]

Linda M. Penfold,[2,3] Lorna Jost,[4] Donald P. Evenson,[4] and David E. Wildt[3]

Conservation and Research Center, Smithsonian National Zoological Park,[3] Front Royal, Virginia 22630
Department of Chemistry and Biochemistry,[4] South Dakota State University, Brookings, South Dakota 57007

ABSTRACT

Teratospermia (>60% of morphologically abnormal spermatozoa) is well documented in felids. Even morphologically normal spermatozoa from teratospermic ejaculates have reduced ability to undergo tyrosine phosphorylation, acrosome react, and bind and penetrate oocytes compared with normospermic (<40% abnormal spermatozoa) counterparts. However, it is unknown whether fertilization deficiencies originate at a nuclear level. This study examined whether fertilization failure also was attributable to abnormal sperm chromatin, using the sperm chromatin structure assay (SCSA), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI). Aliquots of unprocessed and swim-up-processed (to isolate morphologically normal spermatozoa) spermatozoa from teratospermic and normospermic domestic cats were analyzed by the flow cytometric SCSA. Swim-up-processed sperm were incubated with in vivo-matured oocytes or used for ICSI. Teratospermic ejaculates expressed more ($P < 0.05$) chromatin heterogeneity (abnormal chromatin structure) than their normospermic counterparts, both in unprocessed and swim-up-processed samples. Fertilization success in vitro was higher ($P < 0.05$) from normo- compared with teratospermic inseminates. Similar ($P > 0.05$) proportions of oocytes fertilized after ICSI using spermatozoa from normo- and teratospermic cats. Results reveal that teratospermia in the cat is expressed at the nuclear level as increased sperm chromatin heterogeneity, but ICSI showed that this does not apparently affect fertilization rates if the zona pellucida and oolemma can be bypassed.

assisted reproductive technology, gamete biology, in vitro fertilization, sperm

INTRODUCTION

During mammalian fertilization, the sperm nucleus is incorporated into the oocyte's cytoplasm through a complex series of events that include capacitation, the acrosome reaction, binding to the zona pellucida (ZP), and penetrating the oocyte vestments. The oocyte ZP is species specific (preventing penetration by spermatozoa from other species), but also can deter spermatozoa from the homologous species, especially where they may be malformed or otherwise defective [1–7]. Under natural conditions, a functional spermatozoon must complete all of the above processes to accomplish fertilization.

The presence of pleiomorphic spermatozoa is a common feature in felid ejaculates, with the incidence being species- and population-specific [7–9]. The domestic cat (*Felis catus*) is an excellent model for comparative studies of teratospermia because, although the species is usually normospermic, some males consistently produce high proportions of malformed spermatozoa [7, 8]. Adult males known to consistently produce high (>60%/ejaculate) numbers of abnormal spermatozoa are defined as teratospermic, and males known to consistently produce low numbers of abnormal spermatozoa (<40%/ejaculate) are defined as normospermic [4–7].

Early studies demonstrated that these abnormally shaped spermatozoa fail to participate in fertilization and are excluded by the outer ZP layer [5]. Even normal-appearing spermatozoa, which can be isolated from the abnormal spermatozoa by swim-up processing, from teratospermic ejaculates, are compromised in their fertilization capacity in vitro [5, 8]. These morphologically normal spermatozoa, when compared with morphologically normal sperm isolated in the same manner from normospermic donors, take longer to capacitate in vitro [10], are compromised in their ability to undergo tyrosine phosphorylation [11], and have a reduced ability for acrosomal reaction [10]. In ZP-piercing studies, whereby domestic cat oocytes (stored in salt) were incubated with spermatozoa isolated by swim-up processing from normospermic and teratospermic ejaculates, spermatozoa numbers within the perivitelline space (PVS) increased for both cat types but failed to produce similar numbers of sperm within the PVS [12]. Mechanically piercing the ZP partially compensated for reduced sperm-zona interactions but emphasized that inherent differences remained in the ability of morphologically normal spermatozoa to penetrate oocytes. Thus, spermatozoa function in teratospermic individuals is not solely expressed at the sperm-zona level.

In this study, the influence of teratospermia on sperm nuclear integrity was examined using the sperm chromatin structure assay (SCSA [13]). This flow cytometric (FCM) procedure uses the metachromatic properties of the nuclear dye, acridine orange (AO) to evaluate chromatin soundness by in situ denaturation of sperm nuclear DNA [13, 14]. Previous studies have shown that sperm containing DNA strand breaks have a proportional increase in susceptibility to acid denaturation [13–16]; thus, the degree of chromatin fragmentation can be quantified. AO differentially stains native, double-stranded DNA that fluoresces green from denatured single-stranded DNA that fluoresces red when the spermatozoa are stimulated by blue laser light. Increased susceptibility of DNA to denaturation corresponds to the heterogeneity of chromatin structure, which in turn has

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[2]Correspondence: Linda M. Penfold, White Oak Conservation Center, 3823 Owens Rd., Yulee, FL 32097. FAX: 904 225 3337;
e-mail: Lindap@wogilman.com

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been associated with disturbances in spermiogenesis, high proportions of morphologically abnormal spermatozoa in certain cases, and infertility [15, 16]. There is evidence that chromatin structure can be independent of nuclear shape. In one study, although 70% of mouse spermatozoa were morphologically abnormal, only 5% of those abnormal spermatozoa were susceptible to denaturation, and fertility success was comparable with that of other inbred mice [17]. In contrast, induction of mouse sperm-head abnormalities by chemical treatment has been associated with increased DNA fragmentation [18], although fertility data were unavailable. In some cases, the SCSA data correlated with loss of embryos in utero [19]. Last, it is known that, compared with normospermic cats, spermatozoa from teratospermic counterparts have an increased susceptibility to undergoing nuclear decondensation and decreased sulphur concentrations in the head region [20]. This could be indicative of faulty protamine deposition and/or decreased disulfide bond formation. This was one of the incentives for using the SCSA in this study to determine if spermatozoa from teratospermic donors had decreased DNA integrity.

Intracytoplasmic sperm injection (ICSI) is a tool usually considered most useful for offspring production (e.g., cattle [21], mouse [22, 23], human [24], and domestic cat [25]) but can also be useful for studying sperm function [26, 27]. ICSI was used here to gain more details of sperm function in the normo- versus teratospermic cat by eliminating the barrier or filtering role [6] of the ZP and oolemma and placing selected spermatozoa directly into the ooplasm. Functionality was based on comparing results achieved by standard IVF versus ICSI. Thus, normo- versus teratospermic ejaculates were used to determine if there were differences in sperm chromatin heterogeneity between the two male donor types and if morphologically normal spermatozoa from the teratospermic male expressed conventional fertilization when the ZP and oolemma were bypassed.

MATERIALS AND METHODS

Animals

Adult female domestic cats (n = 9) were used as donors for in vivo-matured oocytes and were housed together in indoor enclosures. Adult males, housed separately and in individual cages, were from the same colony and were known to consistently produce high (>60%/ejaculate; normospermic; n = 3 males) or low (<40%/ejaculate; teratospermic; n = 3 males) proportions of structurally normal spermatozoa [4–7]. All cats were exposed to a 12L:12D cycle and were provided dry cat food (Purina Cat Chow; Ralston Purina Co., St. Louis, MO) and water ad libitum on a daily basis. Project approval was secured from the Conservation & Research Center's Animal Care and Use Committee.

Semen Collection, Processing, and Evaluation

Both normo- and teratospermic ejaculates were tested on each day of the study to eliminate day effects. Semen was collected from anesthetized males [5] that had previously been designated as normospermic or teratospermic based on repeated analyses (data not shown) using a standard electroejaculation protocol [28]. Three cats from each group (normospermic and teratospermic) were selected as semen donors. The number of males in each group was considered representative of the groups, based on previous studies investigating the incidence of teratospermia that showed statistical differences in semen or sperm parameters. Semen was evaluated subjectively for the percentage of motile spermatozoa by microscopy at 10×. A 10-μl aliquot of unprocessed semen was fixed in 0.3% glutaraldehyde and examined at 1000× for morphological assessment of sperm morphology [28]. To assess acrosomal integrity, 2 μl unprocessed semen from each ejaculate was incubated in Popes stain [29] for 2 min before air drying a smear on a microscope slide. Spermatozoa were assessed as acrosome intact if a uniform blue stain was present over the acrosomal region. Partial or no blue staining was indicative of a damaged or missing acrosome [29].

Ejaculates collected for ICSI and IVF experiments were diluted 1:3 with Ham F10 medium supplemented with 2 mM glutamine, 1 mM pyruvate, 100 IU penicillin, and 5% fetal calf serum (FCS) (unless otherwise stated, all chemicals were from Sigma Chemical Co., St. Louis, MO). After centrifugation (300 × *g*, 7 min), seminal plasma was decanted and the sperm pellet was resuspended, overlaid with 50 μl Ham F10 containing 5% FCS, and incubated for 30 min to allow the spermatozoa to swim up into the medium [8]. This swim-up processing technique has been previously and adequately shown to increase the numbers of morphologically normal sperm from ejaculates of teratospermic individuals [8, 10]; thus, post-swim-up sperm morphology was not assessed. However, in this study, similar proportions of morphologically normal spermatozoa to those found in previous studies were expected after swim-up processing (unprocessed 71.6% ± 2.3% versus swim-up processed 65.9% ± 2.3%, normospermic and unprocessed 33.8% ± 2.3% versus swim-up processed, 56.4% ± 2.3%, teratospermic; [8]). Aliquots of swim-up-processed spermatozoa were added to 100-μl drops of Ham F10 (under mineral oil) containing 5% FCS in 33-mm Petri dishes at a final concentration of 2×10^5 sperm/ml (for IVF, see below), or were diluted 1:10 with 12% polyvinylpyrrolidine (molecular weight, 360 000) in HEPE's buffered Minimal Essential Medium (HMEM) supplemented with 2 mM glutamine and 1 mM pyruvate and containing 0.4% BSA (Fraction V; for ICSI, see below).

Unprocessed (n = 16; ~10–50 μl) and swim-up-processed ejaculates (n = 6; ~50–100 μl) also were collected and snap frozen in liquid nitrogen in 1.8-ml microcentrifuge vials (Fisher Scientific, Fairlawn, NJ) and kept frozen until SCSA measurements were made.

Sperm Preparation and Staining for Flow Cytometry

Frozen aliquots of unprocessed semen and swim-up-processed spermatozoa (normospermic, n = 16 unprocessed and 6 swim-up-processed samples; teratospermic, n = 16 unprocessed and 6 swim-up-processed samples) were thawed at 37°C. Thawed semen was diluted with TNE (0.01 mol/L Tris[hydroxymethyl]aminomethane [Tris]-HCl, 0.15 mol/L NaCl, and 1 mmol/L EDTA, pH 7.4) buffer to a concentration of 1–2 × 10^6 cells. Details of the SCSA are described elsewhere [13]. In brief, diluted semen was treated with 0.1% Triton X-100, 0.08 N HCl, and 0.15 M NaCl to permeabilize the cell membrane for AO dye uptake and to potentially denature the DNA of abnormal chromatin. Thirty seconds later, 1.2 ml AO staining solution (0.2 M Na_2HPO_4, 1 mM di-sodium EDTA, 0.15 M NaCl, 0.1 M citric acid monohydrate), pH 6.0, with 6.0 μg/ml AO (Polysciences, Inc., Warrington, PA) was added, and flow cytometric measurements were begun 3 min after starting this procedure. The red and green fluorescence of each AO-stained spermatozoa was measured using a Cytofluorograf II (Ortho Diagnostic Systems, Westwood, MS) unit equipped with a Lexel 100-mW argon laser operated at 35-mW output and 488-nm wavelength and interfaced to an Ortho Diagnostics 2150 Data Handling System. The SCSA parameters are the mean and SD of the DNA fragmentation index (mean DFI and SD DFI), the percentage of sperm with fragmented DNA (% DFI), and the percentage of sperm with high DNA stainability (% HDS).

Increased red fluorescence was indicative of increased DNA fragmentation and was quantified using the DNA fragmentation index (DFI; a ratio of red:total (red + green) fluorescence of each individual spermatozoa) [13]. Analysis of the resultant DFI histogram composed of 5000 spermatozoa per sample determined the mean and standard deviation of the distribution and the % of spermatozoa lying in pertinent regions of the plot (mean DFI, SD DFI, and % DFI, respectively). Each of these values is an important indicator of the extent of abnormal chromatin structure (with a higher number indicative of increased DNA denaturation) [13]. Higher green fluorescence is indicative of immature chromatin that permits a high level of DNA stainability.

Inducing Ovarian Activity and Collecting In Vivo-Matured Follicular Oocytes

Donors of in vivo-matured oocytes were treated with a conventional gonadotropin therapy [30, 31] by i.m. injection of 150 IU of equine chorionic gonadotropin (eCG) to stimulate ovarian follicular development. Final oocyte maturation was induced by an i.m. injection of 100 IU human chorionic gonadotropin given 84 h post-eCG. Twenty-four to 26 h later, oocytes were recovered from ovarian follicles via a standard laparoscopic procedure for the cat [32]. In brief, a surgical plane of anesthesia was induced, and the contents of each visible ovarian follicle were aspirated transabdominally under laparoscopic observation into sterile collection

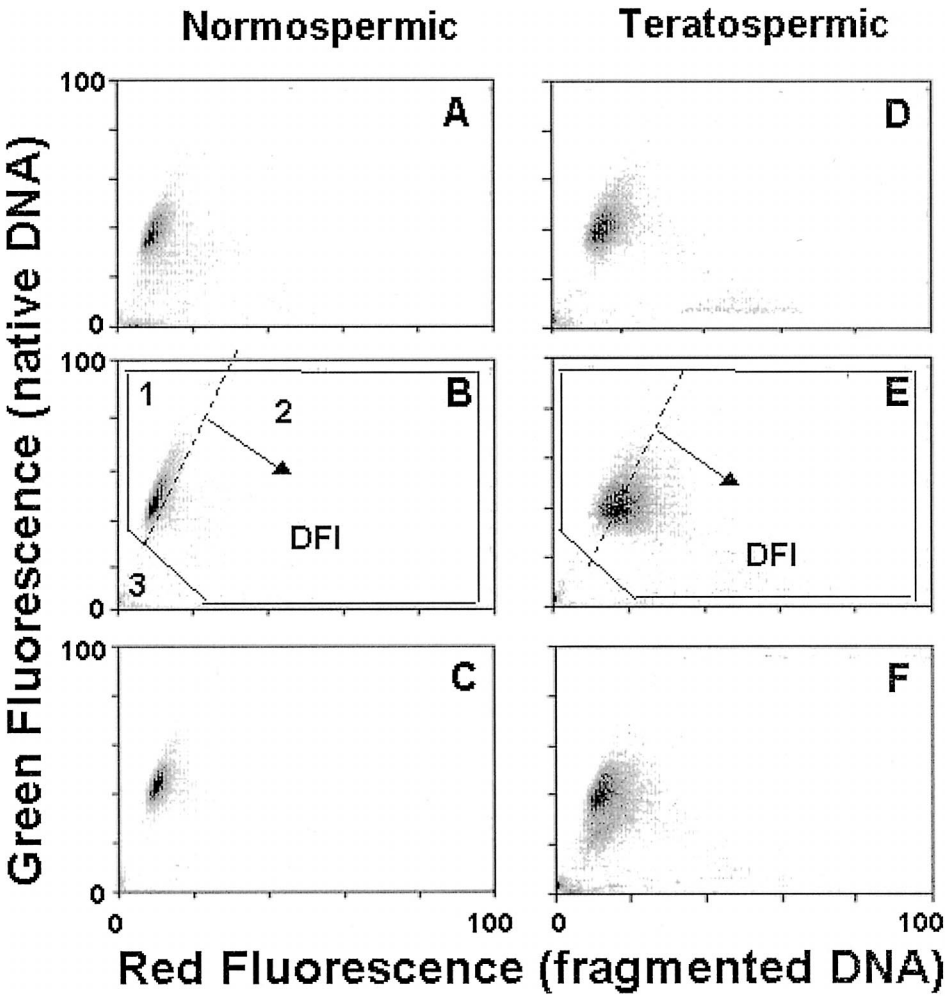

FIG. 1. Native DNA (green fluorescence) versus fragmented DNA (red fluorescence) cytograms of selected normo- and teratospermic felid individuals. Each cytogram contains 5000 sperm, each represented by a dot. The location of each sperm represents the amount of native and fragmented DNA each sperm contains. Sperm falling further to the right are more heterogeneous and contain larger amounts of fragmented DNA.

tubes containing Ham F10 plus 5% FCS and heparin (40 IU/ml). Tube contents were emptied into a sterile Petri dish and examined by stereomicroscopy. Mature oocytes (expanded corona radiata and cumulus mass; [32]) were maintained at 38°C, washed in Ham F10, and transferred to HMEM for cumulus removal (see below). Denuded oocytes were divided into equal groups for IVF or ICSI.

Oocyte Processing

After aspiration, oocytes were incubated in HMEM containing 0.2% hyaluronidase at 38°C for 10 min, and cumulus cells were removed by gently pipetting the oocytes up and down using a fine-bore, sterile borosilicate glass pipette. After cumulus removal, oocytes were transferred to drops of HMEM under oil (IVF oocytes) for the duration of the ICSI procedure or transferred to 5-μl drops of HMEM for ICSI (below). Oocytes were distributed in equal numbers between IVF and ICSI treatments and for both normo- and teratospermic donors.

In Vitro Fertilization

To control for the time the ICSI oocytes were maintained in HMEM during the injection procedure, IVF oocytes also were incubated in HMEM until all ICSI oocytes were sperm injected. Oocytes then were washed in Ham F10 containing 5% FCS and transferred to 100-μl drops of Ham F10 containing 5% FCS and 2×10^5 sperm/ml and incubated at 38°C in 5% CO_2 in air [32]. After 16–18 h, spermatozoa were washed from IVF oocytes by transferring them to fresh Ham F10 drops. Oocytes were assessed for cleavage ~32 h after incubating with spermatozoa. On each day, ~5% of oocytes were incubated in Ham F10 containing 5% FCS in the absence of spermatozoa as a parthenogenetic control. Both fertilization (the presence of a cleaved embryo) and development (cell growth to the morula stage) were monitored by brief examination daily.

Intracytoplasmic Sperm Injection

A 5-μl sperm aliquot in PVP from a normospermic sample and one from a teratospermic sample were placed separately in the center of a 90-mm Petri dish lid, and each was surrounded with up to 10 drops of 5 μl HMEM (for maintaining individual oocytes). The lid was flooded with mineral oil and maintained at 38°C on a slide warmer. A single denuded oocyte was placed into each 5-μl HMEM drop and the lid was transferred to the heated (38°C) stage of a Leica DM-IL Inverted Microscope (Meyer Instruments, Inc., Houston, TX) equipped with Leitz Micromanipulators (W-Nuhsbaum, Inc., McHenry, IL) and microtools. Holding pipettes had

TABLE 1. Comparative data for the sperm chromatin structure assay (SCSA) from unprocessed and swim-up-processed spermatozoa from normospermic and teratospermic cats.*

Comparative data (±SEM)	Unprocessed semen (n = 16 ejaculates)		Swim-up-processed semen (n = 6 ejaculates)	
	Normospermic	Teratospermic	Normospermic	Teratospermic
Mean DFI	223.1 ± 4.4[a]	294.7 ± 5.6[b]	220.9 ± 5.4[a]	284.6 ± 5.4[b]
% DFI	13.8 ± 2.4[a]	48.5 ± 6.0[b]	7.7 ± 1.3[a]	21.7 ± 1.5[b]
SD DFI	69.6 ± 2.9[a]	121.5 ± 4.5[b,A]	57.8 ± 9.0[a]	148.3 ± 9.0[b,B]

* Row means with different lowercase superscripts differ ($P < 0.001$). Column values with different uppercase superscripts differ ($P < 0.05$).

an internal diameter of 10–11 μm and an outside diameter of 25–28 μm and were made using a Kopf 700-D Vertical Pipette Puller (David Kopf Instruments, Tujunga, CA) and a de-Fonbrune Microforge (Bunton Instrument Co., Rockville, MD). Each injection pipette had an external diameter of 6–7 μm and an internal diameter of 4–5 μm with a beveled end (Humagen Fertility Diagnostics, Inc., Charlottesville, VA). The holding pipette was attached to a manometer syringe using silicon tubing filled with mineral oil. The injection pipette was attached to the micromanipulator and silicon tubing was connected to a disposable plastic mouthpiece to form a mouth pipette. The tubing did not contain mineral oil, but did allow very fine control of the injection pipette and facilitated insertion of the spermatozoa. A morphologically normal, motile spermatozoon was selected from a sperm drop (from a normo- or teratospermic donor), immobilized by drawing the injection pipette across the midpiece, and aspirated into the injection pipette, flagellum first. Using the holding pipette, each oocyte was held and the injection pipette was inserted into the middle of the oocyte. Ooplasm was aspirated into the injection pipette until it was observed that the oolemma had been broken (the ooplasm moved relatively slowly into the pipette with some resistance until the oolemma ruptured and the ooplasm rushed into the pipette). The spermatozoon was injected into the oocyte, and positive pressure was maintained on the injection pipette as it was withdrawn from the oocyte. Order of injection alternated with spermatozoa from normo- versus teratospermic males to eliminate a time effect. Ten percent of oocytes were sham injected (with no spermatozoon) to control for activation due to the injection procedure. Oocytes that appeared lysed by the injection procedure (<5%) were excluded from the study. All others were considered successfully injected and were transferred to Ham F10 containing 5% FCS and cultured (as for IVF oocytes; 38°C in 5% CO_2 in air). Fertilization was assessed 32 h later as cleavage to the two- to four-cell stage, and developing embryos were permitted to grow in vitro as for IVF counterparts. Unfertilized oocytes were stained to confirm correct placement of spermatozoa within the ooplasm and were identified as having an uncondensed or partially condensed spermatozoa within the ooplasm (data not shown). No uncleaved oocytes revealed the presence of the two pronuclei within the ooplasm.

Statistical Analyses

Mean ± SEM were calculated. FCM data were analyzed using one-way analysis of variance (ANOVA) and a Tukey test for all pairwise multiple comparisons [33]. Data not normally distributed were analyzed by Kruskal-Wallis ANOVA on ranks [33] and Dunn method for all pairwise comparisons [33]. Differences in sperm characteristics and fertilization and embryo development in vitro to the morula stage between normo- and teratospermic donors and within the IVF versus ICSI groups were assessed by a Student *t*-test (after testing the data for normality and equal variance) or by the Mann-Whitney rank sum test (for data that were not normally distributed [33]).

RESULTS

Semen Collection, Processing, and Evaluations

No differences (± SEM; $P > 0.05$) in average sperm motility (63.1% ± 9.6% versus 52.5% ± 4.9%), total spermatozoa ($\times 10^6$) per ejaculate (19.8 ± 4.2 versus 20.5 ± 5.2), or percent intact acrosomes (86.9% ± 0.3% versus 79.5% ± 1.4%) were observed between normo- and teratospermic samples, respectively. Unprocessed normospermic ejaculates averaged 68.5% ± 5.5% structurally normal spermatozoa compared with 21.3% ± 1.5% for teratospermic counterparts ($P < 0.001$), confirming normospermic and teratospermic status, respectively. Overall, the proportions of pleiomorphisms in the domestic cat were consistent with those reported in previous publications [4–8].

SCSA Measurements

Spermatozoa from teratospermic cats had more ($P < 0.05$) abnormal chromatin (increased mean, SD, and % DFI) than their normospermic counterparts (Fig. 1, Table 1). This occurred within both unprocessed and swim-up-processed ejaculates. Swim-up processing did not alter SD and % DFI ($P > 0.05$) in normospermic ejaculates (Table 1). Although swim-up processing decreased the % DFI in teratospermic counterparts (unprocessed = 48%, swim-up ≤ 21%; $P > 0.05$), the SD DFI increased ($P < 0.05$) after swim-up, suggesting that the swim-up procedure caused an increase in DNA fragmentation in some cells, possibly from oxidative stress.

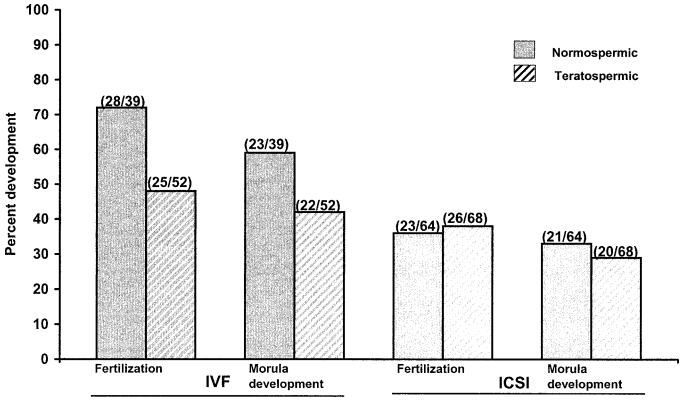

FIG. 2. Development of oocytes fertilized by IVF or ICSI with spermatozoa from normospermic or teratospermic cats.

IVF and ICSI of In Vivo-Matured Oocytes

For the IVF control, no (0%) oocytes cultured in the absence of spermatozoa underwent parthenogenetic cleavage. Of 13 sham-injected oocytes, one produced a two-cell stage structure with unevenly sized blastomeres.

Inseminating oocytes in vitro with structurally normal spermatozoa from normospermic ejaculates resulted in a higher ($P < 0.05$) fertilization percentage (71.8%; 28/39 oocytes) than when spermatozoa from teratospermic donors were used (48.1%; 25/52; Fig. 2). However, regardless of source of sperm, once fertilization occurred, a similar ($P > 0.05$) percentage of embryos developed into morulae in vitro (82%, 23/28 and 88%, 22/25, respectively; Fig. 2). Conventional IVF was superior ($P < 0.05$) to ICSI in producing fertilized oocytes using spermatozoa from normospermic males (Fig. 2), but comparable proportions of oocytes were fertilized following IVF and ICSI with teratospermic ejaculate ($P > 0.05$). Similar proportions of IVF- and ICSI-fertilized oocytes from normospermic ejaculates developed to the morula stage (23/28 = 82% and 21/23 = 91%, respectively; $P < 0.05$) and likewise, similar numbers of fertilized oocytes developed to the morula stage for oocytes fertilized by ICSI (77%) and IVF (88%) using spermatozoa from teratospermic ejaculates.

DISCUSSION

This study revealed two important findings. Using conventional SCSA technology, we found increased DNA fragmentation in spermatozoa from teratospermic donors compared with normospermic counterparts. This variance in integrity was significant because it occurred in spermatozoa that overtly appeared normal on the basis of structural morphology. Second, although there were abnormalities in normally shaped spermatozoa of teratospermic males, there was no evidence that this anomaly adversely affected fertilization or very early embryo development if the ZP was bypassed by assisted sperm injection. However, it is important to note that the morulae stage is a very early stage (5 days), when perhaps very few paternal genes may be called to action. Another study [34] has shown that sperm

with defective DNA fertilize as efficiently as normal sperm but that later embryo development is seriously compromised.

Morphologically normal-appearing spermatozoa from teratospermic cats have been studied extensively. It is clear that these cells have functional deficits, including a compromised ability to undergo capacitation and the acrosome reaction [10], an increased sensitivity to cold-induced acrosomal damage [7], and an increased vulnerability to hypotonic stress [7]. Thus, to date, most investigations have identified the integrity of the sperm membrane as the primary source of difference between a normospermic and teratospermic donor. The current study demonstrated that there also was variance in the nuclear component of the gamete, even in morphologically normal spermatozoa from teratospermic donors. Furthermore, although swim-up processing has been found to have significant value for enriching morphologically normal spermatozoa from teratospermic donors [7, 8], the present study clearly demonstrated that these morphologically normal spermatozoa have a significant degree of DNA fragmentation.

Sperm chromatin normally is highly condensed and highly organized [35, 36]. To achieve this complex packaging, nuclear histones are replaced by sperm-specific protamines during spermiogenesis [35, 36], and disulfide cross linkages between protamines are associated with the packaging of DNA into maximally condensed chromatin [37, 38]. Sperm chromatin is fully condensed at spermiation, but disulfide bonds are formed progressively during transit through the epididymis [38]. Disulfide bond formation is not associated with changes in sperm nuclear shape, but there is a relationship with the mechanical and chemical stability of the spermatozoon [39]. Bedford and Calvin [40] have suggested that a high degree of nuclear condensation is required for penetration of the ZP. Figure 1 shows, however, that increased DNA staining is not a feature of teratosperm that would occur if the chromatin were not fully condensed [41]. Howard et al. [6] have demonstrated that both malformed and normal-appearing spermatozoa from teratospermic cats fail to penetrate beyond the inner layer of the homologous oocyte's inner ZP layer. A similar situation may be present in the case of teratospermic human males. In this case, these spermatozoa also experience poor fertilizing capacity related to an inability to undergo the acrosome reaction and to bind to the ZP [41, 42].

High proportions of all oocytes inseminated with spermatozoa from normospermic donors fertilized in vitro, most of which developed to morulae. Direct sperm injection into the same quality oocytes was not nearly as efficient as conventional IVF. Visualization of the short-lived first polar body in mature cat oocytes was rare. This precluded the accepted method of injecting oocytes with the first polar body at 90 degrees to the injection pipette to avoid disrupting chromosomes aligned on the metaphase plate [43]. This may have resulted in the overall lower fertilization success with ICSI compared with IVF. Thus, the lower proportion of oocytes fertilized after ICSI using spermatozoa from normospermic and teratospermic donors compared with IVF oocytes was not unexpected. If the ICSI technique was partially compromised by a disrupted metaphase plate, then a byproduct of bypassing the ZP by direct sperm injection allowed comparable fertilization success between the two cat populations.

Although ICSI was less effective than a standard IVF protocol for both the normospermic and teratospermic domestic cat, the value of the technique in future studies should not be ignored. Felids not only tend to produce high proportions of pleiomorphic spermatozoa, but virtually all felid species produce low sperm-density ejaculates (in the low millions rather than high millions or billions of spermatozoa with many mammalian species [4]). Thus, in some cases, a male felid may produce too few viable spermatozoa for conventional IVF [7]. The ability to produce felid offspring has been demonstrated by Gomez et al. [44] in the production of domestic cat kittens following ICSI into in vitro-matured oocytes, embryo culture, and transfer into a surrogate domestic cat dam. Thus, ICSI might be a valuable alternative tool to IVF in cases where genetic management dictates the need for propagating a male felid with combined oligo/teratospermia. During the course of the present study, we had the opportunity to recover five oocytes 24 h postmortem from a mature female cheetah (*Acinonyx jubatus*). Using standard in vitro maturation procedures [45] followed by processing of thawed cheetah spermatozoa and ICSI into five oocytes (using procedures described in this article), one embryo cleaved and formed a five-cell embryo. Jaguarundi (*Herpailurus yaguarondi*) embryos also have been generated following ICSI in an independent laboratory [25].

In conclusion, there are some anomalous characteristics of the chromatin of normally shaped spermatozoa from the teratospermic domestic cat. It is of special interest that normal morphological sperm from teratospermic cats can have >20% of sperm with fragmented DNA. The full significance of this increased chromatin heterogeneity remains unclear, although injecting these spermatozoa directly into the cat oocyte results in very early embryo formation and development similar to that using sperm from normospermic donors; however, the extent of paternal genome activity by the morulae stage may be critical. Further experiments are needed to determine implantation rates and pregnancy outcome. Because it is well known that both malformed and even normal-appearing spermatozoa from teratospermic donors fail to penetrate the homologous cat ZP, we speculate the abnormal chromatin contributes to this functional defect. Due to the commonality of teratospermia in both felids and the human, the cat ICSI model may be useful for understanding the mechanism of male-factor infertility as well as exploring options for producing embryos from rare felids that usually produce mostly malformed and/or few total spermatozoa per ejaculate.

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