

## Osmotic Effects on Feline Spermatozoa from Normospermic versus Teratospermic Donors

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Effects of osmolality stresses on the sperm of normospermic (>60% normal sperm/ejaculate) versus teratospermic (<40% normal sperm) domestic cats and the normospermic leopard cat and the teratospermic clouded leopard were studied. Spermatozoa were exposed to various anisotonic solutions in a single step or returned to near isotonic conditions in a single step after exposure to anisotonic solutions. The percentage of sperm motility was measured subjectively, and dual fluorescent stains were used to assess membrane integrity by flow cytometry. The percentage of sperm motility declined ( $P < 0.05$ ) in domestic cat sperm exposed to osmolalities <200 and >450 mOsm. Spermatozoa from all felines underwent marked ( $P < 0.05$ ) membrane disruption following a hypotonic stress, but sperm from teratospermic donors experienced greater ( $P < 0.05$ ) membrane disruption in response to decreased osmolality. While feline spermatozoa appeared to be highly resistant to hypertonic (600, 1200, and 2400 mOsm) conditions, with >85% of the cells maintaining intact membranes, severe membrane disruption occurred when cells were returned to isotonicity in a single step. There was no difference ( $P > 0.05$ ) between a 1- and 5-min exposure to various anisotonic solutions. Similarly, sperm from normospermic and teratospermic domestic cats responded identically after exposure to ionic or nonionic solute. Results demonstrate that: (1) spermatozoa from teratospermic males are more vulnerable to a hypotonic stress than sperm from normospermic counterparts; (2) in response to small deviations in osmolality, feline sperm experience a more rapid decline in motility than membrane integrity; and (3) an abrupt return to isotonicity after a hypertonic stress causes extensive sperm membrane damage regardless of ejaculate quality. © 2000 Academic Press

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Gamete cryopreservation can be used to insure, preserve, and distribute valuable genetic traits. One of this laboratory's long-term goals is the development of artificial insemination (AI) with cryopreserved sperm as a tool for managing rare (endangered) feline species and domestic cat models for human diseases (24, 35). Organized banking of germplasm from such species and genotypes could facilitate exchange of genetic material between geographically disparate populations and overcome com-

monly encountered sexual incompatibility problems. Although pregnancies have been produced in several feline species such as the domestic cat, leopard cat (*Prionailurus bengalensis*), ocelot (*Leopardus pardalis*), and cheetah (*Acinonyx jubatus*) after AI with cryopreserved sperm, there remain serious inadequacies in the present methods of sperm cryopreservation (1, 11, 13, 32, 38). Clearly, current protocols for sperm cryopreservation are inadequate, and improved methods are needed to enhance cryosurvival of feline spermatozoa.

Factors to be considered in developing an optimum cryopreservation protocol include: (1) cryodiluent composition; (2) type of permeating cryoprotectant agent (CPA); (3) final CPA concentration; (4) rate of addition of CPA; (5) rate of cooling to freezing temperatures; and (6) rate of CPA removal after thawing (4). Optimizing these factors together enhances the chance that

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sperm can survive a cryostress. Current methods to cryopreserve feline sperm were developed empirically, rather than by measuring biophysical properties of the specific gametes to be cryopreserved. Although such an empirical approach eventually may yield success, an increasing number of reports focus on understanding the cryobiological properties of cells to be cryopreserved (2, 3, 9, 10, 21, 22). This strategy relies on developing a database first on crucial information, ranging from the membrane permeability to ionic impacts on the ability of cells to survive cryopreservation.

Sperm cryopreservation involves equilibration of cells in relatively high concentrations of CPA that minimize ice crystal formation within the cell (5). The CPAs that are most commonly used are glycerol and dimethyl sulfoxide, with ethylene glycol and propylene glycol used less often. During a typical cryopreservation protocol, spermatozoa are exposed to solutions that are hypertonic with respect to the permeating CPA and isotonic with respect to impermeable salts. In response to such osmotic challenges, the cell initially shrinks due to osmotic efflux of intracellular water and then swells and returns to its near isotonic volume as the CPA and water enter (5). After thawing, it is necessary to remove the CPA from the sperm before it is used for AI or *in vitro* fertilization. Under such circumstances, cells with high intracellular CPA concentrations are exposed to near isotonic solutions resulting in a rapid increase in cellular volume due to osmotic influx of extracellular water. As a function of time, the intracellular CPA diffuses out and water follows to achieve near isotonic volume. Thus, the osmotic stress arising from both the freeze-thaw process and the CPA imposes extreme osmotic stress that is potentially injurious or lethal to sperm survival or function.

To better understand the cryobiological properties of cat gametes, we have initiated a series of studies, including examining the sensitivity of sperm to cold shock and osmotic challenges. Recently, we demonstrated that domestic cat spermatozoa are easily damaged when exposed to a fast rate of cooling (4°C/min) to tempera-

tures above 0°C (28). Sperm from the teratospermic males (<40% morphologically normal sperm/ejaculate) experience more disruption than sperm from normospermic (>60% morphologically normal sperm) males. We further demonstrated that decreasing the cooling rate to 0.5°C/min or slower minimizes the structural damage to spermatozoa from both cat populations.

In this report, we take the next step in understanding the biophysical properties of cat sperm by examining the osmotic tolerance limits of these cells to swelling and shrinking in hypotonic and hypertonic solutions, respectively. Studies were designed to simulate the osmotic environment of sperm from the normospermic domestic cat and leopard cat and the teratospermic domestic cat and clouded leopard (*Neofelis nebulosa*) during freezing and thawing. This was accomplished in a series of experiments in which sperm were exposed abruptly (in a single step) to various anisotonic solutions or allowed to equilibrate to various anisotonic solutions and then abruptly returned to isotonic conditions in a single step. Thus, the specific aim was to assess the impact of hypo- and hypertonic stress as well as ionic (sodium chloride; NaCl) versus nonionic (sucrose) nonpermeating solutes on sperm motility and membrane integrity. Maximal information was generated by evaluating these variables across domestic cat populations and feline species that routinely ejaculate different proportions of structurally normal spermatozoa.

## MATERIAL AND METHODS

### *Animals*

Adult male domestic cats (normospermic,  $n = 3$ ; teratospermic,  $n = 3$ ), leopard cats ( $n = 4$ ), and clouded leopards ( $n = 4$ ) were housed at one of three institutions: the National Zoological Park (Washington, DC, U.S.A.), the Conservation & Research Center (Front Royal, VA, U.S.A.), or the National Institutes of Health Animal Center (Poolesville, MD, U.S.A.). Each male was designated as normospermic or teratospermic on the basis of at least five semen evaluations during the previous 6 months. Do-

mestic cat males were maintained under 12 h per day artificial lighting in individual cages and provided dry cat food (Purina Cat Chow; Ralston-Purina Co., St. Louis, MO, U.S.A.) and water ad libitum. Leopard cats and clouded leopards were exposed to natural lighting with variable periods of artificial illumination. Animals were fed a commercial, nondomestic feline diet (Nebraska Brand Feline Diet, North Platte, NE, U.S.A.) daily with occasional whole prey (rabbit or chicken) supplementation and water ad libitum. All investigations were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

#### *Semen Collection, Analysis, and Processing*

Domestic cat and leopard cat males were anesthetized with an intramuscular injection of tiletamine hydrochloride-zolazepam (Telazol; 4.5 mg/kg, A.H. Robins, Richmond, VA, U.S.A.), and clouded leopard males were anesthetized with ketamine hydrochloride (Ketaset; 11.0 mg/kg, Bristol Laboratories, Syracuse, NY, U.S.A.) (12, 14). Electroejaculation was conducted following a standard protocol using a 60-Hz sine wave stimulator (P.T. Electronics, Boring, OR, U.S.A.) and a rectal probe with three longitudinal electrodes (P.T. Electronics) (14). The diameter of the rectal probe used in the domestic cat, leopard cat, and clouded leopard was 0.6, 0.6, and 1.6 cm, respectively. Eighty stimulations at 2–6 V were given over a 30-min interval. Ejaculates (3 ejaculates/domestic cat male and 1 ejaculate/leopard cat and clouded leopard male) were immediately examined for volume, sperm concentration, sperm percentage of motility, and forward progressive motility (scale, 0 to 5; 5 = best) before processing (14). Ejaculates from each male were maintained separately and utilized in the various experiments. Ejaculates from the domestic cat and leopard cat were immediately diluted 1:1 in Hepes buffered Ham's F-10 medium (Gibco-BRL, Gaithersburg, MD, U.S.A.) containing 5% fetal calf serum (FCS) (HyClone Laboratories, Logan, UT, U.S.A.) (osmolality,  $300 \pm 5$  mOsm). Clouded leopard ejaculates were processed as above except that FCS was replaced

with heat-inactivated 5% clouded leopard serum (12). All samples were centrifuged (300g, 8 min), the supernatant was discarded, and sperm pellets were resuspended in 0.6–0.7 ml of the corresponding medium and maintained at room temperature until further analysis. Ejaculates from all males contained 70–85% motile sperm with >85% viability. A 10- $\mu$ l sample of the raw semen was fixed in 0.3% glutaraldehyde for morphological examination (200 sperm/aliquot) by phase-contrast microscopy (1000 $\times$ ) (14).

#### *Flow Cytometric Analysis of Plasma Membrane Integrity*

Plasma membrane integrity was assessed using the dual fluorescence staining described by Garner *et al.* (8). Spermatozoa were stained using the sperm viability kit (Molecular Probes, Eugene, OR, U.S.A.) by adding 0.27  $\mu$ l SYBR-14 (0.01 mg/ml) and 2  $\mu$ l propidium iodide (PI; 2 mg/ml) to each 500- $\mu$ l cell suspension. The percentage of cells exhibiting SYBR-14 (green) and PI (red) fluorescence was measured for each treatment. Data were obtained using an Epics Profile II (Coulter Corp., Inc., Hialeah, FL, U.S.A.) flow cytometer (8). A minimum of  $1 \times 10^4$  spermatozoa/treatment were counted within 30 min of staining using an air-cooled argon laser operated at 488 nm and 15 mW. All samples were analyzed at room temperature (22–25°C).

#### *Single Exposure to Anisotonic Conditions*

All hypotonic solutions were prepared by diluting  $1 \times$  Hepes-buffered Ham's F-10 with reagent grade water, whereas hypertonic solutions were prepared by adding 5 M NaCl or 5 M sucrose stock solutions to Ham's F-10. Osmolalities of all solutions were determined using a vapor pressure osmometer (Wescor, Inc. Logan, UT, U.S.A.) that was calibrated daily against 100, 290, and 1000 mOsm NaCl standards (Wescor, Inc.) for accuracy within  $\pm 5$  mOsm.

To determine the effects of osmotic stress on motility, domestic cat sperm aliquands were pelleted by centrifugation (300g; 8 min) and resuspended in 100  $\mu$ l of the respective test solutions (hypotonic: 0, 35, 75, and 150 mOsm;

hypertonic: 450, 600, 1200, and 2400 mOsm). After a 10-min equilibration at room temperature, samples were assessed for sperm percentage of motility and forward progressive status.

In experiments assessing the effect of a single exposure of spermatozoa to anisotonic solutions, sperm aliquands (10  $\mu$ l) were added in one step to 500  $\mu$ l of the respective test solution and incubated at room temperature for 10 min. At the end of incubation, sperm were stained with SYBR-14 and PI (as described above) and evaluated for membrane integrity using flow cytometry. The critical tonicity, the hypotonic concentration that results in 50% cell lysis, was determined from these data by extrapolation.

#### *Exposure to Anisotonic Conditions and Return to Isotonicity*

After washing, sperm aliquands (10  $\mu$ l) were pipetted into 200  $\mu$ l of each anisotonic solution and thoroughly mixed. After a 1- or 5-min incubation at room temperature, spermatozoa in anisotonic Ham's F-10 solutions of 0, 35, 75, 150, 600, 1200, and 2400 mOsm were returned to isotonicity (300  $\pm$  10 mOsm) by a single-step addition of 200  $\mu$ l of 600, 565, 525, and 450 mOsm of Ham's F-10 or 200, 600, and 1400  $\mu$ l of reagent grade water, respectively. Isotonic (300 mOsm) controls were run in parallel for each donor on the day of analysis. All samples were stained with SYBR-14 and PI for assessment of membrane integrity by flow cytometry, as described above.

#### *Effect of NaCl versus Sucrose on Domestic Cat Sperm Membrane Integrity*

Anisotonic solutions (600, 1200, and 2400 mOsm) were prepared by adding NaCl or sucrose to Ham's F-10 medium. A 10- $\mu$ l aliquant of washed spermatozoa was added to 500  $\mu$ l of each hypertonic solution and equilibrated at room temperature for 10 min before staining for membrane integrity. The effect of resuspending spermatozoa in solutions that were approximately isotonic after they had first been exposed to hypertonic solutions was determined by adding 10- $\mu$ l aliquants of washed spermatozoa to 200  $\mu$ l of each test solution (600, 1200, and

2400 mOsm). The samples were allowed to equilibrate for 1 or 5 min, and then respective volumes of 200, 600, and 1400  $\mu$ l of reagent grade water were added to them. After a 10-min incubation, sperm were stained with SYBR-14 and PI and analyzed by flow cytometry, as described above.

#### *Statistical Analysis*

Data were subjected to arcsine transformation and normalized to isotonic values. Data on critical tonicity and return to isotonicity following exposure to various anisotonic solutions were analyzed using standard analysis of variance procedures. Mean comparisons were conducted using the least significant difference multiple range test (30). All analyses were performed using SAS procedures. Means are expressed as mean  $\pm$  SEM.

## RESULTS

#### *Ejaculate Traits and Sperm Morphology*

Ejaculates from normospermic domestic cats contained >60% morphologically normal spermatozoa compared to <40% morphologically normal sperm in teratospermic ejaculates (Table 1). Ejaculates from leopard cats also contained >60% normal sperm compared to <25% normal sperm in clouded leopard ejaculates. Most abnormalities in teratospermic domestic cat ejaculates were midpiece and flagellar defects compared to acrosomal and flagellar defects in the clouded leopard.

#### *Osmotic Stress on Domestic Cat Sperm Motility*

The motility of domestic cat spermatozoa exposed to either hypertonic or hypotonic solutions decreased rather sharply (Fig. 1). No differences ( $P > 0.05$ ) were observed between normospermic and teratospermic donors. However, decline in sperm motility was more pronounced after a hypotonic compared to a hypertonic stress. Forward progressive motility was maintained only within a narrow range of osmolality (range, 150--450 mOsm; data not shown) in which motility was observed. At other osmolalities close to this range, the pri-

TABLE 1  
Seminal Traits and Incidence of Morphologically Normal and Abnormal Sperm in Felines<sup>a</sup>

	Domestic cat			
	Normospermic (n = 9 ejaculates)	Teratospermic (n = 9 ejaculates)	Leopard cat (n = 4 ejaculates)	Clouded leopard (n = 4 ejaculates)
Ejaculate volume (ml)	0.2 ± 0.2	0.2 ± 0.1	0.5 ± 0.1	1.5 ± 0.8
Sperm concentration (×10 <sup>6</sup> /ml)	172.0 ± 13.2	196.0 ± 15.3	53.0 ± 5.8	58.3 ± 9.3
Sperm motility (%)	87.5 ± 3.2	83.6 ± 5.1	77.5 ± 2.1	74.3 ± 2.2
Morphologically normal sperm (%)	75.2 ± 3.2	36.1 ± 3.5	76.4 ± 2.5	15.3 ± 2.3
Morphologically abnormal sperm (%)				
Head defects	0.2 ± 0.1	1.6 ± 0.2	2.4 ± 0.6	42.5 ± 3.6
Midpiece defects	11.3 ± 3.2	28.6 ± 2.4	13.9 ± 2.3	19.5 ± 4.1
Flagellum defects	13.3 ± 2.1	33.7 ± 3.1	7.3 ± 1.5	22.7 ± 3.5

<sup>a</sup> Values represent means ± SEM.

mary pattern was either flagellar motility alone and/or lateral head displacement. Spermatozoa exposed to osmolalities below 35 mOsm and above 1200 mOsm were completely immotile.

#### Single Exposure to Hypotonic and Hypertonic Stress

Spermatozoa from all of the males exhibited a marked decrease in membrane integrity when they were exposed to hypotonic solutions. However, significantly higher percentages ( $P < 0.05$ ) of spermatozoa from the normospermic domestic cats and leopard cats had intact membranes compared to those of the teratospermic

domestic cats and clouded leopards (Figs. 2a and 2b). In contrast, feline spermatozoa appeared to be highly resistant to hypertonic stress with >85% of the cells maintaining intact membranes after a single exposure (Figs. 2a and 2b). There were no differences ( $P > 0.05$ ) between the two domestic cat populations or the leopard cat and clouded leopard in sensitivity to hypertonic stress.

Calculated critical tonicities (osmolality that produced 50% cell lysis) were similar for the normospermic domestic cat and leopard cat (<60 mOsm; Table 2). In contrast, the teratospermic domestic cat and clouded leopard were

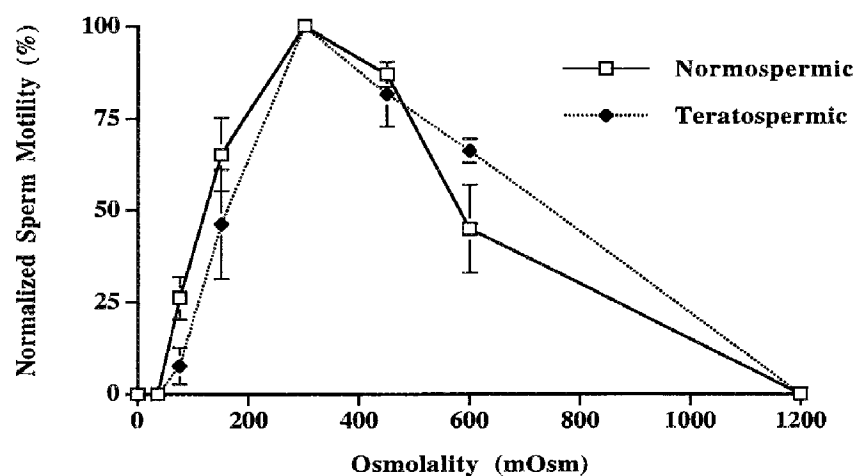
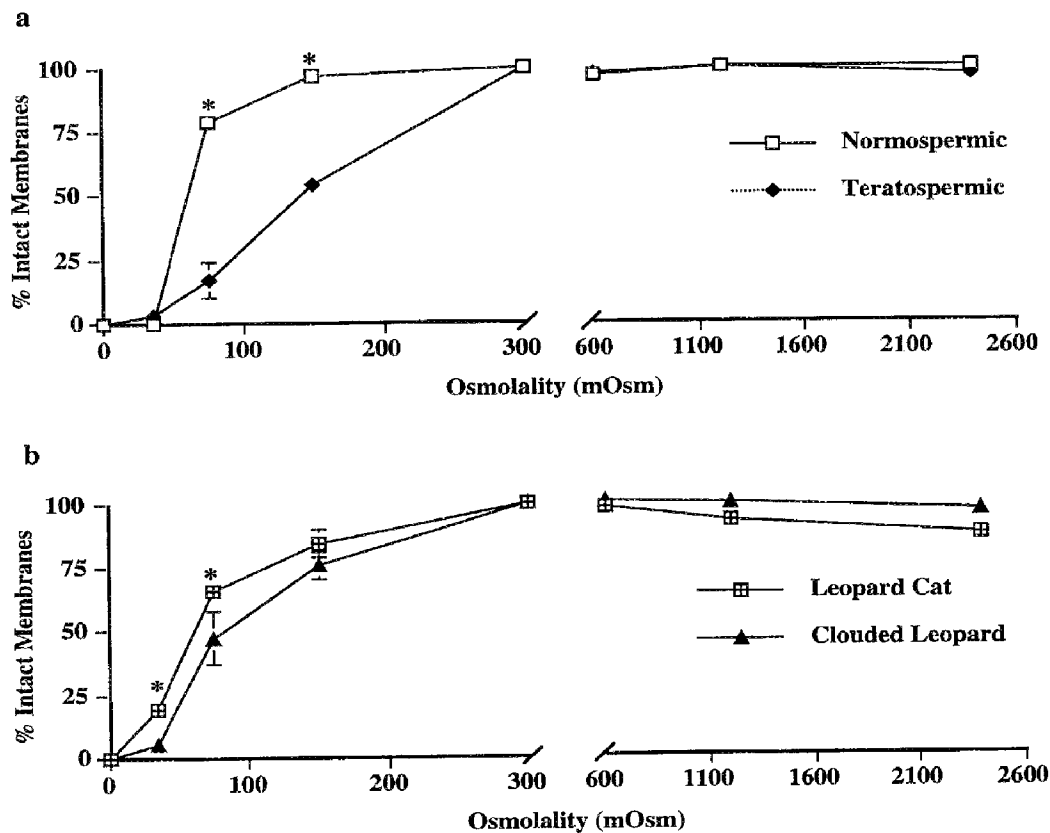


FIG. 1. Effect of a single-step exposure to various anisotonic solutions on sperm motility in normospermic versus teratospermic domestic cats. Values are means ± SEM. Data are normalized to the percentage of motile cells at 300 mOsm.



**FIG. 2.** Effect of a single-step exposure to various anisotonic solutions on sperm plasma membrane integrity: (a) normospermic versus teratospermic domestic cats; and (b) leopard cats versus clouded leopards. Values are means  $\pm$  SEM. Data are normalized to the percentage of intact cells at 300 mOsm. An asterisk indicates differences ( $P < 0.05$ ) between populations of domestic cats (a) or leopard cats and clouded leopards (b) at corresponding osmolalities.

more sensitive to hypotonic stress and demonstrated cell lysis at higher osmolalities (142 and 82 mOsm, respectively; Table 2).

#### *Return to Isotonic Conditions after a Hypo- or Hypertonic Stress*

Spermatozoa from all felines experienced a rapid decline in membrane integrity when cells exposed to these anisotonic solutions were returned in a single step to isotonicity ( $300 \pm 10$  mOsm; Figs. 3a and 3b). Spermatozoa from normospermic domestic cats experienced extensive membrane damage ( $>50\%$ ) after exposure to 75 mOsm and lower, followed by rapid return to isotonic conditions (Fig. 3a). Leopard cat spermatozoa also showed high levels of membrane disruption upon returning the cells to isotonic conditions after exposure to 35 mOsm

solutions (Fig. 3b). Interestingly, no further membrane damage was observed in sperm from teratospermic donors, compared to the exten-

**TABLE 2**  
Critical Tonicities for Spermatozoa at Room Temperature

Species	Critical tonicity (mOsm)	Reference
Mouse	34	(20)
Rabbit	45.6	(3)
Bull	36	(16)
Stallion	47	(22)
Human	57	(23)
Normospermic domestic cat	58	
Teratospermic domestic cat	142	
Leopard cat	60	
Clouded leopard	82	

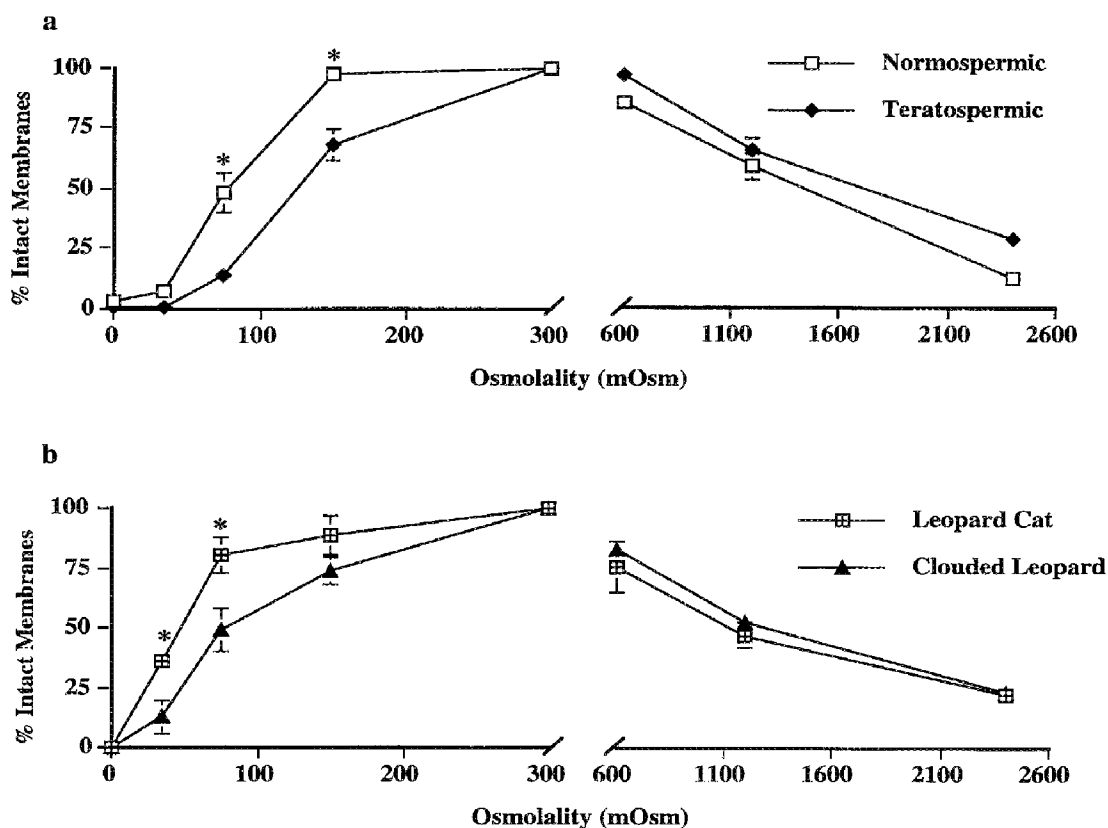


FIG. 3. Effect of abruptly returning spermatozoa to isotonicity in a single step after exposure to various anisotonic solutions on sperm plasma membrane integrity: (a) normospermic versus teratospermic domestic cats; and (b) leopard cats versus clouded leopards. Values are means  $\pm$  SEM. Data are normalized to the percentage of intact cells at 300 mOsm. An asterisk indicates differences ( $P < 0.05$ ) between populations of domestic cats (a) or leopard cats and clouded leopards (b) at corresponding osmolalities.

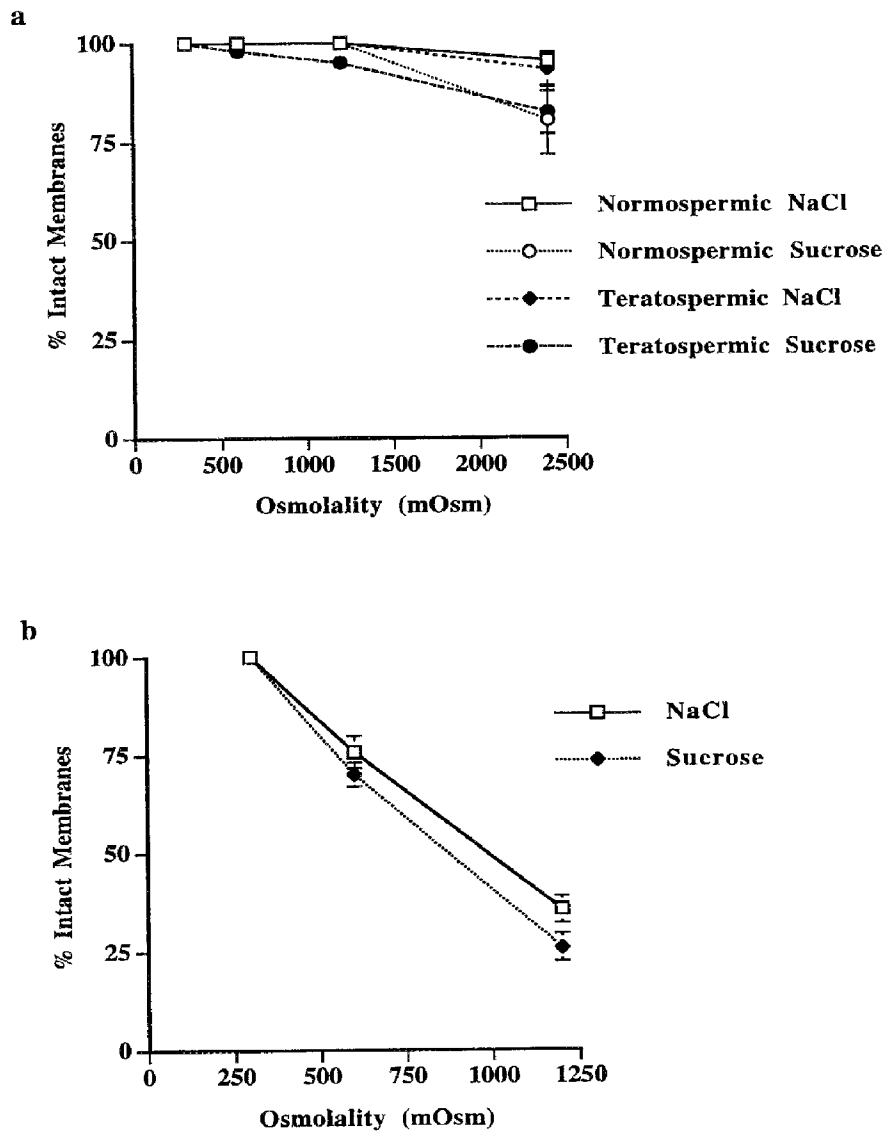
sive damage that resulted from the initial exposure to these anisotonic conditions.

Although spermatozoa from all felines maintained intact membranes after a single exposure to hypertonic conditions, returning the cells to isotonic conditions in a single step severely disrupted ( $P < 0.01$ ) membranes (Figs. 3a and 3b). Membrane integrity decline was most rapid for cells preexposed to osmolalities 600 mOsm and higher, and the magnitude of damage was greater for cells preexposed to higher osmolalities. There were no differences ( $P > 0.05$ ) in the proportion of intact membranes between normospermic versus teratospermic domestic cats, as well as between the leopard cat and clouded leopard donors. Furthermore, there were no differences ( $P > 0.05$ ) in membrane integrity when spermatozoa were returned to

isotonic conditions after 1- or 5-min intervals (data not shown).

#### *Ionic versus Nonionic Solutes on Domestic Cat Sperm Membrane Integrity*

A single-step addition of a sperm aliquot to hypertonic solutions of NaCl or sucrose maintained high proportions of intact membranes (Fig. 4a). There were no differences ( $P > 0.05$ ) between spermatozoa exposed to NaCl or sucrose both within and between domestic cat groups. When sperm exposed to NaCl or sucrose solutions were returned to isotonic conditions in a single step,  $>65\%$  of the sperm underwent membrane disruption at the highest osmolality tested (Fig. 4b). There were no differences ( $P > 0.05$ ) in normospermic versus



**FIG. 4.** Effect of a single exposure (a) and an abrupt return to isotonicity (b) after exposure to various anisotonic solutions of NaCl or sucrose on domestic cat spermatozoa. Values are means  $\pm$  SEM. Data are normalized to the percentage of intact cells at 300 mOsm. Data from both normospermic and teratospermic domestic cats were combined (b).

teratospermic domestic cats between the 1- and 5-min treatments (data not shown).

#### DISCUSSION

Conventional feline sperm cryopreservation involves adding glycerol at the 4% (v/v) level before cooling and removing glycerol in one step after thawing (26). It now is clear that these cells from diverse cat species and populations experience extensive structural damage when

exposed to such osmotic stress. Four important observations were made from the present study. First, susceptibility to hypotonic stress depended, in part, on sperm quality of the donor (i.e., teratospermic males were more vulnerable to gamete damage than normospermic counterparts). Second, feline sperm motility was more sensitive than membrane integrity to changes in osmolality. Third, although spermatozoa appeared to be resistant to hypertonic stress, a



return to isotonic solution caused extensive membrane damage. Finally, the changes observed were not due to the direct effects of the solute on sperm, but rather to osmotic stress, because sperm exposed to hypertonic NaCl or sucrose solutions experienced similar membrane damage.

This report represented a first among carnivores in clearly identifying differences in osmotic tolerance in spermatozoa from normo- versus teratospermic males. Our most interesting discovery was the biophysical differences in membranes between spermatozoa of normo- versus teratospermic donors. This finding was significant because it implied that sperm quality in terms of morphology and membrane integrity likely influences the conditions required for cryopreserving sperm from specific donor types. Thus, cryopreservation conditions for males typically ejaculating high proportions of morphologically abnormal spermatozoa perhaps may have to be significantly modified to account for their increased susceptibility to membrane damage. It is possible, however, that this phenomenon may extend beyond cats to include humans, another species in which high proportions of sperm pleiomorphisms are common (25, 33).

Similar to mouse (37), ram (2), boar (10), bovine (16), and human (2, 7) spermatozoa, cat sperm motility was more sensitive to changes in osmolality than membrane integrity. Sperm motility markedly declined in response to small deviations from isotonicity. Exposure to either a 150- or a 600-mOsm solution resulted in a 40% decline in motility, compared to a complete loss of motility at osmolalities below 35 mOsm and greater than 1200 mOsm. For all species investigated to date, exposure of spermatozoa to hypotonic solutions causes the cells to swell beyond their isotonic volumes, resulting in loss of sperm motility and ultimately cell lysis. Drevius and Eriksson (4) first reported that exposing rabbit, bull, or human spermatozoa to hypotonic conditions caused the swelling and looping of the sperm tail. Similar observations were reported in mouse sperm, wherein exposure to hypotonic conditions caused the sperm flagel-

lum and head to curl toward the midpiece, thereby impairing progressive motility (37). A hypertonic environment also reduces sperm motility, but by causing severe dehydration and extensive shrinkage (5). More study is needed to document the cellular modifications that occur in cat sperm after exposure to an osmotic stress.

Another interesting observation from the present study was that spermatozoa from normospermic donors maintained a high proportion of intact membranes through a series of hypotonic challenges only to experience a sharp decline at <75 mOsm. Similar findings have been reported in fowl (35) and human spermatozoa (2, 6). In contrast, spermatozoa from our teratospermic cat donors experienced a steep decline in intact membranes following exposure to hypotonic conditions similar to ram, bull, and boar spermatozoa (2, 10). These observations clearly highlight the value of our domestic cat model that suggests that differences in membrane sensitivity may exist even between genotypes within a species.

Interestingly, there also were population and species variations in critical tonicities at room temperature. Sperm membranes from teratospermic domestic cats were inherently more vulnerable to damage following a single-step exposure to hypotonic stress than normospermic counterparts. This finding is consistent with earlier studies from our laboratory demonstrating that even normally shaped sperm from teratospermic males are compromised in fertilization ability *in vitro* (15), onset of capacitation (17), and regulation of sperm protein phosphorylation (27). During a hypotonic stress, cells typically respond by swelling, a response that can result in lysis if a maximum volume is exceeded. Critical tonicities for the normospermic domestic cat and leopard cat spermatozoa are similar to that reported for human spermatozoa, but are higher than that for the mouse, rabbit, bull, and stallion (Table 2). However, the values for critical tonicity of spermatozoa from the teratospermic domestic cat and clouded leopard far exceeded the values for other cat species/populations or other mammals studied

to-date (Table 2). Surprisingly, there was a 2.4-fold magnitude increase between the teratospermic and normospermic domestic cat, which reinforced our previous assertions that gamete structure and function are markedly different in these two types of sperm donors (15, 17, 27).

When sperm are exposed to hypertonicity, the first response is to shrink from intracellular water loss (5), with extent of shrinkage depending on intracellular free water volume. Human sperm apparently have an innate resistance to hypertonic stress (2, 6). In contrast, ram spermatozoa express a resistance up to 1000 mOsm, but then experience membrane damage at osmolalities greater than 1000 mOsm (2). Likewise, little or no membrane damage is detectable when bull sperm are exposed to osmolalities up to 800 mOsm, with higher osmolalities causing extensive membrane damage (16). This contrasted with our present observations in felines, where sperm maintained high proportions of intact membranes even in solutions up to 2400 mOsm irrespective of species/domestic cat group. Although little is known about the physicochemical properties of feline sperm membranes, the extreme resistance to hypertonicity observed suggests that feline spermatozoa are remarkably different from species examined to date.

Gao *et al.* (6) reported that the plasma membrane of the human spermatozoon undergoes extensive reorganization during hypertonic shrinkage that places excessive demands on the cellular cytoskeleton, with most of the related damage being irreversible. Like mouse (29, 37), bovine (16), and human spermatozoa (2, 6), feline sperm appeared resistant to hypertonic stress, but experienced extensive loss in membrane integrity after return to isotonicity. This overall response is similar to posthypertonic hemolysis, first reported in human red cells (18, 39). There are at least two models proposed explaining this phenomenon. First, when cells are exposed to hypertonic solutions, it may cause a net leak/influx of nonpermeating solutes into the cells. This alteration is presumed to arise from the solute's ability to change the conformation and stability of the hydrophilic

portions of the plasma membrane. Subsequently, when cells are returned to isotonic solutions, the difference in the osmolality within the cell and the external environment triggers the cells to swell beyond their normal isotonic volume and lyse (19). Alternatively upon shrinkage, cells lose portions of membrane, reducing the effective area of the cell membrane, which (upon return to isotonic conditions) results in cell lysis before their normal volume is recovered (31). It remains unclear which of these mechanisms is responsible for the cellular damage observed in the present study. Interestingly, Songsasen and Leibo (29) recently examined the osmotic tolerance of spermatozoa from three strains of mice and demonstrated a genetic basis for its susceptibility. Our findings further confirm this relationship between genotype and susceptibility to osmotic stress.

To fully understand the cryobiological properties of cells, it is critical to: (1) assess the effect of permeating cryoprotectants (glycerol and dimethylsulfoxide) on sperm motility and membrane integrity; and (2) determine the plasma membrane permeability to water (hydraulic conductivity) and cryoprotectant, as well as the respective dependence of these variables upon temperature (5). Knowledge of these biophysical properties would allow calculating the number of steps, as well as the volumes of the diluent to be added, to minimize the effects of osmotic stress on cells to be cryopreserved. In the present study, similar to ram spermatozoa, feline sperm responded similarly to both ionic and nonionic solutes based on membrane integrity assessments (34). This contrasts to a recent report where human spermatozoa exposed to NaCl solutions were found to be more susceptible to membrane damage than those exposed to hypertonic sucrose solutions (6). Although future studies may allow a better understanding of the physiological basis for these differences, the present study clearly demonstrates the need to thoroughly examine the cryobiological properties of sperm from each species to be cryopreserved. It now seems logical to develop a cryopreservation protocol for normospermic cats and then either modify or de-

velop a different cryopreservation protocol for spermatozoa from teratospermic cats. As observed in the present study, a common approach across a range of feline species/populations can identify a host of mutually supportive findings to strengthen hypothesis testing. Studies are in progress examining additional characteristics of feline spermatozoa, including lipid and fatty acid composition of sperm membranes, effect of permeating cryoprotectants on sperm motility and membrane integrity, and permeability characteristics of water and cryoprotectants. This is the next logical step in developing a consistently effective sperm cryopreservation method for feline models.

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