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Stimulation of Cryopreserved Epididymal Spermatozoa of the Domestic Cat Using the Motility Stimulants Caffeine, Pentoxifylline, and 2'-Deoxyadenosine

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ABSTRACT: We have investigated the effects of caffeine, pentoxifylline, and 2'-deoxyadenosine on the motion characteristics and longevity of domestic cat spermatozoa. Freshly collected or cryopreserved domestic cat epididymal sperm were incubated with 0.01–20 mM caffeine, pentoxifylline, or 2'-deoxyadenosine for 15 minutes at 23°C. The percent motility (MOT), curvilinear velocity (VCL), linearity (LIN), straight line velocity (VSL), and amplitude of lateral head displacement (ALH) were determined for each group using computer-assisted semen analysis. Freshly collected domestic cat sperm exhibited a strong forward progressive movement, and treatment with caffeine, pentoxifylline, or 2'-deoxyadenosine did not consistently alter sperm motion. Following cryopreservation, spermatozoa exhibited decreased ($P < 0.05$) MOT, VCL, VSL, and ALH. Caffeine and pentoxifylline increased ($P < 0.05$) the MOT, VSL, VCL, and ALH of cryopreserved sperm at 0.01–20 mM, in a dose-dependent manner. 2'-Deoxyadenosine also increased ($P < 0.05$) both VSL and VCL at 1.0 mM, and MOT, VSL, VCL, and ALH at 10 mM. All treatments shifted the percentage of nonhyperactive sperm to either a transitional or hyperactivated state. The motility indices of cryopreserved samples were examined during a 6- hour incubation to assess the effects of caffeine, pentoxifylline, and 2'-deoxyadenosine on sperm longevity. Compared to untreated control samples, the longevity of stimulated cryopreserved sperm was not reduced. These results indicate that motility stimulants may prove useful for enhancing the fertility of cryopreserved cat sperm by increasing their motility and producing hyperactivated motion.

Key words: Domestic cat, motility stimulants, hyperactivation, computer-assisted semen analysis, spermatozoa.

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Domestic cats tend to be highly fertile, whereas many nondomestic felids are reproductively compromised (Wildt, 1990). Strategies employing artificial reproductive techniques have been developed to help circumvent the decreased fecundity of several endangered felid species (Wildt, 1990, 1991). However, a high incidence of teratozoospermia, low sperm motility, and decreased longevity of semen samples have, in part, contributed to poor *in vitro* fertilization (IVF) rates in nondomestic felids (Howard et al, 1984, 1990, 1991; Donoghue et al, 1992a,b). These characteristics are analogous to male-factor infertility in humans (Aitken et al, 1982b; Mahadevan et al, 1983; Mahadevan and Trounson, 1984; Cohen et al, 1985; Jeulin et al, 1986; Chan et al, 1989). Therefore, methods that improve sperm function in felids will be essential in attaining fertilization rates necessary for the production of offspring.

Many studies have shown computer-assisted semen analysis (CASA) to be valuable for monitoring sperm motion characteristics and understanding the relationship between sperm movement and fertilization ability. In humans, reduced motion characteristics in either fresh or cryopreserved specimens result in lower fertilization rates, compared to sperm exhibiting normal motion characteristics (Mahadevan and Trounson, 1984; Jeulin et al, 1986; Steinberger et al, 1980; Davis et al, 1991). In general, an elevation of percent motility (MOT), curvilinear velocity (VCL), or amplitude of lateral head displacement (ALH) has been correlated with increased sperm penetration of human oocytes. Sperm hyperactivation, characterized by a rapid, nondirectional trajectory (Burkman, 1984), is associated with mammalian capacitation and is thought to facilitate fertilization (Robertson et al, 1988). Hyperactivation has been shown to result in elevated values for the CASA variables MOT, VCL, and ALH (Burkman, 1984; Robertson et al, 1988; Yanagimachi, 1988; Mortimer and Mortimer, 1990).

Cyclic adenosine 3',5'-monophosphate (cAMP) is an important cellular second messenger involved in various aspects of sperm function including capacitation and fertilization (Hoskins and Casillas, 1975; Fraser, 1979, 1981; Garbers and Kopf, 1980; Tash and Means, 1983; Pariset et al, 1983, 1985; White and Aitken, 1989). In a recent study, Fraser and Monks (1990) induced sperm capaci-

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tation by elevating cAMP levels with adenosine analogues and observed an associated hyperactivation of sperm motility. Intracellular concentrations of cAMP have been elevated using 2'-deoxyadenosine, as well as the methylxanthines, caffeine and pentoxifylline, that inhibit phosphodiesterase activity. Using exogenous motility stimulants, numerous investigators (Garbers et al, 1971; Aitken, 1983; Barkay et al, 1984; Ruzich, 1987; Yovich et al, 1988; Hammitt et al, 1989; Imoedemhe et al, 1992) have enhanced the motion characteristics, respiration, longevity, and fertilization ability of human spermatozoa from both normospermic and teratospermic individuals. In addition to teratospermic samples, cryostored human sperm exhibits reduced motility that is reversible upon the addition of reagents like caffeine and pentoxifylline (Schill et al, 1979; Aitken, 1983). These studies have provided helpful strategies for alleviating male-factor infertility in certain individuals. If motility stimulants can elevate sperm motion characteristics of cat sperm without compromising longevity this strategy may also be useful with reproductive technologies employed in breeding endangered felids.

Sustaining optimal genetic diversity in endangered felids through the distribution of gametes may require assisted reproductive technologies using fresh, as well as cryopreserved, spermatozoa. Modern reproductive techniques, including artificial insemination (AI) and IVF, represent a plausible strategy to circumvent sexual incompatibility, eliminate the risks associated with animal transport, and provide a major avenue for using cryopreserved germ plasm to infuse genes from wild stocks into captive breeding populations. Because cryopreservation will play an important role in the storage of genetic material and its distribution, it is necessary to develop methods of optimizing the usage of cryopreserved gametes. Thus, the purpose of the present study was to examine methods for stimulating sperm motility and determine their utility with cryopreserved cat spermatozoa. In a previous study, we demonstrated the ability of CASA to track domestic cat sperm accurately (Stachecki et al, 1993). Using the domestic cat as a model for nondomestic felids, we have now determined the effects of caffeine, pentoxifylline, and 2'-deoxyadenosine on the motion characteristics, longevity, and hyperactivation of fresh and cryopreserved spermatozoa.

Materials and Methods

Sperm Collection, Processing, and Cryopreservation

Immediately following castration, testes from 8–36-month-old toms provided by local veterinary hospitals were placed in Eagle's media (Sigma Chemical Co., St. Louis, Missouri) supplemented with 25 mM Hepes and 4 mg/ml bovine serum albumin (BSA, Sigma, Fraction V), and maintained at 23°C until processing. Spermatozoa were collected as previously described (Stachecki et al, 1993). Briefly, epididymides were washed in Ham's F10 medium (Sigma) containing 4 mg/ml BSA, and spermatozoa were released into 2 ml of fresh medium through punctures made with a 30-gauge needle. Sperm were then concentrated by centrifugation (300 × *g*, 8 minutes) and resuspended in medium to a working concentration of 40–70 million sperm/ml.

For cryopreservation, an aliquot of each epididymal sperm sample was concentrated by centrifugation, diluted in Ham's F10 to a concentration of approximately 100 million sperm/ml, and then mixed 1:1 with Test Yolk Buffer containing 10% glycerol (Irvine Scientific, Santa Ana, California). The sperm solution was transfered to a 1.5-ml cryotube (Nunc Inc., Naperville, Illinois), held above liquid nitrogen vapors for 12 minutes, and then plunged into liquid nitrogen, where it remained until analysis. Samples were removed from liquid nitrogen after storage for 1–6 months, incubated for 5 minutes in a 37°C water bath, diluted 1:1 with Ham's F10, centrifuged, resuspended to a working concentration of 40–70 million sperm/ml, and kept at 23°C until analysis.

Motility Evaluation

For CASA, a Cell Track/s System (Motion Analysis Corp., Santa Rosa, California) was adjusted to track felid spermatozoa as indicated in our previous study (Stachecki et al, 1993). Briefly, a video digitizing rate of 60 frames per second was used to gather 30 frames of data for calculating kinematics and 5 frames of data for determining MOT. All examinations were performed at 23°C using an Olympus BH2 microscope (Olympus; New York, New York; 100×) with a positive phase-contrast objective. Microscopic images were relayed to the Cell Track/s system using a NEC (Woodale, Illinois) TI-23A CCD video camera and recorded on video tape using a JVC (Elmwood Park, New Jersey) HR-D940U video cassette recorder. A 5-μl aliquot of each sample was loaded into a 12-μm-deep MicroCell chamber (Conception Technologies, Inc., San Rafael, California) and the VCL (microns/second), straight line velocity (VSL; microns/second), linearity (LIN; 1-100%; VSL/VCL), ALH (microns), as well as MOT were determined for at least 200 motile sperm.

Effect of Motility Stimulants on Motion Characteristics

In order to analyze the effect of various motility stimulants on the motion characteristics of fresh sperm and to test our hypothesis that the motility of compromised cat sperm can be enhanced using motility stimulants, we treated cryopreserved sperm with caffeine, pentoxifylline, or 2'-deoxyadenosine. A 10-μl aliquot of a fresh or cryopreserved sperm sample was mixed 1:1 with Ham's F10 containing either 0, 0.02, 0.2, 2.0, 20, or 40 mM caffeine or pentoxifylline, or 0.02, 0.2, 2.0, or 20 mM 2'-deoxyadenosine. The samples were incubated for 15 minutes at 23°C and analyzed for short-term changes in motion characteristics (Hammitt et al, 1989). A total of nine epididymal samples were used in these studies, either fresh or following cryopreservation. All nine samples were used to compare motion

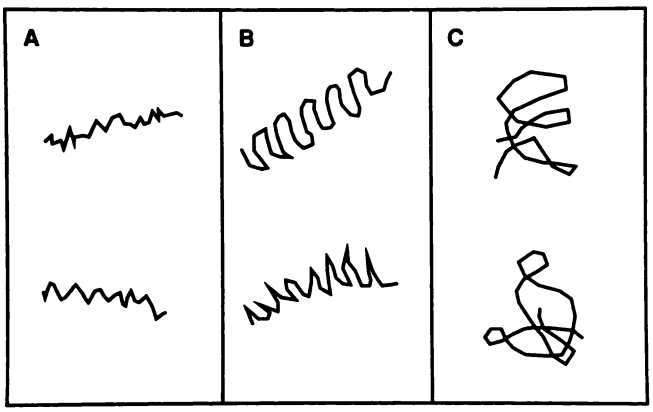

FIG. 1. Representative tracks from **(A)** nonhyperactivated, **(B)** transitional, and **(C)** hyperactivated domestic cat sperm. Sperm tracks were selected by visual inspection of CASA-generated sperm paths using the criteria outlined in the text. The trajectories represent the paths taken by the sperm heads during 0.5 second of tracking at 60 Hz.

characteristics of fresh and cryopreserved spermatozoa. Seven of the nine samples were used to study the effects of caffeine, pentoxifylline, or 2′-deoxyadenosine on freshly collected sperm. In three studies examining the stimulation of cryopreserved sperm, all nine samples were treated with pentoxifylline, eight of the nine samples were treated with caffeine, and eight of the nine samples were treated with 2′-deoxyadenosine. The initial analyses of fresh samples were completed within 3 hours of castration.

Effect of Motility Stimulants on Spermatozoa Longevity

A 20-μl aliquot of a cryopreserved (n = 10) sample was mixed 1:1 with Ham's F10 containing either 0, 0.02, 0.2, 2.0 mM caffeine, or 0.2, 2.0, 20 mM pentoxifylline or 2′-deoxyadenosine. Samples were incubated for 6 hours at 37°C. At hourly intervals, a 5-μl aliquot was placed on a microscope slide, covered with an 18-mm × 18-mm coverslip, and visually analyzed at 37°C. Upon visual analysis, the percent motility and speed of progression (SOP; based on the type of forward movement of the sperm cell: 1 = no movement, 2 = slow, nonprogressive motility, 3 = slow forward progression, or 4 = steady, rapid forward progression) were recorded (World Health Organization, 1992). These values were used to calculate a sperm motility index (SMI), used as an overall evaluation of sperm motility characteristics (Howard et al, 1990). SMI = [percent motility + (SOP × 25)] ÷ 2. The 10 specimens used for this study were different than those examined in the preceding experiments.

Effect of Motility Stimulants on Hyperactivation

Five of the nine samples used in the previous study comparing the motion characteristics of fresh and cryopreserved spermatozoa were analyzed for hyperactivation. Video recordings of cryopreserved sperm exposed to 1 mM caffeine, pentoxifylline, or 2′-deoxyadenosine for 15 minutes at 23°C were examined using CASA. Individual sperm were subjectively classified into one of three groups depending on their swimming pattern (Fig. 1). The CASA-generated paths of 200 motile sperm per sample were characterized using the criteria of Robertson et al (1988) and Mortimer and Mortimer (1990). Briefly, sperm moving in a short, straight line were classified as nonhyperactivated (Fig.

Table 1. *Kinematic characteristics of freshly collected domestic felid spermatozoa treated with caffeine, pentoxifylline, or 2′-deoxyadenosine**

Stimulants	% MOT	VCL	LIN	VSL	ALH
Control	79.1 ± 3.1	178.4 ± 2.9	32.6 ± 1.2	58.9 ± 2.8	9.7 ± 0.3
Caffeine					
0.01 mM	82.3 ± 2.0	178.4 ± 4.0	31.0 ± 0.8	54.4 ± 1.3	10.4 ± 0.3
0.10 mM	80.3 ± 2.6	177.3 ± 5.9	32.3 ± 1.5	55.0 ± 2.7	10.2 ± 0.3
1.00 mM	80.0 ± 2.7	169.0 ± 12.6	35.1 ± 2.0	54.9 ± 4.3	9.6 ± 0.6
10.0 mM	75.3 ± 2.5	151.4 ± 15.8†	35.3 ± 2.2	47.1 ± 4.2†	8.9 ± 0.5
20.0 mM	77.0 ± 3.1	140.4 ± 14.7†	36.0 ± 1.7†	45.7 ± 6.4†	8.5 ± 0.6†
Pentoxifylline					
0.01 mM	79.6 ± 2.4	188.4 ± 7.7	30.6 ± 0.8	56.4 ± 3.0	10.4 ± 0.4†
0.10 mM	79.7 ± 1.4	182.9 ± 4.0	32.0 ± 1.4	57.1 ± 2.0	10.2 ± 0.3
1.00 mM	79.7 ± 2.2	182.3 ± 8.7	29.7 ± 1.2†	52.7 ± 3.8	10.0 ± 0.4
10.0 mM	80.7 ± 1.4	181.7 ± 7.3	27.3 ± 0.6†	45.1 ± 2.7†	10.5 ± 0.4†
20.0 mM	82.7 ± 2.3	181.8 ± 8.0	24.1 ± 1.0†	41.6 ± 2.7†	9.9 ± 0.5
2′-Deoxyadenosine					
0.01 mM	79.0 ± 3.8	184.9 ± 6.6	31.1 ± 1.7	56.3 ± 2.9	10.2 ± 0.4
0.10 mM	81.7 ± 2.6	190.6 ± 6.5	29.6 ± 1.0†	55.0 ± 2.7	10.4 ± 0.3
1.00 mM	77.6 ± 3.3	168.0 ± 8.5	31.0 ± 1.2	50.3 ± 4.0†	9.4 ± 0.5
10.0 mM	78.6 ± 3.2	168.6 ± 7.9	31.6 ± 1.3	49.7 ± 2.9†	9.5 ± 0.4

* Values shown are the mean ± SEM for each characteristic determined. Data represent the average of seven samples, each containing at least 200 sperm.

† Values are different ($P < 0.05$) from controls.

Table 2. *Comparison of the kinematic characteristics of freshly collected and cryopreserved domestic felid spermatozoa**

Treatment	% MOT	VCL	LIN	VSL	ALH
Fresh	79.1 ± 2.3	178.3 ± 2.2	32.6 ± 0.9	59.0 ± 2.2	9.7 ± 0.2
Cryopreserved	52.9 ± 2.5†	107.8 ± 4.1†	33.2 ± 0.8	34.4 ± 1.9†	7.7 ± 0.2†

* Values shown are the mean ± SEM for each characteristic determined. Data represent the average of nine samples, each containing at least 200 sperm.

† Values are lower ($P < 0.05$) than fresh.

1A). Sperm exhibiting heightened velocity and angular head displacement, determined by the length and width of the path, respectively, were classified as transitional (Fig. 1B). Spermatozoa swimming vigorously, in a random thrashing pattern, demonstrated by star-shaped tracks, were classified as hyperactivated (Fig. 1C).

Statistical Analysis

CASA generates VCL, VSL, LIN, and ALH values averaged over a population of sperm. These values and MOT of fresh and cryopreserved domestic cat spermatozoa treated with and without motility stimulants were recorded as means ± SEM. Differences between the means were analyzed using repeated measures ANOVA. Differences between the sperm motility indices at each time point and the percentage of nonhyperactivated, transitional and hyperactivated sperm between control, caffeine, pentoxifylline, and 2′-deoxyadenosine-treated samples were analyzed using ANOVA. P values <0.05 were considered significant.

Results

Stimulation of Fresh Epididymal Sperm

Motion characteristics of freshly collected domestic cat sperm treated with caffeine, pentoxifylline, or 2′-deoxyadenosine are presented in Table 1. None of the motility stimulants consistently altered sperm motion. Caffeine treatment of fresh sperm decreased VCL and VSL at 10 and 20 mM and reduced LIN and ALH at 20 mM. Treatment with 2′-deoxyadenosine lowered LIN at 0.1 mM and VSL at both 1 and 10 mM. Pentoxifylline treatment lowered LIN at 1, 10, and 20 mM, lowered VSL at 10 and 20 mM, and increased ALH at 0.01 and 10 mM. Overall, there were no specific trends in sperm motion following the exposure of fresh sperm to any motility stimulant.

Stimulation of Cryopreserved Epididymal Sperm

All motion characteristics, except LIN, were reduced in cryopreserved sperm as compared to fresh sperm (Table 2). Motion characteristics of cryopreserved domestic cat sperm treated with caffeine, pentoxifylline, or 2′-deoxyadenosine are presented in Tables 3–5. Addition of the motility stimulants resulted in a consistent and dose-dependent increase of the motion characteristics. Caffeine increased the MOT, VCL, VSL, and ALH of treated sperm at all of the concentrations tested, with the exception of ALH when using 20 mM (Table 3). Pentoxifylline also had a profound stimulatory effect, increasing MOT, VCL, VSL, and ALH at the majority of concentrations tested (Table 4). 2′-Deoxyadenosine-treated spermatozoa were also enhanced in their motion characteristics but to a much lesser extent than either caffeine or pentoxifylline treatment (Table 5). MOT, VCL, VSL, and ALH were elevated using 10 mM 2′-deoxyadenosine. Additionally, 1 mM 2′-deoxyadenosine increased both VCL and VSL. Due to the constancy of the VSL/VCL ratio, LIN did not change, except when exposed to 20 mM pentoxifylline (Tables 3–5).

Effect of Motility Stimulants on Sperm Longevity

Stimulation of cryopreserved cat spermatozoa using caffeine, pentoxifylline, or 2′-deoxyadenosine was not detrimental to the maintenance of motility (Fig. 2A–C). Mo-

Table 3. *Kinematic characteristics of cryopreserved domestic felid spermatozoa treated with caffeine**

Caffeine concentration	% MOT	VCL	LIN	VSL	ALH
0 mM	52.6 ± 2.8	108.3 ± 4.6	32.8 ± 0.8	34.1 ± 2.1	7.6 ± 0.3
0.01 mM	59.8 ± 5.5†	125.8 ± 9.7†	33.1 ± 0.9	39.1 ± 2.7†	8.6 ± 0.3†
0.10 mM	62.3 ± 3.5†	126.9 ± 6.8†	33.4 ± 0.9	40.0 ± 2.1†	8.7 ± 0.2†
1.00 mM	65.3 ± 2.1†	133.3 ± 5.0†	33.4 ± 0.8	42.5 ± 2.1†	8.5 ± 0.3†
10.0 mM	66.4 ± 3.2†	139.5 ± 3.7†	32.0 ± 1.1	41.3 ± 1.5†	8.3 ± 0.3†
20.0 mM	68.3 ± 3.5†	137.9 ± 6.1†	32.1 ± 1.0	40.0 ± 3.1†	8.2 ± 0.4

* Values shown are the mean ± SEM for each characteristic determined. Data represent the average of 6 samples, each containing at least 200 sperm.

† Values are greater ($P < 0.05$) than control group.

Table 4. *Kinematic characteristics of cryopreserved domestic felid spermatozoa treated with pentoxifylline**

Pentoxifylline concentration	% MOT	VCL	LIN	VSL	ALH
0 mM	52.9 ± 2.5	107.8 ± 4.1	33.2 ± 0.8	34.4 ± 1.9	7.7 ± 0.2
0.01 mM	54.6 ± 1.7	120.2 ± 5.6†	34.2 ± 0.9	39.3 ± 2.8†	8.3 ± 0.4†
0.10 mM	61.0 ± 3.2†	123.8 ± 3.7†	33.7 ± 0.9	40.2 ± 1.5†	8.1 ± 0.3
1.00 mM	62.1 ± 3.0†	132.1 ± 3.4†	34.1 ± 1.6	42.8 ± 2.2†	8.4 ± 0.3†
10.0 mM	66.4 ± 3.5†	142.7 ± 3.0†	31.8 ± 1.4	42.1 ± 2.2†	8.7 ± 0.3†
20.0 mM	70.0 ± 3.8†	143.0 ± 4.1†	27.8 ± 1.4†	36.1 ± 2.2	9.0 ± 0.2†

* Values shown are the mean ± SEM for each characteristic determined. Data represent the average of nine samples, each containing at least 200 sperm.
† Values are different ($P < 0.05$) from control group.

tility indices of stimulated sperm paralleled those of controls over the 6-hour incubation period. Most importantly, no concentration of motility stimulant resulted in a significant decrease of sperm longevity.

Enhancement of Sperm Hyperactivation Using Motility Stimulants

Based on previous reports that showed hyperactivation to be associated with an increase in VCL and ALH (Robertson et al, 1988; Mortimer and Mortimer, 1990) and our finding in the present study that both VCL and ALH of previously cryopreserved spermatozoa were increased by treatment with motility stimulants, we determined whether exposure to motility stimulants was causing the sperm to become hyperactivated. Therefore, we examined the proportion of sperm that exhibited either nonhyperactivated, transitional, or hyperactivated swimming characteristics (Fig. 1) in control and stimulated samples (Table 6). Spermatozoa were treated with either 1 mM caffeine, pentoxifylline, or 2′-deoxyadenosine. This concentration of each stimulant consistently produced SMI values following a 6-hour incubation that were equal to or greater than ($P > 0.05$) the controls (Fig. 2). All motility stimulant treatments significantly altered the hyperactivation state of the spermatozoa (Table 6). The percentage of nonhyperactivated sperm was reduced following caffeine and pentoxifylline treatment as compared to controls (38.0 ± 2.1% and 34.0 ± 4.0% vs. 53.8 ± 5.7%), and the proportion of sperm that exhibited transitional motion was increased in the caffeine group (54.0 ± 1.7%) as compared to the control (42.6 ± 5.2%). Both pentoxifylline and 2′-deoxyadenosine increased the proportion of hyperactivated sperm (13.2 ± 2.1% and 8.2 ± 1.6%) as compared to controls (3.6 ± 0.8%).

Discussion

This study examined the effects of motility stimulants on the motion characteristics of felid spermatozoa in order to assess their potential for enhancing the reproductive efficacy of this taxon. All of the motility stimulants used in this study have previously been shown to increase MOT, VCL, VSL, and ALH and improve the fertilization ability of mammalian sperm (Fraser, 1979; Barkay et al, 1984; Pomeroy et al, 1988; Yovich et al, 1988; Hammitt et al, 1989; Hellstrom, 1989; Imoedemhe et al, 1992). The results of this study demonstrate that cryopreserved sperm motion characteristics can be significantly stimulated by caffeine, pentoxifylline, or 2′-deoxyadenosine, as assessed using CASA. These results also extend previous reports that cryopreservation decreases felid sperm motility (Platz et al, 1978; Byers et al, 1989; Donoghue et al, 1992b). Cryopreserved domestic cat sperm may thus provide a better model of poorly functioning sperm obtained from nondomestic felids than freshly collected sperm. Our data suggest that motility stimulants can alter the swimming pattern of previously cryopreserved sperm toward a more

Table 5. *Kinematic characteristics of cryopreserved domestic felid spermatozoa treated with 2′-deoxyadenosine**

2′-Deoxyadenosine concentration	% MOT	VCL	LIN	VSL	ALH
0 mM	52.2 ± 2.7	105.5 ± 3.8	33.4 ± 0.9	33.7 ± 2.1	7.7 ± 0.3
0.01 mM	53.9 ± 3.5	111.0 ± 5.8	34.0 ± 0.8	35.6 ± 2.1	7.9 ± 0.2
0.10 mM	50.7 ± 1.9	113.0 ± 3.9	34.5 ± 0.8	37.1 ± 2.2	7.8 ± 0.3
1.00 mM	57.4 ± 1.9	117.7 ± 4.2†	35.4 ± 1.3	40.6 ± 2.1†	8.0 ± 0.3
10.0 mM	59.2 ± 3.3†	130.7 ± 3.8†	32.9 ± 0.8	40.1 ± 1.9†	8.3 ± 0.2†

* Values shown are the mean ± SEM for each characteristic determined. Data represent the average of eight samples, each containing at least 200 sperm.
† Values are greater ($P < 0.05$) than control group.

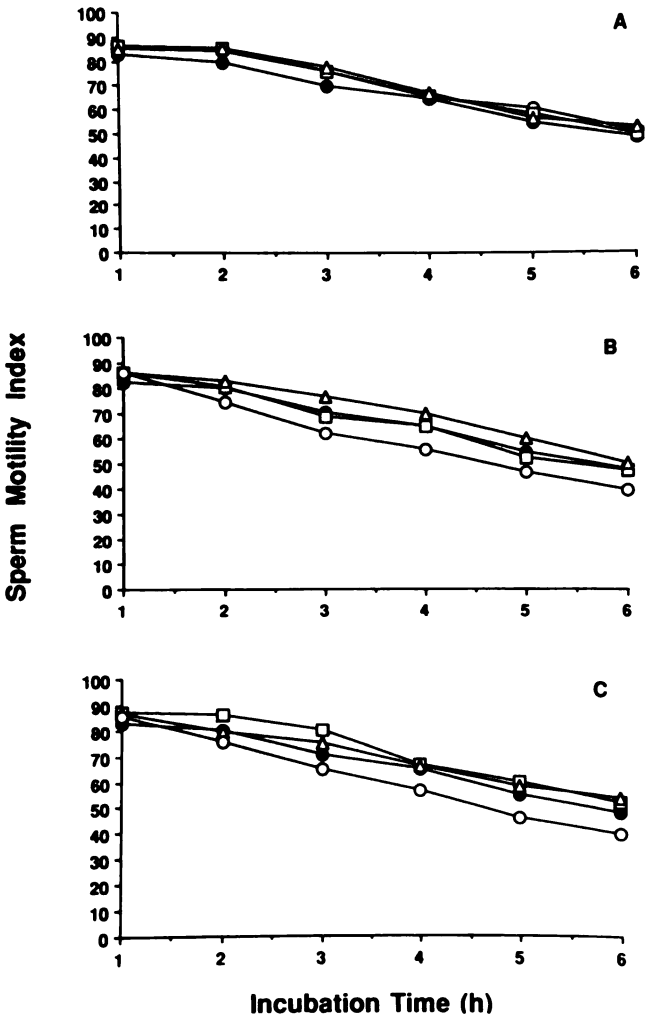

FIG. 2. Effect of motility stimulants on cryopreserved domestic cat sperm longevity. Spermatozoa were recovered from cat epididymides, incubated at 37°C in medium containing (A) 0 mM (closed circles), 0.01 mM (open circles), 0.1 mM (open squares), 1.0 mM (open triangles) caffeine; (B) 0 mM (closed circles), 0.1 mM (open squares), 1.0 mM (open triangles), 10.0 mM (open circles) pentoxifylline; or (C) 0 mM (closed circles), 0.1 mM (open squares), 1.0 mM (open triangles), 10.0 mM (open circles) 2'-deoxyadenosine, and analyzed each hour for 6 hours. MOT and SOP were determined manually, based on the type of forward movement of the sperm cell: 1 = no movement to 4 = steady, rapid forward progression. These values were used to calculate SMI, used as an overall evaluation of sperm motility characteristics. Error bars are not shown. All SEM values are within 5% of the mean.

hyperactivated state without decreasing their longevity. Furthermore, these data lend support to a method for improving the reproductive utility of cryostored sperm that has proven effective in other mammalian species.

We have demonstrated (Stachecki et al, 1993) that freshly collected epididymal cat spermatozoa exhibit high swimming velocities (VCL 178.4 μm/second) and an elevated ALH (9.7 μm), as compared to ejaculated human sperm (VCL 52.0 μm/second; ALH 3.0 μm) (Davis et al, 1992). Although we expect washed ejaculated sperm to be similar to epididymal sperm, there may be differences in their motion characteristics. In the present study, consistent stimulation of fresh cat sperm with motility stimulants was not observed (Table 1), possibly because endogenous cAMP levels were already high in these sperm. In contrast to fresh sperm, treatment of previously frozen domestic cat sperm with motility stimulants resulted in a dose-dependent increase in sperm motion characteristic values (Tables 2–4). Nearly all concentrations of caffeine and pentoxifylline resulted in elevated values for MOT, VCL, VSL, and ALH, whereas only the highest doses of 2'-deoxyadenosine were stimulatory.

CASA has been used to ascertain the motion characteristics that are most closely associated with fertilization ability (Aitken et al, 1982a; Mahadevan and Trounson, 1984; Holt et al, 1985; Jeulin et al, 1986; Chan et al, 1989; Davis et al, 1991). These studies indicate that MOT, VCL, and ALH are the most important characteristics associated with IVF rates in humans. On the basis of these reports, other investigators have used motility stimulants that elevate MOT, VCL, and/or ALH, to increase fertilization rates of sperm compromised by cryopreservation or teratospermia (Aitken et al, 1983; Yovich et al, 1988; Imoedemhe et al, 1992). Therefore, it is possible that exposure of fresh or cryopreserved felid sperm to caffeine, pentoxifylline, or 2'-deoxyadenosine may improve fertilization ability by altering sperm motion characteristics. The impact of motility stimulants on fertilization ability can be quite impressive. Pomeroy et al (1988) demonstrated that fresh capacitated murine spermatozoa treated with caffeine achieved an 89% fertilization rate using a brief, 15-minute insemination period, whereas the fertilization rate of the control was only 27%.

Freshly collected, washed epididymal (unpublished observations) or ejaculated (Wildt, 1991) domestic cat spermatozoa remain motile for approximately 30 hours with a gradual reduction in their motility over time, whereas cryopreserved sperm motility is greatly reduced after only 6 hours (Fig. 2). In a recent study, Donoghue et al (1992a) hypothesized that, in cheetahs, the etiology of poor reproductive performance is more male than female related and demonstrated a correlation between fertilization rates and longevity of spermatozoa. In a similar study, Donoghue et al (1992b) revealed that fertilization rates were reduced among cryopreserved tiger spermatozoa that exhibited decreased longevity patterns. We were concerned that in elevating the motion characteristics of cryopreserved sperm longevity may be decreased. Hammitt et al (1989) noted that longevity of human sperm declined following incubation with motility stimulants, while Ruzich et al (1987) reported that caffeine can extend sperm longevity. In our study, however, we observed no significant reduction in sperm longevity following exposure to any of the motility stimulants.

Table 6. *Hyperactivation of stimulated cryopreserved domestic felid spermatozoa**

Stimulants	Nonhyperactivated	Transitional	Hyperactivated
Control	53.8 ± 5.7	42.6 ± 5.2	3.6 ± 0.8
Caffeine	38.0 ± 2.1†‡	54.0 ± 1.7†	8.0 ± 1.2
Pentoxifylline	34.0 ± 4.0†‡	52.8 ± 3.5	13.2 ± 2.1†
2'-Deoxyadenosine	43.6 ± 4.6	49.0 ± 3.1	8.2 ± 1.6†

* Values shown are the mean percentages of five samples ± SEM for each characteristic determined, each sample consisting of 200 sperm. Treated samples were exposed to 1 mM caffeine, pentoxifylline, or 2'-deoxyadenosine for 15 minutes at 23°C.

† Values shown are different ($P < 0.05$) than controls.

‡ Values shown are lower ($P < 0.05$) than the transitional plus hyperactivated values.

Hyperactivation is an additional factor that can influence fertilization rates. Hyperactivated motility was first described in hamster sperm, during capacitation in Tyrode's solution and in the ampullary region of the oviduct around the time of fertilization (Yanagimachi, 1970). During hyperactivation, spermatozoa exhibit a whiplash pattern of movement associated with an increase in VCL and ALH (Burkman, 1984; Mortimer et al, 1984; Robertson et al, 1988; Yanagimachi, 1988; Mortimer and Mortimer, 1990). It has been proposed that hyperactivation aids the sperm in reaching the egg and penetrating its vestments (Suarez et al, 1983; Katz et al, 1989; Suarez, 1991). Our data revealed a shift in the sperm population from a nonhyperactive state to either a transitional or hyperactivated state following a 15-minute exposure to any of the motility stimulants (Table 6). Because hyperactivated sperm were found in both freshly collected (data not shown) as well as in cryopreserved control samples (Table 6) and hyperactivated motion is required for fertilization, these data support the observation by Niwa et al (1985) that freshly collected epididymal domestic cat sperm can fertilize oocytes within 30 minutes of insemination.

The present study provides kinematic values for fresh and frozen domestic cat sperm exposed to motility stimulants, revealing important differences following cryopreservation and stimulation. These results extend previous work (Stachecki et al, 1993) by demonstrating that domestic cat sperm show a significant reduction in their motion characteristics following cryopreservation that is similar to the decreased kinematics found in freshly collected sperm from teratozoospermic cats. Based on the information presented here, it is conceivable that the success of assisted reproductive techniques including IVF and AI could be enhanced by treating felid spermatozoa with caffeine, pentoxifylline, or 2'-deoxyadenosine.

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