Journal of Andrology, Vol. 27, No. 3, May/June 2006
Copyright © American Society of Andrology

Hyperactivated Motility in Rhesus Macaque (*Macaca mulatta*) Spermatozoa

JULIE BAUMBER AND STUART A. MEYERS

From the Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, California.

ABSTRACT: Macaque spermatozoa can be capacitated according to a defined protocol and exhibit hyperactivated motility similar to that described in other species. The aim of this study was to create a method for defining hyperactivation that could be routinely used in the laboratory alongside our existing sperm motility analysis protocol. Percoll-separated macaque spermatozoa were incubated for 2 hours (37°C; 5% CO_2 in air) at a concentration of 20 $\times$ 10^6/mL in bicarbonate (36 mmol)-buffered Biggers, Whitten and Whittingham medium (BWW) containing 30 mg/mL bovine serum albumin (BSA), followed by an additional 30 minutes with (capacitated) or without (incubated) caffeine (1 mmol) and dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP; 1.2 mmol). One hundred and fifty progressive and hyperactivated tracks were selected from each of three monkeys. Thresholds for hyperactivation were based on the 10th (amplitude of lateral head displacement, ALH) and 90th (linearity, LIN) percentiles of the hyperactivated kinematic data set and were LIN less than or equal to 69% and ALH greater than or equal to 7.5 μM; a threshold of greater than or equal to 130 μM/s was also included for curvilinear velocity (VCL). These thresholds were 91% effective at identifying hyperactivated tracks. Capacitation of macaque spermatozoa, by the addition of caffeine and dbcAMP, resulted in a significant increase in ALH, VCL, and beat cross frequency and a significant decrease in total and progressive motility, straight line velocity, straightness, and LIN when compared to incubated spermatozoa, suggesting the expression of hyperactivated motility. Utilizing the above thresholds, hyperactivation was expressed by 5% $\pm$ 0.8% of the incubated sperm population vs 53 $\pm$ 3.7% of the capacitated sperm population ($P < .0001$). Hyperactivation was not observed when dbcAMP and caffeine were added separately and was significantly ($P < .005$) reduced by the addition of H-89. The results of this paper demonstrate that hyperactivation can be reliably estimated for rhesus macaque spermatozoa.

Key words: Primate sperm, capacitation, cyclic adenosine monophosphate, caffeine.

J Androl 2006;27:459–468

Freshly ejaculated mammalian spermatozoa are actively motile but are unable to fertilize an oocyte. Spermatozoa need to spend a period of time in either the female reproductive tract or an appropriate in vitro environment to become competent for fertilization. The complex physiological changes that confer on sperm the ability to fertilize are collectively termed "capacitation" (Yanagimachi, 1994). During capacitation, mammalian sperm develop distinctive motility patterns, known as hyperactivation, characterized by the development of asymmetrical, high-amplitude flagellar beats, causing vigorous, sometimes nondirectional movement of free-swimming spermatozoa (Suarez et al, 1983). Hyperactivation appears to be related to the final stages or completion of capacitation (Katz et al, 1989), with researchers identifying a subgroup of hyperactivated spermatozoa before or at the time of fertilization in vivo (Katz and Yanagimachi, 1980; Suarez and Osman, 1987). Although hyperactivation is a capacitation-associated phenomenon, the two processes do not appear to be coupled. Capacitation and hyperactivation can occur independently from the other (Neill and Olds-Clarke, 1987; Olds-Clarke, 1989; Boatman and Robbins, 1991; Stauss et al, 1995); suggesting that capacitation and hyperactivation should be considered independent events (Suarez, 1996). In this case, researchers prefer to define capacitation as the process that prepares spermatozoa for a physiologically induced acrosome reaction, thereby eliminating the inclusion of hyperactivation.

Several theories have been put forward for the functional significance of hyperactivated motility, including that it enables penetration of mucoid oviductal secretions (Suarez et al, 1991, 1992; Suarez and Dai, 1992), provides a mechanism for escape from pockets of oviductal mucosa (Suarez et al, 1983; Suarez and Osman, 1987), allows detachment of bound spermatozoa from the oviductal epithelium or sperm reservoir (Suarez, 1987; De Mott and Suarez, 1992; Pacey et al, 1995), increases the chances of a spermatozoon finding

Supported by NIH/NCRR RO1 RR16581.

Correspondence to: Stuart A. Meyers, Sperm Biology Laboratory, Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, CA 95616 (e-mail: smeyers@ucdavis.edu).

Received for publication May 23, 2005; accepted for publication October 30, 2005.

DOI: 10.2164/jandrol.05107

the oocyte within the oviduct due to frequent directional changes (Katz et al, 1978; Suarez and Osman, 1987), stirs ampullary fluid to ensure biochemical homogeneity (Katz and Dott, 1975; Katz et al, 1989), facilitates transit through the cumulus matrix (Suarez et al, 1991), and allows sufficient thrust for the spermatozoon to penetrate the zona pellucida (Baltz et al, 1988; Drobnis et al, 1988; Katz et al, 1989; Stauss et al, 1995).

Objective evaluation of hyperactivation has been attempted using computer-assisted sperm analysis (CASA) in mice (Neill and Olds-Clarke, 1987), humans (Mortimer and Swan, 1995), sheep (Mortimer and Maxwell, 1999), bulls (McNutt et al, 1994), rabbits (Young and Bodt, 1994), boars (Abaigar et al, 1999), and rats (Cancel et al, 2000). To evaluate the incidence of hyperactivation using CASA, swimming patterns of spermatozoa incubated in capacitating and noncapacitating conditions are compared and threshold values are set for kinetic parameters that distinguish hyperactivated sperm from progressive sperm.

Macaque spermatozoa can be capacitated according to a defined protocol and exhibit hyperactivated motility similar to that described in other species (Boatman and Bavister, 1985). Thresholds for defining hyperactivation are not available in the rhesus macaque for use with computer-assisted sperm analysis software. The aim of this project was to create a method for defining hyperactivation that could be routinely used in the laboratory alongside our existing sperm motility analysis protocol. Once thresholds for hyperactivation had been established, the percentage of spermatozoa exhibiting hyperactivated motility following capacitation was determined using CASA and the effect of protein kinase A (PKA) inhibitor H-89 on sperm hyperactivation was evaluated.

Materials and Methods

Chemicals and Reagents—HEPES (21 mmol)–buffered Biggers-Whitten-Whittingham (HEPES-BWW) medium was obtained from Irvine Scientific (Irvine, Calif). H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide-dihydrochloride) was obtained from EMD Biosciences (La Jolla, Calif). All other reagents were obtained from Sigma Chemical Company (St Louis, Mo).

Semen Collection, Processing and Capacitation—Semen samples were obtained by electroejaculation from male rhesus macaques (*Macaca mulatta*; n = 7) under chair restraint as described previously (Sarason et al, 1991). Animals were housed at California National Primate Research Center and maintained according to Institutional Animal Care and Use Committee protocols at the University of California. Following collection, semen samples were diluted in 4 mL HEPES-BWW media containing 1 mg/mL bovine serum albumin (BSA; Fraction V, catalog number A-4503) and rocked for 5 minutes. The coagulum was removed and the sample maintained at room temperature for 10 minutes. The upper 3.5 mL of semen was then transferred into a separate tube for determination of initial motility, sperm count and subsequent processing. Two milliliters of semen were placed over 3 mL of 80% Percoll and centrifuged at 300 $\times$ g for 25 minutes. First, a 95% Percoll solution was prepared by adding 20$\times$ HEPES-buffered saline to 100% Percoll (modified from Tollner et al, 2000). This solution was diluted with HEPES-BWW containing 1 mg/mL BSA to produce the 80% Percoll. Following centrifugation, the supernatant was removed and the pellet washed twice in HEPES-BWW + 1 mg/mL BSA (300 $\times$ g, 5 minutes). Sperm were finally resuspended at a concentration of 20 $\times$ 10^6/mL in bicarbonate (36 mmol)-buffered BWW (Bicarb-BWW; Overstreet et al, 1980) containing 30 mg/ml BSA and incubated for 2 hours at 37°C (5% CO_2 in air) followed by an additional 30 minutes with (capacitated) or without (incubated) caffeine (1 mmol) and N^6, 2′-O-dibutyryladenosine 3′, 5′-cyclic monophosphate (dbcAMP; 1.2 mmol) (Yudin et al, 2000).

Motion Analysis

Sperm motility was determined on at least 200 cells/sample in a minimum of four fields by CASA utilizing HTM CEROS (Version 12.2 g; Hamilton Thorne Research, Beverly, Mass). Five microliters of sperm suspension was placed onto a prewarmed microscope slide overlaid with a 22-mm^2 prewarmed cover slip and the slide maintained at 37°C during analysis by a heated slide holder (MiniTherm; Hamilton Thorne Research). Sperm motility was videotaped using a Panasonic AG-DV1000 digital videocassette recorder utilizing a Sony XC-75 CCD video camera mounted on an Olympus CH 30 microscope equipped with a 10$\times$ negative phase-contrast objective and a 10$\times$ projection ocular. The videotape was replayed for analysis at a subsequent time, and the playback feature was used to identify and delete aberrant tracks occurring when trajectories cross over each other or spermatozoa collide.

Instrument settings for the CASA analysis were as follows: frame rate 60 Hz, frames acquired 30, minimum contrast 80, minimum cell size 4 pixels, static average path velocity (VAP) cutoff 20 µ/s, static straight-line velocity (VSL) cutoff 10 µ/s, progressive VAP threshold 25 µ/s, progressive straightness (STR) threshold 80%, static intensity limits 0.6–1.4, static size limits 0.6–2.31, and static elongation limits 0–80. Percentages of total motility (TM) and progressive motility (PM), VSL, curvilinear velocity (VCL), VAP, linearity (LIN), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and STR were determined.

Motility Characteristics

Incubated spermatozoa exhibited predominantly straight tracks (Figure 1a); progressive tracks were also observed similar to Figure 1b but with a much smaller ALH (generally less than 4 µm). Capacitated macaque spermatozoa displayed several types of motility patterns characteristic of hyperactivation (Figure 1b through f). The majority of hyperactivated sperma-

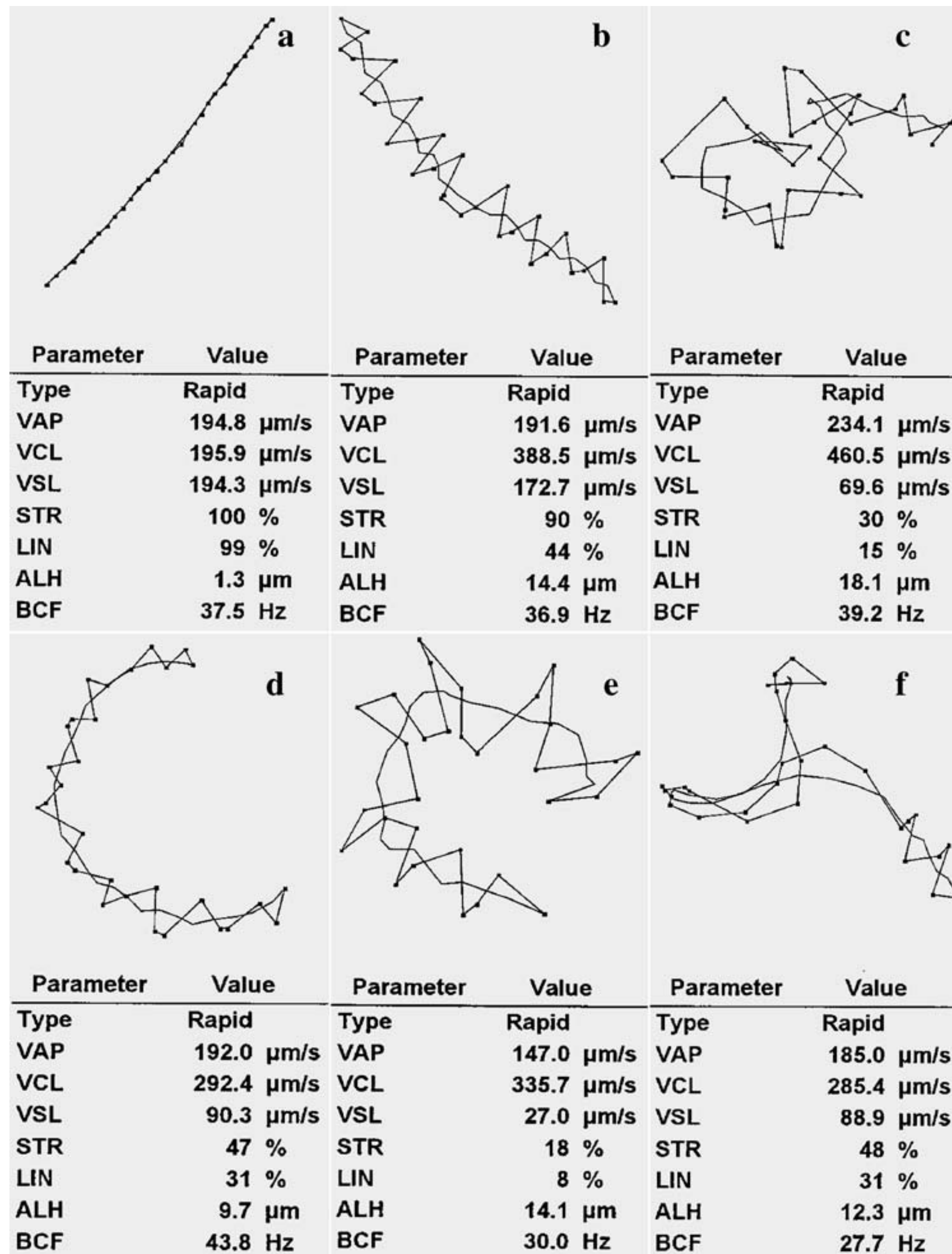

Figure 1. Representative trajectories selected from the computer-assisted sperm analysis program demonstrating progressive **(a)** and hyperactivated **(b–f)** macaque spermatozoa.

tozoa traced circular or star-shaped trajectories (Figure 1d and e) or random paths with abrupt changes in direction (Figure 1f). Commonly, spermatozoa traced progressive tracks but with jerky motion and extreme flagellar bending (Figure 1b), previously described as transitional motility (Burkman, 1984, Robertson et al, 1998; Mortimer and Mortimer, 1990). These transitional tracks were often interspersed with episodes of circular or erratic motion (Figure 1c and f).

Defining Hyperactivated Sperm

Objectively Selected Tracks—In order to objectively establish criteria for the classification of hyperactivation in rhesus macaque sperm, motility parameters were measured with CASA in the incubated and capacitated treatments (Young and Bodt, 1994; Mahony and Gwathmey, 1999; Cancel et al, 2000). One hundred and fifty tracks were selected from the incubated (predominantly progressive) and capacitated (predominantly hyperactivated) treatments for each of three monkeys, that is, 450 tracks total for each treatment. In order to minimize observer bias, tracks were not selected by visual assessment during the CASA analysis but by randomly selecting tracks from the exported data files. Capacitated trajectories showed significantly ($P < .0005$, except BCF $P < .05$) different motility characteristics than incubated tracks for

Table 1. *Kinetic motility data representing the mean (± standard deviation) of 450 tracks (150 from each of 3 monkeys) selected objectively or subjectively from capacitated or noncapacitated samples. Objectively selected tracks represent a sample of all tracks within a treatment, whereas subjectively selected tracks were identified as being either progressive or hyperactivated*

		VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
Objective*	Incubated	147 ± 74	140 ± 74	184 ± 82	4.4 ± 3	40 ± 12	93 ± 13	75 ± 23
	Capacitated	150 ± 44	125 ± 45	266 ± 75	9.5 ± 4	42 ± 7	84 ± 17	48 ± 15
Subjective†	Progressive	217 ± 45	215 ± 45	222 ± 47	2.3 ± 1	36 ± 8	99 ± 1	97 ± 3
	Hyperactivated	172 ± 41	131 ± 55	280 ± 60	10.6 ± 3	34 ± 8	75 ± 23	47 ± 18

* Capacitated trajectories showed significantly ($P < .0005$, except BCF $P < .05$) different motility characteristics than incubated tracks for VSL, VCL, ALH, BCF, LIN, and STR.

† There was a significant difference ($P < .0001$, except BCF $P < .05$) across all parameters between the progressive and hyperactivated tracks.

VSL, VCL, ALH, BCF, LIN, and STR (Table 1). There was a significant ($P < .05$) difference between monkeys for VAP, VSL, VCL, BCF, and LIN.

Preliminary experiments determined that ALH and LIN were the best combination of parameters to accurately define hyperactivated sperm. Classification potential of each motility parameter was assessed graphically by comparing parameter distributions for incubated and capacitated sperm. ALH, LIN, and VCL were predominantly focused on because of their suggested use in classifying hyperactivated human sperm (Robertson et al, 1998; Mortimer and Mortimer, 1990; Burkman, 1991). Percentile rank values were used to determine thresholds for defining hyperactivated sperm, and the accuracy of parameter combinations were also compared as described below. Thresholds for hyperactivation were based on the 10th (ALH) and 90th (LIN) percentiles of the capacitated kinematic data set and were LIN less than or equal to 67% and ALH greater than or equal to 5.0 μm. The accuracy of these thresholds was evaluated by applying them back to subjectively selected hyperactivated tracks and determining the percentage of tracks correctly identified. These thresholds were found to be 91% effective at identifying hyperactivated tracks, with no false positives.

Subjectively Selected Tracks—One hundred fifty progressive and hyperactivated tracks were selected by the same observer from each of 3 monkeys utilizing the incubated and capacitated treatments. The visually selected tracks were identified as hyperactivated based on criteria defined by Burkman (1984, 1991) and Mortimer and Mortimer (1990). Hyperactivated trajectories showed significantly ($P < .0001$, except BCF $P < .05$) different motility characteristics than progressive tracks across all kinetic parameters (Table 1). There was a significant ($P < .05$) difference between monkeys for VAP, VCL, ALH, BCF, and LIN. Thresholds for hyperactivation were based on the 10th (ALH) and 90th (LIN) percentiles of the hyperactivated kinematic data set and were LIN less than or equal to 69% and ALH greater than or equal to 7.5 μm. These thresholds were found to be 91% effective at identifying hyperactivated tracks, with no false positives.

Thresholds for defining hyperactivation were very similar between the objective and subjective methods of track selection. In the subjectively selected tracks, as opposed to the objectively selected tracks, there was no overlap in the percentile rank values between progressive and hyperactivated tracks for ALH, LIN, and STR. For example, with ALH the 10th percentile of hyperactivated tracks (7.5) utilized as the threshold for identifying hyperactivated sperm was much greater than the 90th percentile of progressive tracks (3.8). To clarify, 10% of hyperactivated tracks had an ALH smaller than 7.5, whereas 90% of progressive sperm had an ALH smaller than 3.8. For objectively selected tracks the 10th percentile of capacitated tracks (5.0) overlapped the 90th percentile of incubated tracks (8.0). Therefore, we decided to enter the thresholds from the subjectively selected tracks into the sort criteria of the CASA software for identifying hyperactivated motility.

Although VCL is commonly used for the determination of hyperactivation in other species (Robertson et al, 1988; Grunert et al, 1990; Mortimer and Mortimer, 1990; Burkman, 1991), we found that it did not increase the accuracy of our thresholds. The variation in VCL between monkeys necessitated a lower threshold in order to identify hyperactivated trajectories from all monkeys. The threshold VCL value for identifying hyperactivated trajectories (10th percentile, 230 μm/s) overlapped with the 90th percentile (275 μm/s) of progressive spermatozoa; this was not the case for STR, LIN, and ALH. Furthermore, although there was a high correlation between all other motility parameters in the hyperactivated tracks, VCL did not correlate with BCF, LIN, or STR. Spermatozoa in the capacitated treatments can occasionally be observed to be shaking in situ with minimal progression, but nonetheless describing definite trajectories. These spermatozoa have a VAP/VSL exceeding that which defines nonmotile spermatozoa and may also have a high ALH. A minimum VCL threshold of 130 μm/s was included in our analysis to exclude these spermatozoa. A similar situation has been reported by Mahony and Gwathmey (1999) in cynomolgus macaque spermatozoa and Mazzilli et al (2001) in human spermatozoa.

In conclusion, the thresholds utilized in the CASA software for defining hyperactivated motility were VCL greater than or equal to 130 μm/s, LIN less than or equal to 69%, and ALH greater than or equal to 7.5 μm. Spermatozoa exhibiting motility characteristics outside all 3 thresholds and a trajectory with a minimum of 15 data points were subsequently counted as hyperactivated. A supplementary threshold for STR less than or equal to 97% could also be included in the analysis without a significant effect on the percentage of hyperactivated motility.

Table 2. *Kinetic motility data representing 50 transitional and 50 nonprogressive hyperactivated (star spin or random) tracks selected from each of 3 monkeys (150 total tracks for each type of motility pattern)*

	VAP (μm/s)	VSL* (μm/s)	VCL (μm/s)	ALH* (μm)	BCF* (Hz)	STR* (%)	LIN* (%)
Transitional	179 ± 31	164 ± 32	295 ± 40	10.3 ± 2	39 ± 7	91 ± 5	56 ± 10
Nonprogressive	172 ± 31	101 ± 44	303 ± 50	13.1 ± 3	35 ± 8	58 ± 22	34 ± 15

* Means (± standard deviation) within columns differ ($P < .0001$).

Transitional vs Nonprogressive Hyperactivated Tracks

In a separate experiment, 50 sperm tracks representing the 2 predominant types of hyperactivated motility, transitional and nonprogressive (star spin and random trajectories) hyperactivation, were selected from each of 3 monkeys as described above. Nonprogressive hyperactivated tracks showed a significant ($P < .0001$) increase in ALH and a significant ($P < .0001$) decrease in VSL, BCF, STR, and LIN when compared to transitional tracks (Table 2). There was a significant ($P < .05$) difference between monkeys across all kinetic parameters. Thresholds based on the 10th/90th percentile of this kinematic data set were for transitional motility, STR less than or equal to 97%, LIN less than or equal to 69%, and ALH greater than or equal to 7.5 μm (VCL ≥ 130 μm/s), and nonprogressive (star spin/random) motility, STR less than or equal to 85%, LIN less than or equal to 55%, and ALH greater than or equal to 10 μm (VCL ≥ 200 μm/s). The thresholds for transitional hyperactivated motility matched exactly with the thresholds derived from the previous data set, confirming the inclusion of all types of hyperactivated motility.

Microscope Slide vs Chamber of Fixed Depth—The aim of this project was to create methodology for defining hyperactivation that could be routinely used in the laboratory alongside our existing sperm motility analysis protocol. According to ESHRE guidelines (ESHRE Andrology Special Interest Group, 1996), human spermatozoa should be evaluated for sperm hyperactivation at a depth of at least 30 μm (Mortimer, 1997). Unfortunately, at this depth the CASA software has a difficult time tracking macaque spermatozoa as they move up and down within the field of view, resulting in tracks with missing data points and consequently inaccurate data. Our current methodology (placing 5 μL of spermatozoa on a microscope slide overlaid with a cover slip) creates a depth of approximately 10 μm but does not create a field of uniform depth. To evaluate whether this would impact the results, 85 progressive and hyperactivated tracks were selected from each of 3 ejaculates visualized on microscope slides as described above or in chambers of a fixed depth (20 μm; 2X-CEL, Hamilton Thorne Research). In a separate experiment, sperm motility and hyperactivation of incubated and capacitated samples were compared across 3 ejaculates evaluated by microscope slide or chamber.

When hyperactivated trajectories were selected from spermatozoa visualized by either microscope slide or chamber, there was a significant difference in VCL ($P < .0001$; 328 ± 4.3 vs 351 ± 3.4 μm/s respectively), ALH ($P < .05$; 12.3 ± 0.2 vs 12.8 ± 0.2 μm) and BCF ($P < .0001$; 36 ± 0.5 vs 41 ± 0.5 Hz). Again, there was a significant ($P < .005$) difference between monkeys across all kinetic parameters. Although ALH was significantly different between spermatozoa visualized by slide or chamber, the mean difference was small (0.5 μm) and would not affect our thresholds. Even at a depth of 20 μm the CASA software still had problems tracking some trajectories and missing data points could have contributed to the increased value for ALH. When the motility of incubated and capacitated samples was evaluated and compared using microscope slides or chambers, there were no significant differences in kinetic values or in the percentage of total, progressive, or hyperactivated spermatozoa (data not shown).

Evaluation of Sperm Hyperactivation With Capacitation in Macaque Spermatozoa

Sperm motility and the percentage of hyperactivated spermatozoa were determined in incubated and capacitated samples, replicated across 5 ejaculates from each of 5 monkeys. In a separate experiment (replicated across 4 ejaculates), sperm motility and hyperactivation were evaluated at times 0, 10, 20, 30, 45, 60, 90, and 120 minutes after the addition of caffeine and dbcAMP.

Influence of Caffeine and dbcAMP Alone on Hyperactivation—Spermatozoa (1 ejaculate from each of 5 monkeys) were processed as described above and incubated for 2 hours at 37°C (5% CO_2 in air) at 20 × 10^6/mL in Bicarb-BWW + 30 mg/ml BSA followed by an additional 30 minutes with caffeine alone (1 mmol), dbcAMP alone (1.2 mmol), or caffeine + dbcAMP. The percentage of hyperactivated spermatozoa was determined as described above.

Influence of PKA Inhibitor H-89 on Hyperactivation—Spermatozoa (1 ejaculate from each of 5 monkeys) were processed as described above and incubated for 2 hours at 37°C (5% CO_2 in air) at 20 × 10^6/mL in Bicarb-BWW + 30 mg/ml BSA alone or with the addition of H-89 (60 μm) or DMSO control (1 μL, final concentration 0.2%). This was followed by an additional 30 minutes with caffeine (1 mmol) and dbcAMP (1.2 mmol). Sperm motility and percentage hyperactivation were determined as described above. In a subsequent experiment (replicated across 4 ejaculates), H-89 or DMSO control was added after the initial 2-hour incubation, at same time as caffeine and dbcAMP.

Statistical Analysis—Data were analyzed by analysis of variance (ANOVA), and comparisons between individual means were performed with Fisher's protected least significant difference test utilizing Statview statistical software (Version 5.0; SAS Institute Inc, Cary, NC). Differences with values of $P < .05$ were considered to be statistically significant.

Table 3. *Motility of macaque spermatozoa in Bicarb-BWW + 30 mg/mL BSA following incubation for 2 hours at 37°C (5% CO_2 in air) followed by an additional 30 minutes with (capacitated) or without (incubated) caffeine (1 mmol) and dbcAMP (1.2 mmol)*

	TM* (%)	PM* (%)	VAP (μm/s)	VSL* (μm/s)	VCL* (μm/s)	ALH* (μm)	BCF* (Hz)	STR* (%)	LIN* (%)
Incubated	70 ± 1.6	64 ± 1.8	182 ± 6.0	177 ± 6.1	204 ± 4.7	3.5 ± 0.15	37 ± 1.0	96 ± 0.3	86 ± 1.4
Capacitated	64 ± 1.6	48 ± 2.0	178 ± 5.3	154 ± 5.4	266 ± 4.0	7.5 ± 0.32	39 ± 0.6	86 ± 0.8	60 ± 2.1

* Means (± SEM) within columns differ ($P < .05$).

Results

Evaluation of Sperm Hyperactivation With Capacitation in Macaque Spermatozoa

The addition of caffeine and dbcAMP resulted in a significant increase in ALH ($P < .0001$), VCL ($P < .0001$), and BCF ($P < .05$) and a significant decrease in TM ($P < .01$), PM ($P < .0001$), VSL ($P < .005$), LIN ($P < .0001$), and STR ($P < .0001$) when compared to incubated spermatozoa; suggesting the expression of hyperactivated motility (Table 3). Hyperactivated motility was expressed by 5% ± 0.8% of the incubated sperm population vs 53% ± 3.7% of the capacitated sperm population ($P < .0001$). In the capacitated samples a mean of 24% ± 2.8% displayed nonprogressive hyperactivated tracks vs 2% ± 0.5% in the incubated samples.

The percentage of hyperactivated motility following the addition of dbcAMP and caffeine increased over time, becoming significant ($P < .0001$) at 10 minutes and reaching a maximum at 1 hour (Figure 2). Although hyperactivated motility could be maintained for up to 4 hours, a significant ($P < .05$) decrease in total motility and percent hyperactivation occurred after 2 hours. Hyperactivated motility was observed to be predominantly transitional during the first 10 to 20 minutes of incubation with caffeine and dbcAMP; subsequently, spermatozoa exhibited increasing numbers of star-spin or random trajectories.

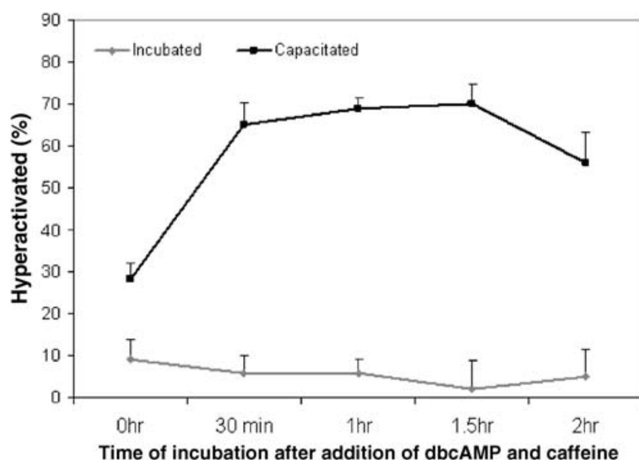

Figure 2. Percentage of macaque spermatozoa exhibiting hyperactivated motility over time. Spermatozoa were incubated in Bicarb-BWW + 30 mg/ml BSA for 2 hours at 37°C (5% CO_2 in air), followed by incubation (37°C; 5% CO_2 in air) with (capacitated) or without (incubated) the addition (at time 0) of caffeine (1 mmol) and dbcAMP (1.2 mmol).

Influence of Caffeine and dbcAMP Alone on Hyperactivation—The addition of caffeine (9% ± 1.8%) and dbcAMP (2% ± 0.6%) alone did not result in a significant increase in hyperactivated motility when compared to the incubated sample (4% ± 1.9%). However, the combination of caffeine and dbcAMP in capacitated samples (53% ± 7.6%) resulted in a significant ($P < .0001$) increase in hyperactivated motility.

Influence of PKA Inhibitor H-89 on Hyperactivation—Capacitation, with (42% ± 8.7%) or without (46% ± 10.5%) the presence of DMSO, resulted in a significant ($P < .005$) increase in hyperactivated motility when compared to the incubated sample (3% ± 1.5%). The addition of H-89 (28% ± 6.2%) at the start of the 2.5-hour capacitation did not significantly decrease the percentage of hyperactivated spermatozoa when compared to capacitated samples. Capacitated samples (with or without DMSO) showed a significant ($P < .005$) increase in VCL and ALH and a significant ($P < .005$) decrease in STR, LIN, and PM when compared to incubated samples (data not shown). However, capacitated DMSO control samples also exhibited a significant ($P < .05$) decrease in total motility (53% ± 2.4%) when compared to incubated (68% ± 3.1%) and capacitated (64% ± 3.1%) samples. Treatment with H-89 resulted in an even greater decrease in total motility (39% ± 5.2%) and negatively impacted other kinetic parameters. Incubation with H-89 also had a significant ($P < .05$) effect on sperm viability (59% ± 6.2% dead) when compared to incubated (25 ± 4.9) or capacitated (37± 7.0) samples.

Since incubation with H-89 for 2.5 hours had a negative impact on sperm motility and viability, a subsequent experiment investigated the effect of adding H-89 at the same time as caffeine and dbcAMP. In this case, H-89 resulted in a significant ($P < .005$) decrease in hyperactivated motility (23% ± 4.3%) when compared to capacitated samples (57% ± 9.2% with DMSO; 64% ± 9.9% without DMSO). The addition of H-89 also resulted in a significant decrease ($P < .005$) in VCL and a significant ($P < .001$) increase in STR, when compared to capacitated samples; without a significant effect on total motility (data not shown).

Discussion

Descriptions of hyperactivated motility vary greatly in the literature. One of the reasons is that the swimming patterns formed by hyperactivated spermatozoa depend on the length of the flagellum and the thickness of the outer dense fibers, which show a certain degree of interspecies variability. Species with a small radius of curvature in flagellar bends (mice, humans, rabbits) tend to have smaller outer dense fibers than do species showing a large radius of curvature in the flagellar bend (rats, hamsters) (Phillips, 1972; Baltz et al, 1990). Even within a species, different types of hyperactivated motility patterns exist; the complexity in human spermatozoa has been described by Burkman (1991). Furthermore, measurement conditions, including chamber type, temperature, culture medium composition, and sperm concentration, can affect the quantification of hyperactivation (Kay and Robertson, 1998). Consequently, there is no agreement on a defined criterion for hyperactivated motility. It is universally agreed, however, that the distinguishing characteristics of hyperactivated spermatozoa are a curvilinear velocity greater than 70 μm/s, irrespective of the frame rate (Robertson et al, 1988; Grunert et al, 1990; Mortimer and Mortimer, 1990; Burkman, 1991; Zhu et al, 1994), and a low linearity, although the threshold values for LIN vary considerably across independent studies, ranging from 19% (Robertson et al, 1988) to 80% (Zhu et al, 1994). ALH has also been widely used to identify hyperactivated motion, and the values for this parameter are generally greater than or equal to 7 μm (Robertson et al, 1988; Grunert et al, 1990; Mortimer and Mortimer, 1990; Burkman, 1991; Zhu et al, 1994). Thresholds defined in this study for hyperactivated rhesus macaque sperm (VCL $\geq$ 130 μm/s, LIN $\leq$ 69%, and ALH $\geq$ 7.5 μm) are similar to those described by Burkman (1991) for human sperm (VCL $\geq$ 100 μm/s, LIN $\leq$ 65%, and ALH $\geq$ 7.5 μm) and by Mahony and Gwathmey (1999) for cynomolgus macaque spermatozoa (VCL $\geq$ 150 μm/s, LIN $\leq$ 60%, and ALH $\geq$ 8.0 μm).

Differences in the thresholds used to define hyperactivation may be partly due to conflicting views over whether spermatozoa exhibiting transitional motility should be classified as hyperactivation or whether they should be grouped separately. It has been suggested that transitional motility may be considered a progressive form of hyperactivation (Zhu et al, 1994). However, it also has been shown that it is not necessary for human spermatozoa to pass through a transitional phase in order to show the star-spin phase (Mortimer and Swann, 1995). Furthermore, transitional motility did not correlate significantly with IVF rates, in contrast to nonprogressive hyperactivated motility (Sukcharoen et al, 1995). Consequently, several researchers have described additional criteria for identifying subtypes of hyperactivated spermatozoa (Robertson et al, 1998; Mortimer and Mortimer, 1990; Burkman, 1991; Griveau and Le Lannou, 1994; Sukcharoen et al, 1995; Cancel et al, 2000; Mazzilli et al, 2001). In this study, 45% of the hyperactivated spermatozoa were exhibiting star spin or erratic motion.

Transitional motility has also been shown to be related to the depth of the chamber used for sperm analysis, with deeper chambers giving a higher proportion of star spin rather than transitional phase patterns of motility (Le Lannou et al, 1992; Mortimer and Swan, 1995). Deeper chambers are less likely to constrain flagellar movement, but it is more difficult to keep the spermatozoa in focus. The aim of this study was to create methodology for defining hyperactivation that could be routinely used in the laboratory alongside our existing sperm motility analysis protocol. Analysis of spermatozoa by our current methodology, utilizing simple microscope slides, was compared to chambers of a fixed depth. Although differences in kinetic parameters of hyperactivated sperm tracks were observed between the 2 types of slides, these were not sufficient to affect our thresholds, and the percentages of hyperactivated motility identified under the 2 types of slides were similar. However, due to the limitations of accurately tracking spermatozoa in a deeper chamber we did not evaluate macaque spermatozoa at a depth greater than 20 μm and therefore cannot confirm that a greater depth would influence the percentage of transitional or star-spin motility patterns.

Macaque spermatozoa do not capacitate spontaneously in vitro but require the addition of caffeine and dbcAMP to stimulate hyperactivated motility, binding to the zona pellucida and fertilization (Boatman and Bavister, 1984; Vandevoort et al, 1992, 1994). In the current study, hyperactivation was observed in 53% of rhesus macaque spermatozoa incubated under conditions known to promote capacitation. An increase in sperm hyperactivation, or changes in sperm motion parameters suggestive of hyperactivation, with the addition of cyclic AMP analogues or phosphodiesterase inhibitors (such as caffeine and pentoxifylline) has also been reported in human (Burkman, 1984; Nassar et al, 1999; Bajpai and Doncel, 2003), hamster (Mrnsy and Meizel, 1980), mouse (Fraser, 1981), bovine (Ho and Suarez, 2001; Marques and Suarez, 2004), and cynomolgus macaque (Mahony and Gwathmey, 1999) spermatozoa. In the latter study, hyperactivation was observed in 58% of capacitated cynomolgus macaque spermatozoa and was associated with a significant

increase in tyrosine phosphorylation of sperm tail proteins. We have also demonstrated an increase in tyrosine phosphorylation with capacitation in rhesus macaque spermatozoa (Baumber and Meyers, unpublished data). As described by Mahony and Gwathmey (1999) we also observed a high percentage of the sperm population exhibiting hyperactivation. In vitro capacitation of human spermatozoa is asynchronous (Austin, 1952; Bedford, 1983), as evidenced by the low proportion of spermatozoa exhibiting hyperactivation at any given time (Oehninger et al, 1997). Macaque sperm treated with dbcAMP and caffeine display more synchrony; this may be due in part to these exogenous stimulators bypassing the initial receptor-mediated events that would elicit the appropriate signaling cascade. The fact that macaque spermatozoa do not spontaneously capacitate in vitro may be advantageous in utilizing these sperm as a model to elucidate the requirements for stimulation of hyperactivated motility and sperm-zona pellucida interaction.

Both caffeine and dbcAMP are presumed to elicit the same effect, namely an increase in intracellular levels of cAMP. If caffeine and dbcAMP were acting through similar mechanisms in macaque sperm, both compounds should not be necessary to promote hyperactivation. However, in this study, the addition of caffeine and dbcAMP individually did not result in sperm hyperactivation, and this finding is supported by Vandevoort et al (1994) with cynomolgus macaque spermatozoa. It is possible that caffeine and dbcAMP together are more efficient at raising cAMP levels than either alone. Perhaps a longer incubation time or a higher concentration may be required to promote hyperactivation when the activators are used separately. Si and Okuno (1999) demonstrate a significant increase in hyperactivation of hamster spermatozoa following a 90-minute incubation with dbcAMP. Preliminary experiments in our laboratory have demonstrated that increasing the concentration of dbcAMP to 10 mmol did not promote hyperactivation, whereas 10 mmol caffeine did, supported by Boatman and Bavister (1984).

Caffeine has also been reported to promote hyperactivation in bull (Ho and Suarez, 2001; Marques and Suarez, 2004) and human (Mbizvo et al, 1993) spermatozoa. Experiments with bovine sperm have demonstrated additional effects of caffeine on other cellular processes that may contribute to its effectiveness in enhancing sperm motility and fertilization (Vijayaraghavan et al, 1985; Schoff and Lardy, 1987; Tajik and Niwa, 1998; Ho and Suarez, 2001; Pavlok et al, 2001; Marquez and Suarez, 2004). Caffeine is known for inducing intracellular calcium release by activating ryanodine receptors (Zucchi and Ronca-Testoni, 1997) and has also been found to activate calcium-permeable cation channels in the plasma membrane to increase intracellular calcium (Guerrero et al, 1994; Schoppe et al, 1997). Calcium is a key player in the initiation of sperm hyperactivation (Suarez et al, 1993; Yanagimachi, 1994; Ho and Suarez, 2003). These studies suggest that the difference in the response of spermatozoa to caffeine and dbcAMP alone may involve effects of caffeine that are unrelated to an increase in intracellular cAMP. A discussion of the mechanisms regulating sperm hyperactivation is beyond the scope of this manuscript; the authors refer readers to excellent reviews on this topic (Mortimer, 1997; Kay and Robertson, 1998; Suarez and Ho, 2003).

H-89 is a specific PKA inhibitor that operates by competing with ATP at the catalytic subunit of the kinase (Engh et al, 1996). Evidence of its PKA-inhibiting properties have been reported in sperm cells (Leclerc et al, 1996; Galantino-Homer et al, 1997; Harrison and Miller, 2000). Visconti et al (1995) showed that mouse spermatozoa treated with H-89 displayed decreased capacitation-associated tyrosine phosphorylation, acrosome reactivity, and IVF potential. The dramatic effects of H-89 on sperm motility during the longer (2.5-hour) incubation period in this study demonstrate the importance of cAMP/PKA pathways in maintaining sperm motility. A decline in sperm motion parameters following treatment with PKA inhibitor has also been reported for human (Leclerc et al, 1996) and hamster (Si and Okuno, 1999) spermatozoa. The effect of H-89 on sperm motility may be specifically related to the activation of PKA during capacitation. In a report by Bajpai and Doncel (2003), incubation of mouse spermatozoa in capacitation media for 6 hours in the presence of H-89 only slightly decreased motion parameters, and hyperactivation was not affected. However, when mouse spermatozoa were incubated for 90 minutes in capacitation media plus dbcAMP and pentoxifylline to stimulate PKA, the addition of H-89 resulted in a significant decrease in motility parameters and a significant decrease in hyperactivation. Although H-89 reduced the percentage of hyperactivated spermatozoa during the shorter (30-minute) incubation period without an effect on total motility, we assume this reflects an overall negative impact of H-89 on the ability of spermatozoa to swim rather than the specific inhibition of the execution of sperm hyperactivation.

The results of this paper demonstrate that hyperactivation can be initiated in macaque spermatozoa following incubation with dbcAMP and caffeine, and the percentage of hyperactivated spermatozoa can be reliably estimated for rhesus macaque spermatozoa utilizing CASA software.

References

Abaigar T, Holt WV, Harrison RA, del Barrio G. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. *Biol Reprod*. 1999;60:32–41.

Austin CR. The capacitation of the mammalian sperm. *Nature*. 1952; 170:326.

Bajpai M, Doncel GF. Involvement of tyrosine kinase and cAMP-dependent kinase cross-talk in the regulation of human sperm motility. *Reproduction*. 2003;126:183–195.

Baltz JM, Katz DF, Cone RA. Mechanics of sperm-egg interaction at the zona pellucida. *Biophys J*. 1988;54:643–654.

Baltz JM, Williams PO, Cone RA. Dense fibers protect mammalian sperm against damage. *Biol Reprod*. 1990;43:485–491.

Bedford JM. Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol Reprod*. 1983;28:108–120.

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

Boatman DE, Robbins RS. Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. *Biol Reprod*. 1991;44:806–813.

Burkman LJ. Characterization of hyperactivated motility by human spermatozoa during capacitation: comparison of fertile and oligozoospermic sperm populations. *Arch Androl*. 1984;13:153–165.

Burkman LJ. Discrimination between nonhyperactivated and classical hyperactivated motility patterns in human spermatozoa using computerized analysis. *Fertil Steril*. 1991;55:363–371.

Cancel AM, Lobdell D, Mendola P, Perreault SD. Objective evaluation of hyperactivated motility in rat spermatozoa using computer-assisted sperm analysis. *Hum Reprod*. 2000;15:1322–1328.

Demott RP, Suarez SS. Hyperactivated sperm progress in the mouse oviduct. *Biol Reprod*. 1992;46:779–785.

Drobnis EZ, Yudin AI, Cherr GN, Katz DF. Hamster sperm penetration of the zona pellucida: kinematic analysis and mechanical implications. *Dev Biol*. 1988;130:311–323.

Engh RA, Girod A, Kinzel V, Huber R, Bossemeyer D. Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulfonyl protein kinase inhibitors H7, H8, and H89. Structural implications for selectivity. *J Biol Chem*. 1996;271:26157–26164.

ESHRE Andrology Special Interest Group. Consensus workshop on advanced diagnostic andrology techniques. *Hum Reprod*. 1996;11: 1463–1479.

Fraser LR. Dibutyryl cyclic AMP decreases capacitation time in vitro in mouse spermatozoa. *J Reprod Fertil*. 1981;62:63–72.

Galantino-Homer HL, Visconti PE, Kopf GS. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3′5′-monophosphate-dependent pathway. *Biol Reprod*. 1997;56:707–719.

Griveau JF, Le Lannou D. Effects of antioxidants on human sperm preparation techniques. *Int J Androl*. 1994;17:225–231.

Grunert JH, De Geyter C, Nieschlag E. Objective identification of hyperactivated human spermatozoa by computerized sperm motion analysis with the Hamilton-Thorn sperm motility analyser. *Hum Reprod*. 1990;5:593–599.

Guerrero A, Fay FS, Singer JJ. Caffeine activates a Ca(2+)-permeable, nonselective cation channel in smooth muscle cells. *J Gen Physiol*. 1994;104:375–394.

Harrison RA, Miller NG. cAMP-dependent protein kinase control of plasma membrane lipid architecture in boar sperm. *Mol Reprod Dev*. 2000;55:220–228.

Ho HC, Suarez SS. An inositol 1,4,5-trisphosphate receptor-gated intracellular Ca(2+) store is involved in regulating sperm hyperactivated motility. *Biol Reprod*. 2001;65:1606–1615.

Ho HC, Suarez SS. Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. *Biol Reprod*. 2003;68:1590–1596.

Katz DF, Dott HM. Methods of measuring swimming speed of spermatozoa. *J Reprod Fertil*. 1975;45:263–272.

Katz DF, Yanagimachi R. Movement characteristics of hamster spermatozoa within the oviduct. *Biol Reprod*. 1980;22:759–764.

Katz DF, Mills RN, Pritchett TR. The movement of human spermatozoa in cervical mucus. *J Reprod Fertil*. 1978;53:259–265.

Katz DF, Drobnis EZ, Overstreet JW. Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. *Gamete Res*. 1989;22:443–469.

Kay VJ, Robertson L. Hyperactivated motility of human spermatozoa: a review of physiological function and application in assisted reproduction. *Hum Reprod Update*. 1998;4:776–86.

Leclerc P, de Lamirande E, Gagnon C. Cyclic adenosine 3′,5′monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol Reprod*. 1996;55:684–692.

Le Lannou D, Griveau JF, Le Pichon JP, Quero JC. Effects of chamber depth on the motion pattern of human spermatozoa in semen or in capacitating medium. *Hum Reprod*. 1992;7:1417–1421.

Mahony MC, Gwathmey T. Protein tyrosine phosphorylation during hyperactivated motility of cynomolgus monkey (*Macaca fascicularis*) spermatozoa. *Biol Reprod*. 1999;60:1239–1243.

Marquez B, Suarez SS. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. *Biol Reprod*. 2004;70: 1626–1633.

Mazzilli F, Rossi T, Delfino M, Dondero F, Makler A. A new objective method for scoring human sperm hyperactivation based on head axis angle deviation. *Int J Androl*. 2001;24:189–196.

Mbizvo MT, Johnston RC, Baker GH. The effect of the motility stimulants, caffeine, pentoxifylline, and 2-deoxyadenosine on hyperactivation of cryopreserved human sperm. *Fertil Steril*. 1993; 59:1112–1117.

McNutt TL, Olds-Clarke P, Way AL, Suarez SS, Killian GJ. Effect of follicular or oviductal fluids on movement characteristics of bovine sperm during capacitation in vitro. *J Androl*. 1994;15: 328–336.

Mortimer ST. A critical review of the physiological importance and analysis of sperm movement in mammals. *Hum Reprod Update*. 1997;3:403–439.

Mortimer ST, Maxwell WM. Kinematic definition of ram sperm hyperactivation. *Reprod Fertil Dev*. 1999;11:25–30.

Mortimer ST, Mortimer D. Kinematics of human spermatozoa incubated under capacitating conditions. *J Androl*. 1990;11:195–203.

Mortimer ST, Swan MA. Kinematics of capacitating human spermatozoa analysed at 60 Hz. *Hum Reprod*. 1995;10:873–879.

Mrnsy RJ, Meizel S. Evidence suggesting a role for cyclic nucleotides in acrosome reactions of hamster sperm in vitro. *J Exp Zool*. 1980;211:153–157.

Nassar A, Mahony M, Morshedi M, Lin MH, Srisombut C, Oehninger S. Modulation of sperm tail protein tyrosine phosphorylation by pentoxifylline and its correlation with hyperactivated motility. *Fertil Steril*. 1999;71:919–923.

Neill JM, Olds-Clarke P. A computer-assisted assay for mouse sperm hyperactivation demonstrates that bicarbonate but not bovine serum albumin is required. *Gamete Res*. 1987;18:121–140.

Oehninger S, Mahony M, Ozgur K, Kolm P, Kruger T, Franken D. Clinical significance of human sperm-zona pellucida binding. *Fertil Steril*. 1997;67:1121–1127.

Olds-Clarke P. Sperm from tw32/+ mice: capacitation is normal, but hyperactivation is premature and nonhyperactivated sperm are slow. *Dev Biol*. 1989;131:475–482.

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

Pacey AA, Davies N, Warren MA, Barratt CL, Cooke ID. Hyperactivation may assist human spermatozoa to detach from intimate association with the endosalpinx. *Hum Reprod*. 1995;10: 2603–2609.

Pang SC, Chan PJ, Lu A. Effects of pentoxifylline on sperm motility and hyperactivation in normozoospermic and normokinetic semen. *Fertil Steril*. 1993;60:336–343.

Pavlok A, Kubelka M, Peknicova J. The effect of various capacitation active compounds and capacitation time on the in vitro fertility and protein tyrosine phosphorylation profiles of bovine sperm. *Zygote*. 2001;9:25–38.

Phillips DM. Comparative analysis of mammalian sperm motility. *J Cell Biol*. 1972;53:561–573.

Robertson L, Wolf DP, Tash JS. Temporal changes in motility parameters related to acrosomal status: identification and characterization of populations of hyperactivated human sperm. *Biol Reprod*. 1988;39:797–805.

Sarason RL, VandeVoort CA, Mader DR, Overstreet JW. The use of nonmetal electrodes in electroejaculation of restrained but unanesthetized macaques. *J Med Primatol*. 1991;20:122–125.

Schoff PK, Lardy HA. Effects of fluoride and caffeine on the metabolism and motility of ejaculated bovine spermatozoa. *Biol Reprod*. 1987;37:1037–1046.

Schoppe J, Hochstrate P, Schlue WR. Caffeine mediates cation influx and intracellular Ca2+ release in leech P neurones. *Cell Calcium*. 1997;22:385–397.

Si Y, Okuno M. Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. *Biol Reprod*. 1999;61:240–246.

Stauss CR, Votta TJ, Suarez SS. Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. *Biol Reprod*. 1995;53:1280–1285.

Suarez SS. Hyperactivated motility in sperm. *J Androl*. 1996;17: 331–335.

Suarez SS, Dai X. Hyperactivation enhances mouse sperm capacity for penetrating viscoelastic media. *Biol Reprod*. 1992;46: 686–891.

Suarez SS, Ho HC. Hyperactivated motility in sperm. *Reprod Domest Anim*. 2003;38:119–124.

Suarez SS, Osman RA. Initiation of hyperactivated flagellar bending in mouse sperm within the female reproductive tract. *Biol Reprod*. 1987;36:1191–1198.

Suarez SS, Katz DF, Overstreet JW. Movement characteristics and acrosomal status of rabbit spermatozoa recovered at the site and time of fertilization. *Biol Reprod*. 1983;29:1277–1287.

Suarez SS, Katz DF, Owen DH, Andrew JB, Powell RL. Evidence for the function of hyperactivated motility in sperm. *Biol Reprod*. 1991;44:375–381.

Suarez SS, Dai XB, DeMott RP, Redfern K, Mirando MA. Movement characteristics of boar sperm obtained from the oviduct or hyperactivated in vitro. *J Androl*. 1992;13:75–80.

Suarez SS, Varosi SM, Dai X. Intracellular calcium increases with hyperactivation in intact, moving hamster sperm and oscillates with the flagellar beat cycle. *Proc Natl Acad Sci U S A*. 1993;90: 4660–4664.

Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Definition of the optimal criteria for identifying hyperactivated human spermatozoa at 25 Hz using in-vitro fertilization as a functional end-point. *Hum Reprod*. 1995;10:2928–2937.

Tajik P, Niwa K. Effects of caffeine and/or heparin in a chemically defined medium with or without glucose on in vitro penetration of bovine oocytes and their subsequent development. *Theriogenology*. 1998;49:771–777.

Tollner TL, Yudin AI, Cherr GN, Overstreet JW. Soybean trypsin inhibitor as a probe for the acrosome reaction in motile cynomolgus macaque sperm. *Zygote*. 2000;8:127–137.

VandeVoort CA, Tollner TL, Overstreet JW. Sperm-zona pellucida interaction in cynomolgus and rhesus macaques. *J Androl*. 1992;13: 428–432.

VandeVoort CA, Tollner TL, Overstreet JW. Separate effects of caffeine and dbcAMP on macaque sperm motility and interaction with the zona pellucida. *Mol Reprod Dev*. 1994;37:299–304.

Vijayaraghavan S, Critchlow LM, Hoskins DD. Evidence for a role for cellular alkalinization in the cyclic adenosine 3′,5′-monophosphate-mediated initiation of motility in bovine caput spermatozoa. *Biol Reprod*. 1985;32:489–500.

Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development*. 1995;121:1139–1150.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction*. New York, NY: Raven Press; 1994;189–317.

Young RJ, Bodt BA. Development of computer-directed methods for the identification of hyperactivated motion using motion patterns developed by rabbit sperm during incubation under capacitation conditions. *J Androl*. 1994;15:362–377.

Yudin AI, Goldberg E, Robertson KR, Overstreet JW. Calpain and calpastatin are located between the plasma membrane and outer acrosomal membrane of cynomolgus macaque spermatozoa. *J Androl*. 2000;21:721–729.

Zhu JJ, Pacey AA, Barratt CL, Cooke ID. Computer-assisted measurement of hyperactivation in human spermatozoa: differences between European and American versions of the Hamilton-Thorn motility analyser. *Hum Reprod*. 1994;9:456–462.

Zucchi R, Ronca-Testoni S. The sarcoplasmic reticulum Ca2+ channel/ ryanodine receptor: modulation by endogenous effectors, drugs and disease states. *Pharmacol Rev*. 1997;49:1–51.