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Temporal Changes in Motility Parameters Related to Acrosomal Status: Identification and Characterization of Populations of Hyperactivated Human Sperm

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ABSTRACT

The occurrence and time course of capacitation, acrosomal loss, and hyperactivated motility require quantitative definition in order to characterize fertile human sperm. In this study, video microscopy and digital image analysis were used to measure curvilinear (VCL) and straight line (VSL) velocity, average linearity of progression (LIN [100 × VSL/VCL]), maximum and mean amplitude of lateral head displacement (ALH), beat-cross-frequency (BCF), DANCE (VCL × meanALH) and DANCEMEAN (meanALH/(LIN/100)). These parameters were measured for sperm in semen and in the swim-up fraction of washed cells during incubation for up to 24 h under in vitro fertilization (IVF) conditions. Acrosomal loss was monitored in the same population of washed cells by an immunofluorescence end-point assay.

The greatest increase in mean values of motility parameters was observed when seminal sperm were washed free of seminal plasma. Increases continued for up to 6 h of incubation. Two subpopulations of hyperactivated sperm were identified; one type, not found in semen, showed star-spin trajectories, and constituted 3.0, 3.8, 4.5, and 4.1% of the swim-up population after 0, 3, 6 and 24 h of incubation. The second type, termed transitional showed a more progressive trajectory and constituted less than 1% in semen. In total, hyperactivated cells constituted 0.8% of cells in semen, 14.5% of the swim-up population with no incubation, and 23.1, 22.7, and 19.4% after 3, 6, and 24 h of incubation, respectively. Acrosomal loss in the swim-up population was delayed during the first 3 h of incubation, then increased from near 5% at 3 h to 7 and 12% at 6 and 24 h, respectively. The kinetics of change in the extent of hyperactivation and in acrosomal loss, although measured in different cell populations, are consistent with an association between these two events.

INTRODUCTION

To extend studies of human male infertility, it has become important to characterize and quantify "normal," fertile human sperm. Sperm require a period of maturation subsequent to ejaculation and exit from seminal plasma, called capacitation, before attaining the ability to undergo an acrosome reaction and fertilize an egg (Austin, 1951; Chang, 1951; Bedford, 1983). Although capacitation kinetics have been established for a number of mammalian species (Rogers, 1978; Yanagimachi, 1981), the characteristics and the time course of capacitation have not been clearly established in humans. With either acrosomal change or the ability to penetrate zona-free hamster oocytes as indicators, capacitation is initiated in some cells almost immediately upon exit from seminal plasma and continues over at least a 24-h time period (Perreault and Rogers, 1982; Byrd and Wolf, 1986; Lee et al., 1987).

Capacitation results in no obvious, easily discernible, morphological alterations. However, sperm of many mammalian species undergo a change in the type of flagellar motility expressed, from progressive, linear motility to less progressive, less linear, more vigorous,

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hyperactivated motility. Hyperactivated motility was first described in hamster epididymal sperm (Yanagimachi, 1970) and subsequently in a number of other mammalian species (Yanagimachi, 1972; Mahi and Yanagimachi, 1976; Fraser, 1977; Cooper et al., 1979; Cummins, 1982; Boatman and Bavister, 1984; Neill and Olds-Clarke, 1987) including humans (Burkman, 1984; Mortimer et al., 1984). Hyperactivation has been characterized by wide amplitude flagellar beat, marked lateral head displacement, "star-spin" trajectory, and low forward progression, and it is considered an integral part of capacitation preceding sperm binding to the zona pellucida and the occurrence of the acrosome reaction. With nonhuman species, more than 80% of sperm show hyperactivated motility (Yanagimachi, 1970; Katz and Yanagimachi, 1980). Initially, human sperm in capacitating medium were not thought to show hyperactivation (Yanagimachi, 1981), although changes in the type of motility displayed in synthetic medium were recognized (Mortimer et al., 1983, 1984). Subsequently, it was demonstrated that approximatley 20% of washed cells developed hyperactivation (Burkman, 1984). Since the development of hyperactivated motility is considered an integral part of the capacitation process and thus prerequisite for fertilization in other mammalian species, it is obviously important to establish the relationship between hyperactivated motility, the acrosome reaction, and fertility in human sperm. The recent validation of a computer-assisted digital image analysis system has allowed the rapid measurement of a range of motility parameters in a large number of individual sperm (Mack et al., 1988). By viewing the trajectories of individual sperm, two distinct subpopulations of hyperactivated sperm have been identified and characterized. Investigation of the temporal relationship between changes in sperm motility and the occurrence of spontaneous acrosomal loss, although measured in different aliquots of sperm, suggests an association between these events. In the population studied, our results revealed that motility changes (including hyperactivation) occurred prior to spontaneous acrosomal loss.

MATERIALS AND METHODS

Semen Collection

Semen samples were collected from a panel of 14 healthy adult donors of presumed or proven fertility participating in an Artificial Insemination Donor (AID) program, showing a normal spermiogram with concentration $> 50 \times 10^6$/ml, > motility, > 60% normal morphology, with at least 48 h abstinence requested. Samples were collected by masturbation and allowed to liquefy for 30–60 min prior to processing.

Sperm Processing

Samples of whole semen were taken for videotaping immediately after liquefaction. The remainder of the ejaculate was washed, at room temperature, by the addition of 3 volumes of Ham's F-10 containing 7.5% maternal serum (heat-treated), pH 7.4, at 280 mOsm/l and centrifugation at $200 \times g$ for 5 min. The supernatant was decanted, replaced with fresh medium, and centrifuged for 10 min. The supernatant was again decanted, and the pellet was carefully overlaid with a volume of fresh medium equivalent to that of the original. The samples were incubated for 24 h at 37°C in 5% CO_2 in air, in a 20° angled rack. Aliquots were removed from the upper quarter of the supernatant to obtain the highly motile swim-up population for videotaping and acrosomal status determination. Analysis was performed immediately after overlaying the pellet with fresh medium (Time 0 = no incubation) and after 0.5, 1, 2, 3, 6, and 24 h of incubation. If both the swim-up population and the whole population were studied, the ejaculate was divided into two tubes after the first wash. One tube was assigned to the "swim-up population" group and treated as above. The second tube was assigned to the "whole population" group and the pellet was thoroughly resuspended immediately before the withdrawal of each aliquot for analysis. Sperm counts were performed in duplicate with a hemocytometer. The percent motility was estimated by subjective scoring.

Videomicroscopy

Sperm motility was analyzed using CellSoft™ Ver. 3.5lc (CryoResources, Ltd., New York, NY) computer-assisted digital image analysis system. This system, previously validated for quantifying specific motility parameters of human sperm (Mack et al., 1988), was set up as shown in Table 1. Samples were viewed through a Nikon Optiphot microscope with a 20× BM phase objective. Aliquots (5 μl) of sample were pipetted into a cell chamber of 32-μm depth (Mack et al., 1988) on a 37°C heated microscope

TABLE 1. CellSoft set-up parameters used for the quantification of human sperm.

Frame rate (Hz)	30
Number of consecutive frames to analyze	23
Minimum sampling for motility	4
Minimum number of consecutive points for VCL, VSL, and LIN*	13
Minimum number of consecutive points for ALH and BCF*	13
Maximum velocity (μm/s)	400
Threshold velocity (μm/s)	10
Threshold gray level	---[a]
Pixel scale (μm/pixel)	0.602
Dilution factor	0.302[b]
Cell size range (pixels)	4–50

[a]Adjusted such that the digitized head images were 3–4 mm in diameter on a 17-inch (43.2-cm) monitor using an underscan feature.

[b]CellSoft assumes a chamber depth of 10 μm for calculation of sperm concentration parameters. This dilution factor was used to compensate for the deeper chamber (32 μm) used in these studies.

*VCL = curvilinear velocity; VSL = straight line velocity; LIN = average linearity of progression; ALH = lateral head displacement; BCF = beat-cross frequency.

stage and videotaped for 2–5 min. Motility parameters did not differ significantly when measured in a 10-μm Makler chamber as opposed to the 32-μm cell chamber (unpublished results). Multiple viewing areas (20–30) on each slide and duplicate slides from each sample (75–100 motile cells) were videotaped at an NTSC standard 30 frames/s and analyzed with CellSoft. The motility parameters curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), linearity of forward progression (LIN; 100 × VSL/VCL), maximum (maxALH) and mean (meanALH) amplitude of lateral head displacement (μm), beat-cross frequency (BCF, Hz) and 2 new, derived parameters called DANCE (VCL × meanALH, $\mu m^2/s$) and DANCEMEAN (meanALH × VCL/VSL), μm/s) were calculated for individual cells: trajectories were viewed by using CellSoft Research Module and the motility parameters of individual cells were calculated with the "Cell Parameters" option.

Acrosomal Status

Acrosomal status was established with an indirect immunofluorescence assay utilizing a monoclonal antibody developed by us (Byrd and Wolf, 1986; Ochs et al., 1986) and commercially available (Humagen, Charlottesville, VA). Aliquots (100–200 μl) of the washed sperm, at each time point, were pelleted by centrifugation at 200 × *g* for 5 min and resuspended in 1% paraformaldehyde in PBS (phosphate-buffered saline). Sperm were washed in 0.2 M glycine and resuspended in Dulbecco's PBS at a concentration of approximately 1×10^6 sperm/ml. Aliquots (5–10 μl) of the suspension were air-dried in duplicate tape wells on a slide. Since fixed sperm did not lose antigenicity with storage over 24 h, samples were scored immediately after processing the 24-h time points. Slides were wet with PBS containing 0.01% (v/v) NP-40, 0.1% 2-mercaptoethanol, and 1% (w/v) bovine serum albumin (Solution A) during processing. Sperm were incubated with hybridoma supernatant (5 μl) at 2.5 mg/ml for 30–60 min, washed with Solution A, then incubated with goat anti-mouse fluorescein isothiocyanate (FITC) for 30–60 min. To avoid bias, slides were assigned a random number prior to scoring: two wells on each slide and a minimum of 100 cells in each well were scored. Cells with acrosomal cap fluorescence were scored acrosome-intact; those showing no cap fluorescence or showing only a band of fluorescence in the equatorial region were scored as acrosome-negative, as described previously (Ochs et al., 1986; Byrd and Wolf, 1986).

Statistical Analysis

Statistical analysis was carried out with the statistics package Systat (Version 3.1, Systat Inc., 2902 Central Street, Evanston, IL) on a Compaq Deskpro 386 microcomputer with 80387 math coprocessor. Data were analyzed by Student's *t*-test and analysis of variance for normally distributed populations as determined by probability plots. Pearson and likelihood ratio chi-square and box-and-whisker plot analyses were used to determine significance between non-normally distributed groups (McGill et al., 1978). Box plots were set to give 95% confidence intervals.

RESULTS

Motility Parameters in Semen and Washed Cells

Motility parameters were measured in semen and washed cells (Table 2). Samples of the swim-up were taken immediately after washing and overlay of medium (0) and after 0.5, 1, 2, 3, 6, and 24 h of incubation, videotaped and analyzed by digital image analysis. These results represent data from multiple ejaculates. All time points were not always analyzed for each sample. Significant increases ($p<0.001$) in all sperm motility parameters were observed upon

TABLE 2. Motility parameters* in seminal and in washed swim-up sperm preparations.

	Donors (n)	Cells (n)	VCL	VSL	LIN	maxALH	meanALH	BCF	DANCE	DANCEMEAN
Semen	10	594	27.6 ± 7.2[a]	11.9 ± 4.9	36 ± 7	2.0 ± 0.5	1.5 ± 0.4	10.5 ± 1.0	73 ± 47	6.8 ± 2.4
Swim-up										
0 hr	13	990	89.1 ± 17.4[bd]	42.9 ± 8.2[bd]	48 ± 8[bd]	6.0 ± 1.5[bd]	4.7 ± 1.2[bd]	14.5 ± 1.8[bd]	590 ± 244[bd]	17.4 ± 8.4[b]
0.5 h	3	235	97.9 ± 23.5	47.1 ± 7.8	50 ± 4	5.1 ± 1.5	4.9 ± 1.3	15.1 ± 0.9	662 ± 287	15.9 ± 6.1
1 h	3	269	98.4 ± 26.8	45.2 ± 7.1	48 ± 5	6.5 ± 1.9	5.3 ± 1.8	14.7 ± 0.6	738 ± 367	17.6 ± 7.2
2 h	3	317	105.0 ± 19.4	50.5 ± 6.5	52 ± 5	6.5 ± 1.0	5.2 ± 0.9	15.7 ± 1.1[e]	753 ± 307	18.8 ± 6.7
3 h	13	1121	108.3 ± 20.9[ce]	47.4 ± 8.1	45 ± 8[e]	7.5 ± 1.7[ce]	6.0 ± 1.4[e]	14.4 ± 1.7	854 ± 316[ce]	21.1 ± 8.8
6 h	10	957	112.8 ± 27.8[ce]	50.7 ± 10.6[e]	48 ± 10	7.4 ± 2.3[e]	5.9 ± 1.9[e]	14.9 ± 1.7	880 ± 397[ce]	20.6 ± 10.3
24 h	9	580	93.8 ± 24.5	41.3 ± 12.0	45 ± 8[e]	6.3 ± 1.8[e]	5.1 ± 1.4	13.2 ± 2.2	612 ± 267	18.0 ± 8.0

[a]All values represent the mean ± standard deviation of the mean determinations for each donor sample processed as described in *Materials and Methods*.

[b]Means are significantly different ($p<0.001$) from values in semen.

[c]Significantly different ($p<0.05$) from wash value at 0 h. Statistical comparisons *a*, *b*, and *c* were made by *t*-test of paired time points.

[d]Medians are significantly different ($p<0.05$) from values for seminal cells.

[e]Medians are significantly different ($p<0.05$) from 0 h wash time.

*VCL = curvilinear velocity; VSL = straight line velocity; LIN = average linearity of progression; ALH = lateral head displacement; BCF = beat-cross frequency; DANCE = VCL × meanALH; DANCEMEAN = meanALH/(LIN/100).

removal of sperm from semen by washing. All parameters increased by 2-fold or greater except for LIN (33%) and BCF (38%). During subsequent incubation under in vitro fertilization (IVF) conditions, VCL, maxALH, and DANCE increased (27%, 25%, and 45%, respectively) to significantly higher values by 3–6 h ($p<0.05$), after which there was an apparent, but not statistically significant, drop in all parameters at 24 h.

Characterization of Hyperactivated Motility

In order to define the trajectory characteristics of hyperactivated sperm, a subpopulation (120 out of a total of 3085 cells; pooled data from 0-, 3-, 6-, and 24-h time points) was identified (by visual inspection) that displayed trajectories similar to the star-spin described by Burkman (1984), with vigorous activity and low net progression (for examples, see Fig. 1). The mean values for the motility parameters of these cells were obtained with the CellSoft Research Module. All motility parameters were significantly ($p<0.001$) different from the corresponding mean values for the nonhyperactivated sperm population (Table 3). Cells with star-spin trajectories were not found in semen but constituted 3.0, 3.8, 4.5, and 4.1% of the washed swim-up population sampled at 0, 3, 6, and 24 h of incubation, respectively (as determined by subjective observation).

From observations of a variety of species, a linearity (LIN, sometimes referred to as progressiveness ratio) of 34 has generally been the upper limit (mean plus one standard deviation) for hyperactivated nonhuman sperm in vivo (Katz and Yanagimachi, 1980). An LIN of 16 ± 11 characterized the star-spin trajectories described in this study. By increasing the acceptable maximum LIN to an upper limit of 34, a second subpopulation of cells could be identified (examples of trajectories shown in Fig. 1). Mean values for the motility parameters of these cells, termed the "transitional" type of hyperactivation, are shown in Table 3. These cells were identified according to the criteria set below and were found to be present in semen at <1% and constituted 11.5, 19.3, 18.2, and 15.3% of the swim-up population sampled, after 0, 3, 6 and 24 h of incubation, respectively. In total, both types of hyperactivated cells accounted for 0.8% of cells in semen and 14.5, 23.1, 22.7, and 19.4% of washed sperm populations after 0, 3, 6, and 24 h of incubation, respectively. Mean values for all of the motility parameters of each of the hyperactivated subpopulations did not change significantly with time. However, the difference in percentage hyperactivation (both types) between no incubation and 3 h of incubation was significant ($p<0.001$). Similar results were obtained when the data were compared by using median values and 95% confidence limits (Table 3). On the basis of the motility values obtained for the star-spin subpopulation, the following criteria were

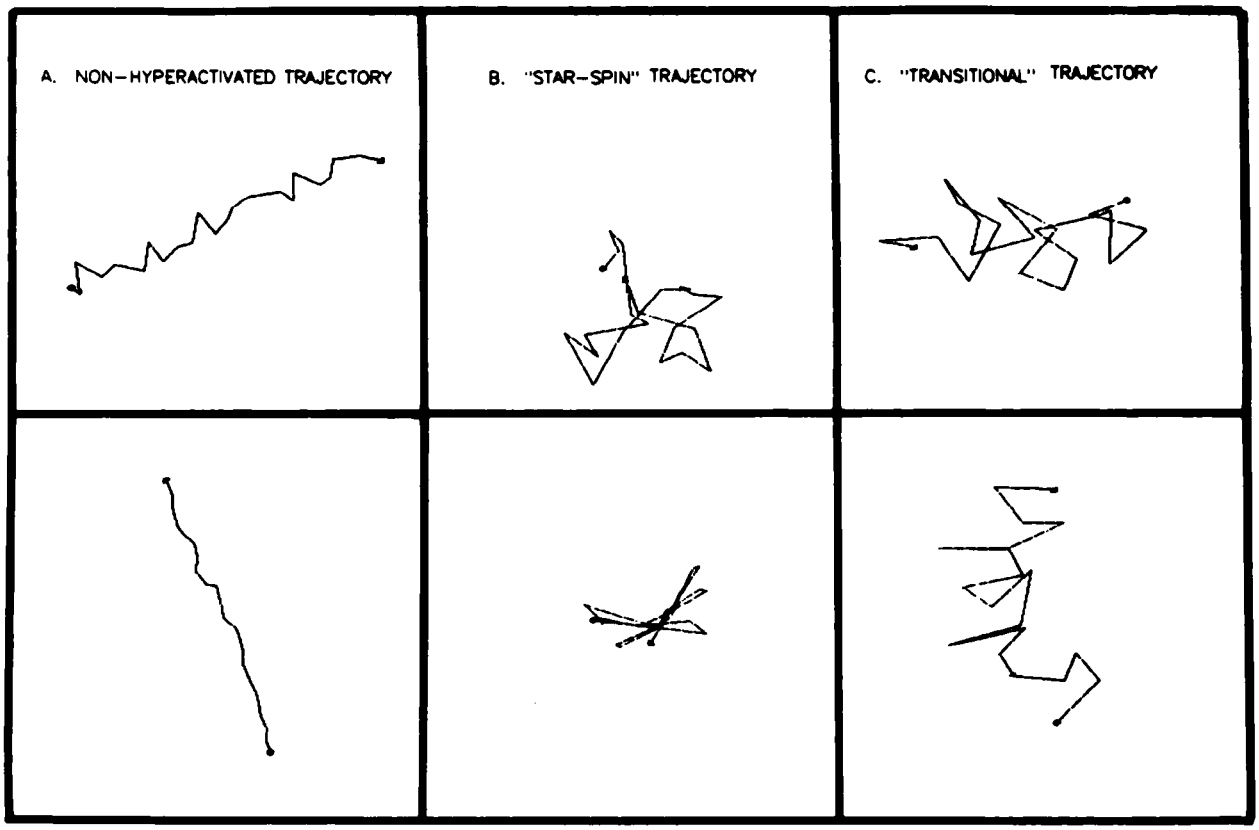

FIG 1. Representative trajectories from nonhyperactivated, star-spin hyperactivated, and transitional hyperactivated human sperm. Examples of nonhyperactivated sperm *(column A)*, star-spin hyperactivated *(column B)*, and transitional hyperactivated *(column C)* sperm were selected by visual inspection and confirmed by the criteria presented in the text. The trajectories were plotted from the original x, y positions with the Research Module of CellSoft™ (CryoResources, NY).

chosen to assign cells to this subpopulation: VCL>80 μm (based on mean −1.5 standard deviations), LIN≤19 (based on mean + 1.5 standard deviations), and DANCEMEAN≥17 μm/s (based on histograms of the distribution of DANCEMEAN values for nonhyperactivated vs. star-spin values, where maximal separation of the two groups was observed). The transitional hyperactivated group used the same limits for

TABLE 3. Motility parameters* of nonhyperactivated, star-spin, transitional, and the total population of hyperactivated human sperm.

	n	VCL	VSL	LIN	maxALH	meanALH	BCF	DANCE	DANCEMEAN
Nonhyperactiated	2409	90.3 ± 50.4[a]	48.9 ± 19.6	53 ± 37	5.8 ± 3.7	4.6 ± 3.1	15.3 ± 5.2	561 ± 589	11 ± 12
All hyperactivated	592	157.6 ± 37.9	36.9 ± 15.1[b]	23 ± 8	11.8 ± 3.1	9.6 ± 2.7	10.4 ± 4.0	1593 ± 779	55 ± 78
Star-spin (visual)	120	149.9 ± 41.1	22.4 ± 14.4[c]	16 ± 11[c]	10.9 ± 3.4[c]	8.4 ± 2.8	8.7 ± 4.1[c]	1362 ± 749[c]	107 ± 163[c]
Star-spin (algorithm)	162	158.1 ± 38.5	19.4 ± 8.9[c]	12 ± 5[c]	12.0 ± 3.5[c]	9.2 ± 3.0	8.5 ± 3.9[c]	1552 ± 825[c]	107 ± 140[c]
Transitional	472[d]	157.4 ± 37.7	42.8 ± 11.8[b]	27 ± 4	11.7 ± 2.9	9.7 ± 2.6	10.9 ± 3.9	1607 ± 763	37 ± 12

[a]All values are mean ± standard deviation for all cells, irrespective of sample (not mean of means). Seminal sperm were excluded from the nonhyperactivated group.

[b]The mean and median values were not significantly different from the values for nonhyperactivated cells; all other values were significantly different (means: $p<0.001$; medians: $p<0.05$).

[c]These medians for the star-spin subgroup were significantly different from the transitional hyperactivation subgroup.

[d]These cells were identified by the criteria described in the *text*.

*See footnote to Table 2 for definitions.

TABLE 4. A comparison of subjective identification and computer-assisted identification of trajectories of hyperactivated human sperm in semen and in swim-up preparations.

	Seminal sperm	Washed sperm
Total no. of cells	754	2789
Hyperactivated trajectories* identified subjectively	8	520
Hyperactivated trajectories* identified by computer program	8	516
% Efficiency	100%	>99%

*Hyperactivated trajectories include both transitional and star-spin subpopulations.

VCL and DANCEMEAN, but LIN was >19 and ≤34. Cells not meeting these criteria were considered nonhyperactivated. With these criteria, a computer program was constructed and tested for its ability to identify hyperactivated sperm in semen and in swim-up populations. Both types of hyperactivated cells were identified with >99% efficiency when compared to visual determination (Table 4). The values for motility parameters of visually identified star-spin trajectories were not significantly different from star-spin trajectories identified by the mathematical criteria (Table 3).

Assessment of Acrosomal Status in Swim-up and Total Sperm Populations

To determine if a relationship exists between motility changes and acrosomal loss in sperm subpopulations, sperm were incubated under IVF conditions. Each sample (at least triplicate) was divided into two groups: 1) an elite swim-up population, prepared as in the previous experiments; and 2) a total-suspension population obtained by mixing the washed sample immediately before taking an aliquot for analysis. Motility parameters, acrosomal status, cell concentration, and percent motility of the swim-up and of the whole population were determined with no incubation and after 0.5, 3, 6, and 24 h of incubation (Table 5). Motility values for the swim-up population (Fig. 2, upper graph) showed time-dependent changes similar to those seen in previous experiments (Table 2). Values for VCL, VSL, LIN, ALH, BCF, and DANCE for the whole population were significantly lower than for the swim-up population with no incubation ($p<0.001$) and remained lower during the entire 24-h incubation (Fig. 2, lower graph). DANCEMEAN and percentage hyperactivation were significantly different from 30 min of incubation onwards ($p<0.05$). The swim-up cells showed a low level of acrosomal loss that remained constant at about 5% through the 3-h time point but increased at 6 and 24 h. All values for acrosomal loss were significantly lower than the whole population at 0, 3, and 6 h of incubation ($p<0.05$). These results suggest that acrosomal loss, DANCEMEAN, and percentage hyperactivation are influenced by the contribution of cells that are not normally capable of swimming up from the pellet.

Temporal Relationship between Motility Changes and Spontaneous Acrosomal Loss

The mean values for percentage of hyperactivated cells at 0, 0.5, 1, 2, 3, 6, and 24 h were calculated from the per cell data used to compile Tables 2 and the percent acrosomal loss (Table 5 plus data from 10 additional donors) were plotted against time of incubation after washing (Fig. 3). The peak rate of increase of hyperactivated motility occurred within the first 2 h, prior to the peak rate of spontaneous acrosomal loss (between 3 and 6 h). The percentage

TABLE 5. Comparison of acrosomal loss, percent motility, and concentration of the swim-up population and the whole population of sperm during 24 h incubation.

	Acrosome loss (%)		Motile (%)		Sperm concentration (× 10^6/ml)	
Incubation time (hr)	Swim-up	Whole	Swim-up	Whole	Swim-up	Whole
0	5.2 ± 1.2[a]	12.0 ± 1.0	83.0 ± 5.5	66.7 ± 3.4	14.3 ± 4.2	68.3 ± 36.8
0.5	5.0 ± 1.5	18.3 ± 4.9	88.0 ± 2.1	67.7 ± 2.8	15.3 ± 5.5	78.0 ± 39.3
3	5.0 ± 1.0	15.0 ± 1.5	93.3 ± 0.7	65.0 ± 1.5	14.3 ± 2.6	73.3 ± 39.3
6	7.0 ± 2.1	14.8 ± 1.4	93.0 ± 0.6	63.3 ± 3.8	11.3 ± 2.0	75.7 ± 43.0
24	11.9 ± 4.8	28.0 ± 5.3	70.7 ± 11.8	27.0 ± 16.3	2.0 ± 0.6	72.3 ± 36.0

[a]Values represent mean ± SEM for each sample.

of cells showing hyperactivation increased significantly by 30 min of incubation ($p<0.01$), whereas spontaneous acrosomal loss measured in a second aliquot of sperm from the same suspension showed a marked lag after washing and did not show a significant increase until 6 h. These results are consistent with the hypothesis that changes in flagellar motility, specifically the occurrence of hyperactivated motility, precedes the occurrence of an acrosome reaction in free-swimming sperm.

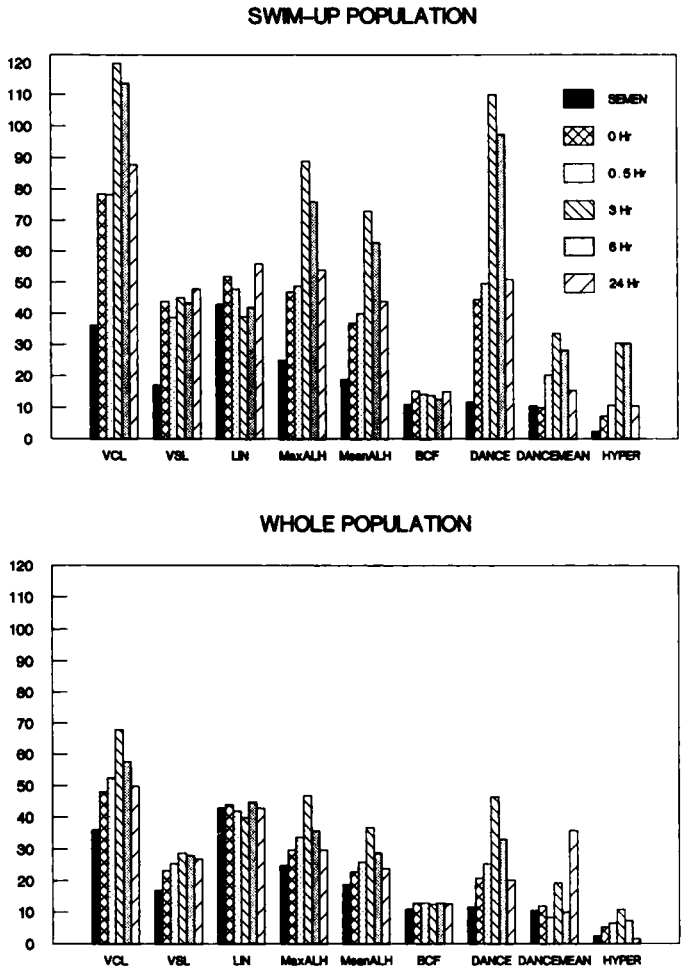

FIG. 2. Comparison of temporal changes in motility parameters of swim-up sperm vs. resuspended total populations of sperm. Motility parameters were measured in swim-up *(upper graph)* sperm samples or in aliquots of the same starting population but resuspended *(lower graph)* immediately prior to sampling for motility analysis. Values for all motility parameters (except DANCEMEAN and percent hyperactivation) in the swim-up population at 0 h were significantly higher than the resuspended population at 0 h ($p<0.001$). DANCEMEAN and percent hyperactivation were significantly higher at 30 min ($p<0.05$). All parameters stayed significantly higher in the swim-up group at subsequent time points, except LIN at 3 h and BCF at 6 h. Units for each parameter are as follows: VCL and VSL (μm/s), LIN (none), Max and MeanALH (μm X 10), BCF (Hz), DANCE ($\mu m^2/s$), DANCEMEAN μm), and HYPER (%). HYPER represents both transitional and star-spin subpopulations. The y-axis is the value for each of the motility parameters (except for ALH, see above). (Abbreviations defined in *Materials and Methods.*)

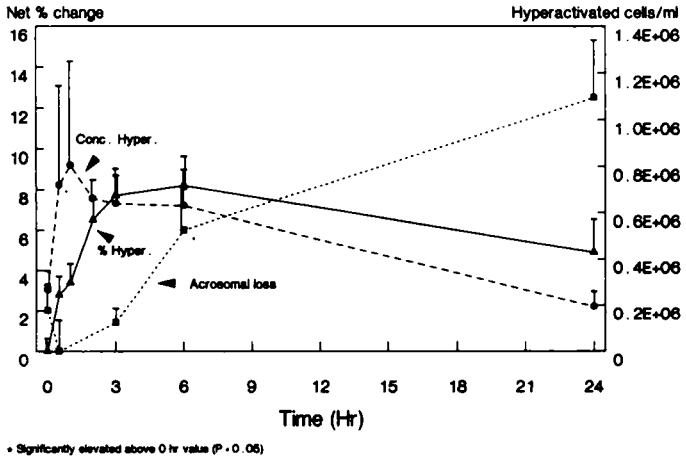

FIG. 3. Temporal relation between percent and concentration of hyperactivated cells and acrosomal loss in human sperm. Values for hyperactivated cells represent both star-spin and transitional subgroups. *Error bars* represent the mean ± SEM for pooled data. Values indicated with (*) represent the first time point significantly ($p<0.05$) elevated above the background value. *Conc. Hyper.* represents the concentration of hyperactivated cells per ml at each time point.

DISCUSSION

In this study, an automated method was developed to identify and count two subpopulations of hyperactivated cells in the swim-up population of washed cells. One population of cells displayed star-shaped trajectories that corresponded to those classified as star-spin by Burkman (1984); a second population, which we termed transitional, more closely approximated hyperactivation as originally described by Yanagimachi (1970). The rationale for using this term is based on the observation that the type 5 (hyperactivated) cell described by Mack et al. (1988), when examined by the criteria set forth in this study, showed both the star-spin and transitional type of hyperactivation, depending on which portion of the trajectory was used for the analysis. Since the major distinguishing feature of the two types of hyperactivated trajectories is LIN, and LIN for transitional cells is intermediate between star-spin and nonhyperactivated trajectories, it is logical to assume that transitional hyperactivation may be an intermediate between star-spin and nonhyperactivation. Further verification that human hyperactivated sperm switch between these two types of motility, as do rabbit sperm (Johnson et al., 1981), remains to be determined. Hyperactivated cells were initially identified subjectively, by inspection of the trajectory. On the

basis of data gathered from the star-spin cells and the LIN established in nonhuman mammalian species, a computer program was written that was 99% effective in identifying hyperactivated sperm when compared with subjective visual inspection of cell trajectories. Although hyperactivated motility has been studied extensively in a wide variety of mammalian species (Yanagimachi, 1972; Mahi and Yanagimachi, 1976; Fraser, 1977; Cooper et al., 1979; Cummins, 1982; Boatman and Bavister, 1984; Neill and Olds-Clarke, 1987) including man (Burkman, 1984; Mortimer et al., 1984), this study represents the first automated method of measuring this important subpopulation of sperm in man solely by objective criteria (patent pending for some of the material presented here that may be applied to the diagnosis of fertility). This method also does not rely on manual selection of cells or manual determination of the x, y position of the cells, as is required by previously published methods (Mortimer et al., 1983, 1984; Neill and Olds-Clarke, 1987).

Analysis of the motility parameters of the swim-up population of human sperm showed that the greatest increase in mean values occurred after washing sperm free from seminal plasma. A significant difference between washed sperm and sperm in seminal plasma has been demonstrated by other workers (Mortimer et al., 1984; Mack et al., 1988). Viscosity might influence motility in seminal plasma. Human sperm in Hank's solution of increased viscosity show reduced rotation of the sperm head about the longitudinal axis, reduced amplitude of flagellar bend, and reduced velocity compared to sperm in normal Hank's solution (Ishijima et al., 1986). Alternatively, the removal of adsorbed seminal plasma components from the sperm surface during washing may trigger changes in regulatory components such as cyclic adenosine 3′, 5′-monophosphate or Ca^{2+}, thereby leading to motility changes. The immediate appearance of hyperactivated motility as an early manifestation of capacitation was reported by Burkman (1986). However, it should be noted that in the present studies additional significant increases in motility parameters were observed 3–6 h after the initial changes that occurred upon washing.

The motility parameters of the swim-up and whole-cell populations were significantly different from each other immediately after washing (Fig. 2), with the important exception of the parameter DANCEMEAN and the percentage hyperactivation, which are significantly different by 30 min of incubation, suggesting that hyperactivation continues to develop over a period of time. Since a far greater proportion of cells develop hyperactivation in the swim-up than in the whole population (3 times as many at 3 h, Fig. 2), there may be a subpopulation of sperm within the general population selected by swim-up that more readily undergoes capacitation. Although hyperactivation may be a key component of capacitation, it is possible that the two may not be coupled (Neill and Olds-Clarke, 1987; Suarez et al., 1987).

Investigation of the temporal relationship between changes in the motility parameters and the occurrence of spontaneous acrosomal loss in the swim-up population revealed that the motility parameters VCL, ALH, DANCE, DANCEMEAN and the percentage of cells showing hyperactivated motility increased initially to plateau levels, whereas spontaneous acrosomal loss as measured in a second sperm aliquot, after an initial lag period, increased for the entire 24-h incubation period. The development of hyperactivation under capacitation conditions may therefore precede the occurrence of an acrosome reaction in free-swimming sperm. ALH, one of the criteria for identifying hyperactivated cells in this study, has already been linked to fertility of human sperm (Aitken, 1985; Jeulin et al., 1986; Mortimer et al., 1986).

Several authors have also demonstrated that a significant increase in acrosomal loss occurs when washed sperm populations are incubated under IVF conditions (Plachot et al., 1984; Mallet et al., 1985; Byrd and Wolf, 1986). The techniques employed for quantifying acrosomal loss cannot readily differentiate between physiologic and degenerative changes. Since spontaneous acrosomal loss over time reflects—at least in part—sperm senescence, this parameter alone may be inadequate for assessing fertilizing potential unless standardized sperm preparation techniques are adopted. Hyperactivation, however, may prove a useful marker of the progression of capacitation prior to the occurrence of acrosomal loss in the normal fertile cell. The quantification of hyperactivation and the measurement of motility parameters of the swim-up population using the methods described here is efficient, rapid, and objective, making it feasible to study individual cell performance in male factor infertility patients on a routine basis. Studies designed to optimize the conditions for monitoring hyperactivation, prerequisite to defining the relationship between hyperacti-

vation and fertilizing ability in the IVF situation, are currently underway.

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