Development of Computer-Directed Methods for the Identification of Hyperactivated Motion Using Motion Patterns Developed by Rabbit Sperm During Incubation Under Capacitation Conditions

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ABSTRACT: Rabbit spermatozoa developed motions that mimicked hyperactivated motility during incubation for 16–20 hours under capacitation conditions and in several other commonly used media. Sperm from some rabbits failed to acquire this type of motility, and sperm from others failed to survive the long incubation time. Four motility patterns developed during incubation for 16–20 hours. Motility parameters measured by the CellSoft and CellTrak motion analysis systems were similar except for the average amplitude of lateral head displacement. Multivariate discriminant analysis with complementary regression analysis, and an unrelated tree structured classification method (CART®), were used to derive rules, based on motility parameters, for the objective classification of sperm into the two motility classes: 1) nonhyperactivated motility and 2) hyperactivated motility or motility that mimicked hyperactivated motility. The motility parameter wobble (WOB) was superior to the commonly used parameter, linearity, as a classifier of motility types. It classified sperm into the two motility groups with 96.6% efficiency and, together with curvilinear velocity (VCL), attained classification efficiencies of 98%. The classification model produced by CART was preferred over the one obtained by discriminant analysis. The rule for motility classification was dependent on the motion analysis system used to measure the motion parameters. The rule for the CellSoft system, WOB ≤0.78 and VCL ≥51 µm/second, classified sperm with an efficiency of 98%, whereas the rule for the CellTrak system, WOB ≤0.69 and VCL ≥55 µm/second, achieved a classification efficiency of 97%. These rules should facilitate the study of sperm hyperactivation and its role in sperm function.

Key words: Hyperactivated motility, multivariate discriminant analysis, CART®, classification, modeling.

J Androl 1994;15:362–377

Mammalian sperm develop distinctive motion patterns during capacitation known as hyperactivated motility. This is characterized by a change from progressive movement to a highly vigorous, nonprogressive random motion with large lateral displacement of the sperm head and wide-amplitude flagella movement (Yanagimachi, 1970, 1988); it is a component of capacitation (Chang, 1984; Yanagimachi, 1988). Studies in the mouse (Fraser, 1977), guinea pig (Flemming and Yanagimachi, 1982), and human (Burkman, 1984; Morales et al, 1988; Robertson et al, 1988; Burkman et al, 1990; Coddington et al, 1991; Wang et al, 1991) have pointed to an association between hyperactivation, *in vitro* fertilization, and the occurrence of the acrosome reaction (Robertson et al, 1988). The role of hyperactivated motility in fertilization has received some attention, but it is still unresolved (Katz et al, 1989; Tesarik et al, 1990; Suarez et al, 1991; Suarez and Dai, 1992; Demott and Suarez, 1992).

The relationship between hyperactivated motility and fertilization suggests its use as an indicator of capacitation and as a tool for the clinical assessment of fertility (Pang et al, 1993). Hyperactivated motility may also be useful for the study of the physiological significance and mechanism of hyperactivation as well as a marker for the investigation of sperm function (Neill and Olds-Clarke, 1987; Grunert et al, 1990; Murad et al, 1992; Olds-Clarke and Johnson, 1993). As a first step an objective method for both the recognition and quantitation of hyperactivated sperm in a mixed population of hyperactivated and non-hyperactivated sperm is required. Analysis of the motility patterns found in human sperm incubated under capacitating conditions (Burkman, 1984, 1991; Hoshi et al, 1988; Morales et al, 1988; Robertson et al, 1988; Mortimer and Mortimer, 1990) with manual or automated computer-assisted motion analysis systems has determined that the motility parameters linearity (LIN), average amplitude of lateral head displacement (AALH), maximum amplitude of lateral head displacement (MALH), curvilinear velocity (VCL) (Robertson et al, 1988; Mortimer and Mortimer, 1990; Grunert et al, 1990; Burkman, 1991), and curvature ratio (CR) (Morales et al,

1988) are suitable criteria for the detection of hyperactivated human sperm.

The wobble parameter (WOB) and VCL were also selected as the criteria for hyperactivated sperm for the CF1 strain of mouse (Neill and Olds-Clarke, 1987), but LIN and VCL were subsequently preferred for sperm from mice carrying the t complex (Olds-Clarke, 1989; Olds-Clarke and Johnson, 1993). Species differences in hyperactivated motility patterns have been noted (Blottner et al, 1989; Olds-Clarke, 1989), and it is not clear whether the motility parameters or their subjectively determined threshold values, used for the classification of human and mouse sperm hyperactivated motility, are applicable to sperm from other species. The selection of LIN and VCL for mouse sperm hyperactivation classification indicates that some degree of commonality does exist between the motility characteristics of hyperactivated mouse and human sperm.

The motility patterns of capacitated rabbit sperm recovered from does several hours after mating have been reported, but there is little information on the motility parameters of such sperm and no information on the hyperactivation of rabbit sperm *in vitro*. Rabbit sperm are capacitated in 6 hours *in vivo* (Bedford, 1970), but they require a minimum of 16 hours of incubation *in vitro* (Brackett et al, 1982). After 6 hours of incubation in a capacitation medium, the quality of rabbit sperm motility has been poor, and sperm with motility patterns characteristic of hyperactivated motility are absent (Young et al, 1992). To determine if motility patterns of rabbit sperm change during long incubation, medium and incubation conditions conducive to the maintenance of rabbit sperm motility during long incubation were investigated. The results show that the motility patterns of rabbit sperm after 6 and 16–18 hours of incubation in capacitation medium are different; those after 16 hours, but not after 6 hours, mimick the motility patterns reported for capacitated rabbit sperm recovered several hours after mating (Johnson et al, 1981; Suarez et al, 1983). When the subjectively determined motility parameter threshold values for identification of hyperactivated mouse and human sperm were applied to rabbit sperm incubated for 2–6 hours, many nonhyperactivated sperm were mistakenly chosen as hyperactivated. To establish criteria suitable for the classification of rabbit sperm with the two different motility patterns in mixed populations, the motility patterns of nonhyperactivated sperm, and those after 16–18 hours of incubation under capacitation conditions (Brackett and Oliphant, 1975; Brackett et al, 1982), were analyzed by the CellSoft and CellTrak motion analysis systems. The motility parameters were analyzed using standard discriminant and complementary regression analysis and newer tree-structured classification methods, CART® and FACT®. Computer modeling and discriminant analysis show that WOB, rather than the commonly used LIN, is the primary classifier of sperm that, after 16–18 hours of incubation under capacitating conditions, developed motility patterns mimicking hyperactivated motility. VCL was a secondary classifier, serving to refine the classification. In this report, and in the absence of an alternate name, hyperactivated motility has been used to describe the motion patterns acquired by rabbit sperm incubated under the conditions defined for *in vitro* capacitation of rabbit sperm (Brackett et al, 1982), although the biological status of sperm in the present experiments is unknown.

Materials and Methods

Animals

Male New Zealand white rabbits (Hazleton Research Products, Denver, Pennsylvania) were individually housed in standard rabbit cages in a room maintained at 20–22°C and 50 ± 10% relative humidity with a 12-hour light/dark cycle. Certified approved laboratory rabbit chow (Zeigler Brothers, Gardners, Pennsylvania) and water were available *ad libitum*.

Media and Incubation

Rabbit semen was collected with an artificial vagina and washed, either by centrifugation through a Percoll gradient, as previously described (Young et al, 1992), or by suspension in medium and centrifugation for 5 minutes at 350 × g at room temperature. The pellet was gently resuspended and washed once with medium. Each experiment was carried out with sperm from a single ejaculate, and the treatment regimen was repeated with sperm from three or more rabbits. Sperm motions were visually assessed by phase-contrast microscopy at a final magnification of 100× after incubation for 1, 3, 5–6, and 18–20 hours.

Incubation Conditions—The ejaculate was divided into several aliquots, and one, the control, was treated as described (Brackett and Oliphant, 1975; Brackett et al, 1978 1982) for capacitation of ejaculated and epididymal rabbit sperm and subsequent *in vitro* fertilization. The crucial step in capacitation and fertilization is incubation of sperm for at least 12 hours (Akruk et al, 1979; Hosoi et al, 1981; Viriyapanich and Bedford, 1981; Brackett et al, 1982). Briefly, the incubation steps and media defined by Brackett and Oliphant (1975) are as follows: semen is washed with defined medium (DM), a simple salts medium containing bovine serum albumin (BSA; 290–305 mosmol/kg), followed by preincubation (15 minutes, 37°C) in high ionic strength (HIS) medium (DM with osmolarity increased to 380 mosmol/kg by addition of NaCl), before washing and resuspension in DM and incubation at 37°C under an atmosphere of 8% O_2, 5% CO_2, and 87% N_2. The other aliquots were treated identically except for the condition under study. Thus, to determine the effect of exposure to HIS or of preincubation, on motility, one aliquot was washed, preincubated in DM instead of HIS, and washed again before incubation, or after two washes in DM the preincubation step was omitted. The effect of incubation

conditions on sperm that had been centrifuged through a Percoll gradient was investigated in the same way.

Effect of Serum—Rabbit, horse, and calf sera were obtained from GIBCO Laboratories (Grand Island, New York). Sera were inactivated by heating at 56°C for 1 hour, or by heating briefly to 85°C and centrifugation at 10,000 × g for 15 minutes at 4°C to remove denatured protein. Aliquots of sperm, washed with DM or by centrifugation through Percoll, were resuspended, without preincubation in HIS or DM, in DM with BSA replaced by 20% rabbit, horse, or calf serum. Control sperm were resuspended in DM. Incubation was carried out at 37°C under 95% air and 5% CO_2.

Alternate Medium—Aliquots of the ejaculate or Percoll-washed sperm were washed and resuspended, without preincubation or exposure to HIS, in either DM, Biggers Whitten Whittingham (BWW) medium (Biggers et al, 1971), the medium (TC) of Toyoda and Chang (1974), or modified medium T6 (Mohr and Trounson, 1984), and incubated at 37°C in 5% CO_2 in air.

Motion Analysis

Motility of sperm was recorded on videotape with the system previously described (Young et al, 1992). A 5- to 7-μl drop of sperm suspension (1–1.3 × 10^7/ml) was placed in a 20-μm-deep chamber (Cryo Resources Ltd., Montgomery, New York), prewarmed to 37°C on a microscope slide warmer, and covered with a 22 × 22 mm coverslip or a 20 × 26 mm hemacytometer cover glass. Videotaping was carried out at 37°C with a Dage NC-67M videocamera mounted on an Olympus BH-2 microscope equipped with a 10× negative phase-contrast objective, a 6.7× projection ocular, and a heating stage. The CellSoft Series 3000 with the ALH, Beat Frequency, Circular Motion and Research modules (Cryo Resources Ltd.) and the CellTrak/S v.3.22B (Santa Rosa, California) CASA systems were used to analyze sperm track trajectories. CellSoft software settings for analysis of nonhyperactivated cell were those determined previously (Young et al, 1992). To accommodate the rapid and random motion of cells with hyperactivated motility and their movement in and out of focus of the videocamera, the maximum velocity setting was increased to 500–1,200 μm/second and the pixel size range changed to 1–120 pixels. The gray level setting was adjusted for each analysis. Even with those settings several attempts were frequently necessary to successfully track a sperm for 15 frames, and it was not possible to track some cells for a minimum of 15 frames (0.5 second). Motility parameters measured by the CellSoft system were VCL, LIN, maximum amplitude of lateral head displacement (MALH), AALH, beat cross frequency (BCF), straight line velocity (VSL), straightness (STR, VSL/VAP), and WOB. Measures for WOB and STR were obtainable only on a sperm-by-sperm basis by laborious use of the Research Module.

The software settings for the CellTrak system were as follows: frame rate, 30 frames/second; duration of frame capture, 30 frames; minimum path length, 15 frames; minimum burst speed, 20 μm/second; maximum burst speed, 500 μm/second; distance scale factor, 1.839 μm/pixel; camera aspect ratio, 1.0; ALH path smoothing factor, 7 frames; centroid X and Y search neighborhood, 4 and 2 pixels, respectively; centroid cell size minimum and maximum, 2 and 25 pixels respectively; maximum path interpolation, 1 frame; and path prediction percentage, 0%. The

threshold was adjusted before each analysis by using all four edges, but only the left edge was used for analysis. The same settings were used for analysis of both hyperactivated and nonhyperactivated cells because it is possible to compensate for the low burst speed of 500 μm/second by changing the search mask size with the system EV language during each analysis. This system was able to track more hyperactivated sperm than the CellSoft system for a minimum of 15 frames at the first attempt. It should be noted the CellTrak system uses VSL rather than VCL to select motile cells for the computation of the percentage of motile sperm and sperm kinematics. Consequently, the values reported for percentage motile sperm and motility parameters are not applicable to sperm samples containing hyperactivated sperm or circularly moving sperm, such as those from rabbit and bull. The system provided information only for AALH, VCL, and LIN on a routine basis, but values for the motility parameters VSL, WOB, and STR for every sperm tracked in each analysis were routinely obtained by modification of the system Funkey6 batch file.

Statistical Modeling

The motility parameters of 322 sperm obtained by incubation of sperm from four rabbits for 16–20 hours under the capacitation conditions of Brackett and Oliphant (1975) and Brackett et al (1982), and 899 nonhyperactivated sperm from another three rabbits incubated for 1 or 2 hours in T6 medium, were used for the statistical analysis. Sperm with motility patterns mimicking those of hyperactivated sperm were visually selected based on criteria defined by Yanagimachi (1970, 1988), Johnson et al (1981), and Suarez et al (1983). These sperm populations contained all four motility types, and these, together with sperm in the progressive phase of biphasic hyperactivated motion and sperm in transition from one phase to the other, were used for statistical analysis. Only sperm that could not be tracked for a minimum of 15 frames (0.5 seconds) were excluded from analysis. The inclusion of the latter two groups of cells as a subset of all the hyperactivated cells used in the modeling process provides a greater challenge to developing an accurate classification model because, in addition to their appearance toward progressiveness, their motility parameters values begin to look more progressive and thus they are harder to classify. A wide range of motion patterns is represented in the 322 cells analyzed. At least 29% of the hyperactivated sperm population consists of the erratic and star classes of hyperactivated sperm—WOB values for these sperm are ≤0.3–0.4 (Figs. 1B and 2)—and the distribution of WOB values in the population is smooth and not skewed to high values (Figs. 7D and 8D). The WOB values of the circular and biphasic classes of sperm are in the range ≥0.5 (Figs. 1A and 4A,B), showing that not all sperm in the high range of WOB values are of the progressive class. The use of a heterogeneous mix of hyperactivated motility patterns enhances the general applicability of the model developed. Earlier studies demonstrated that sperm with the motility characteristics of hyperactivated sperm were absent after 2 hours of incubation. The 899 nonhyperactivated sperm were unselected, being all of the motile sperm present in the fields analyzed. The derived motility parameters Dance (VCL*AALH), and Dancemean (AALH*VCL/VSL or AALH/LIN) (Robertson et al, 1988), to-

gether with the previously defined motility parameters, were used to support hyperactivated motility classification.

The statistical modeling was completed in three stages. The classifying potential of an individual motility parameter was assessed by contrasting its distribution properties under the conditions of hyperactivated and nonhyperactivated motility. Tools included simple univariate statistics, boxplots, histograms, the Mann-Whitney Test, and the Squared Ranks Test. The classifying potential of motility parameter combinations was then explored in a traditional manner, initially using scatter plots generated by STATISTICA/w® 1993 and BMDP 1983, program 6D, followed by model building with stepwise discriminant analysis and binary regression using BMDP statistical software (BMDP 1983, programs 7M, 1R, and 9R). Finally, recent advances in classification methods were utilized using Classification and Regression Trees (CART, version 1.1, 1985 California Statistical Software, Inc.) and a Fast Algorithm for Classification Trees (FACT® version 1.1, 1988, Software Development and Distribution Center, MACC, University of Wisconsin). CART was principally used with the results being corroborated by FACT. The CART routine offers many options; only the defaults were used. Final results for misclassification errors were computed using cross validation.

Classical discriminant analysis can be used to build classification models based on one or more motility parameters. Discriminant analysis forms a one-dimensional index indicating class membership based on a linear compound of the motility parameters. In this two-class environment, discriminant analysis is analogous to performing a regression analysis on a binary (0,1) class variable, assigning an observation to class one if the regression model predicts 0.5 or greater and to class zero otherwise. Both regression and discriminant analysis software were used to develop classification models. Model performance is assessed in terms of the number of misclassified cells, the explained variation r^2 (the proportion of the variation associated with classification that is explained by the motility parameter in the model or equivalently 1 − Wilk's lambda), the degree of collinearity (strong linear dependence between or among motility parameters) in the model, and parsimony (using the least number of motility parameters for classification in the model without penalty to classification efficiency).

Generally, CART works as follows for univariate partitions. Each possible predictor variable (motion parameter) for class is examined individually. For a specific variable, the program searches all values, resting at each to see how efficient it would be to partition the data into hyperactivated and nonhyperactivated classes based on that value. (In our data set this requires over 1,200 assessments of efficiency for each variable.) The routine notes the best value for that variable based on classification efficiency. The variable that partitions the data in the most efficient manner is selected, and its value is used as the first partition of the data, creating two nodes, one each for hyperactivated and nonhyperactivated classes. Within each node, some cells may be misclassified. The routine then searches among the variables looking to further partition the two nodes to increase efficiency. The routine eventually settles on a decision tree for classification with maximum efficiency subject to the constraint that the tree complexity should not be great. An advantage of tree-structured methods is that they employ an objective approach that is not bound by a linear model and may lend itself to easier interpretation and use.

Results

Incubation Conditions

Motility of rabbit sperm was good for the first 3-hour incubation, and a high percentage of sperm were motile under the capacitation conditions described by Brackett and Oliphant (1975). After 5–6 hours of incubation, the percentage of motile sperm, the frequency of head rotation, and the progressive velocity were much reduced. Sperm usually were immotile after 16–20 hours of incubation. Sperm motility after long incubation was specific for individual rabbits; percentage motility of sperm from some rabbits was as high as 60% at 5–6 hours, and 5–25% of sperm from others were still motile at 16–20 hours. Using DM instead of HIS for preincubation, omitting preincubation with HIS or DM, incubation under an atmosphere of 95% air and 5% CO_2 in place of 5% CO_2, 8% O_2, and 87% N_2 with or without preincubation, or washing sperm by centrifugation through a Percoll gradient before incubation did not produce any visually discernible change in sperm motility. In each experiment (N = 3–5) sperm received identical treatments except for the condition under study. Substituting BSA with 20% heat-inactivated rabbit, horse, or calf serum (N = 5 for each serum) did not confer any survival advantage during long incubation in DM. Sperm formed aggregates in the presence of sera, and the range of motile sperm was 15–40% at 5 hours and 0–20% at 16–20 hours. Replacing DM with modified T6 medium (Mohr and Trounson, 1984), medium TC (Toyoda and Chang, 1974), or BWW medium had a positive effect on sperm motility (N = 3–5 for each medium). Improvement of sperm motility was best with T6 and least with BWW. Five to 40% of sperm, when incubated without preincubation for 16–20 hours in T6, under an atmosphere of 5% CO_2 and 95% air, were still motile, whereas the motility range of identically treated sperm suspended in DM was 0–10%. For TC and BWW, the range was 1–30% and 0–15%, respectively.

Small numbers (2–3%) of sperm from some males developed the motility characteristics reported for hyperactivated sperm of the rabbit and other species (Cooper et al, 1979; Johnson et al, 1981; Suarez et al, 1983; Yanagimachi, 1988) after 3–5 hours of incubation in DM under capacitation conditions (Brackett and Oliphant, 1975). Few, if any, sperm with these motility patterns were present when the same sperm suspension was examined after 16–20 hours of incubation. On the other hand, subpopulations of sperm with motility patterns emulating hyperactivated rabbit sperm (Johnson et al, 1981; Suarez et al, 1983) were present at 16–20 hours in suspensions of

sperm from other males. Thirteen to 15 hours earlier these sperm were either quiescent or possessed poor motility, or, although possessing good motility, were devoid of motions mimicking hyperactivated activity. The subpopulation of sperm with hyperactivated motility after 16–20 hours of incubation under the capacitation conditions of Brackett and Oliphant (1975) and Brackett et al (1982) varied with the individual males and was in the range of 10–80% of motile sperm. Acquisition of hyperactivated motility did not require preincubation of sperm in HIS or incubation under 8% O_2 and occurred when DM was replaced by medium T6, medium TC, or BWW medium. Hyperactivated sperm appeared earlier, and their numbers were higher (up to 5%) in T6 incubated under an atmosphere of 95% air and 5% CO_2 than in suspensions of identically treated sperm from the same male incubated in DM. Sperm from up to 40% of the males in the rabbit colony (40–50 males over 3–4 years) did not develop hyperactivated motility. The extent of development of hyperactivated motility in sperm of individual males was relatively consistent, but this varied widely among males. Small subpopulations (4–20%) of sperm, incubated in DM containing 20% rabbit, horse, or calf serum instead of BSA, acquired motions very similar to hyperactivated motility after only 1–3 hours of incubation. The percentage of such sperm in these suspensions after 5–6 or 16–20 hours of incubation was low (1–5%).

Motility Characteristics

The motility patterns of sperm incubated for 16–18 hours under the capacitation conditions defined by Brackett and Oliphant (1975) and Brackett et al (1982) could be grouped into four types. Type I traced essentially circular (Suarez et al, 1983), or star-shaped trajectories (Fig. 1). This pattern was characterized by highly random movements occurring within a confined space that resulted in a multidirectional trajectory with frequent directional changes over 360°. The paths of a second group of sperm (type II) were highly erratic, consisting of a series of bidirectional linear tracks interspersed with abrupt changes in translational movement and/or changes in track orientation, producing an expanded trajectory (Fig. 2). Motion of sperm with type II pattern was three-dimensional and often difficult to track for a minimum of 15 frames. Spermatozoa with type III motility moved progressively but with a jerky motion resulting from a high degree of yawing of the head about the trajectory axis (Fig. 3). Slow videotape playback revealed that these sperm moved with a wide-amplitude flagella beat of variable frequency, the motility characteristics of hyperactivation, but the plane of motion, unlike that in the type I pattern, was not normal to the videocamera. Type IV motility was characterized by combinations of the other three patterns of movement. In this biphasic mode of advancement, first described by Cooper
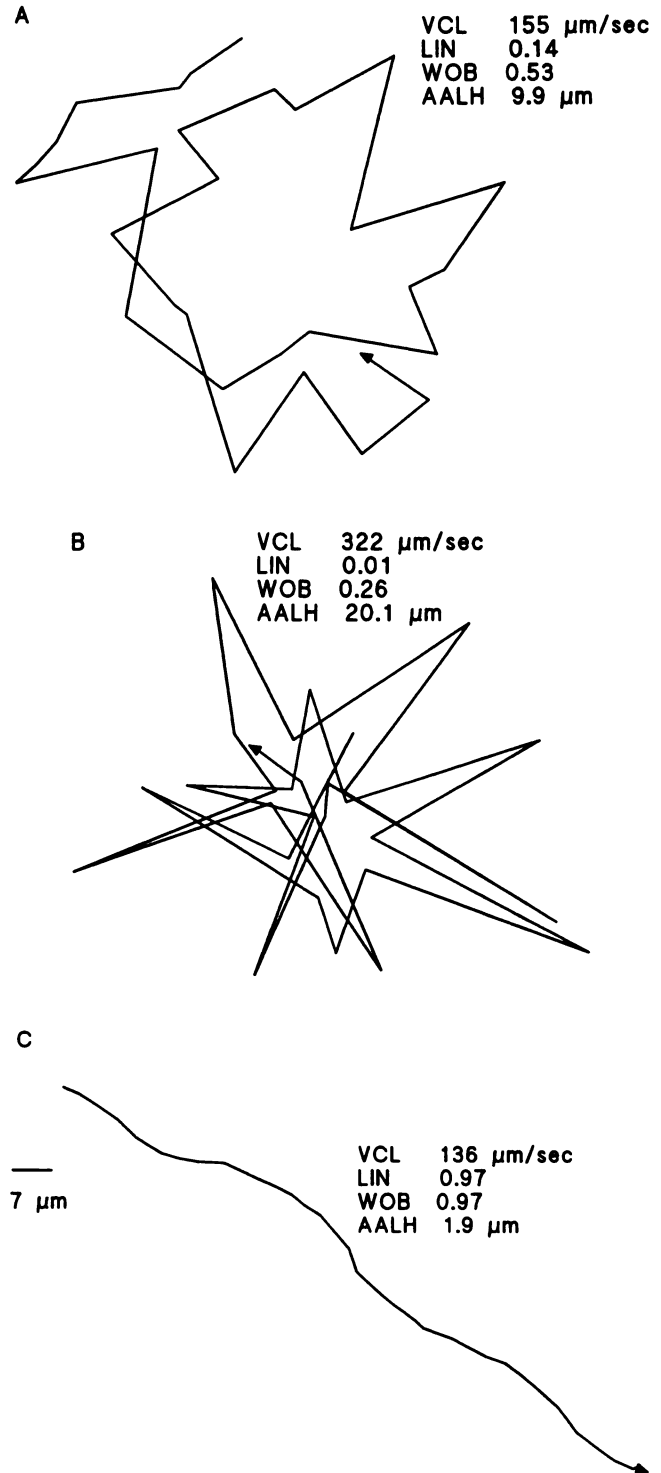

FIG. 1. Type I motility patterns of hyperactivated rabbit spermatozoa. Tracks were obtained with the CellSoft System Research Module. Spermatozoa were tracked at 30 frames/second for a minimum of 15 frames and a maximum of 30 frames. (A) hyperactivated circular patterns; (B) hyperactivated star pattern; and (C) nonhyperactivated sperm track.

A   A

VCL   246 μm/sec
LIN   0.03
WOB   0.08
AALH   10.3 μm

VCL   157 μm/sec
LIN   0.23
WOB   0.36
AALH   8.7 μm

B

VCL   121 μm/sec
LIN   0.06
WOB   0.23
AALH   3.5 μm

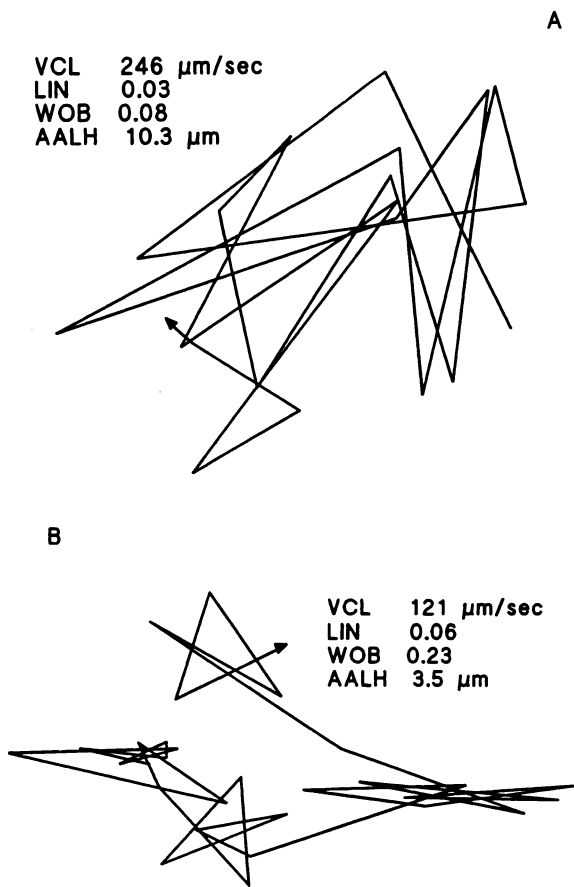B

VCL   123 μm/sec
LIN   0.34
WOB   0.51
AALH   4.0 μm

FIG. 2. Type II erratic motility patterns of hyperactivated rabbit spermatozoa tracked for 30 frames with the CellSoft System. (A) high speed erratic pattern consisting of a series of bidirectional linear tracks with similar orientation but with frequent changes in track orientation. (B) erratic pattern with frequent short translational movement.
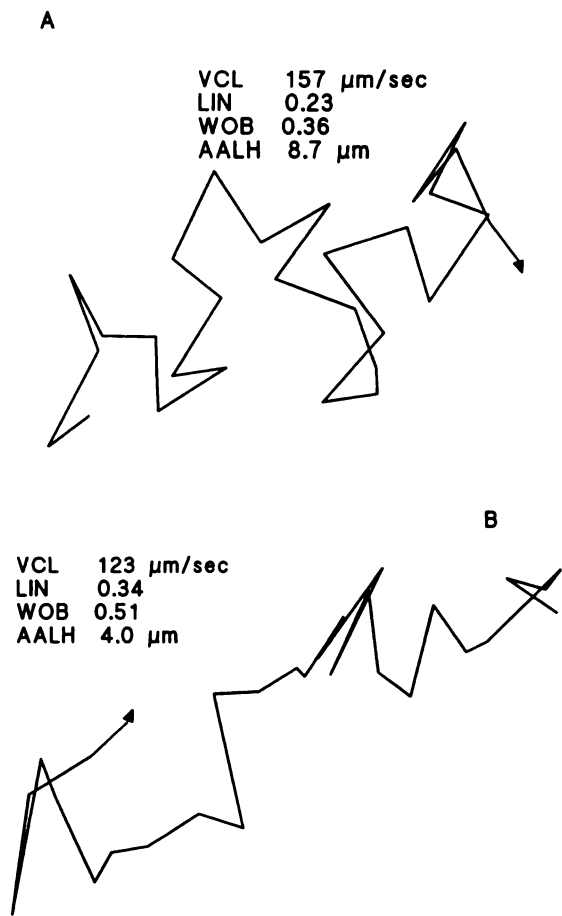
FIG. 3. Type III progressive motility patterns of hyperactivated rabbit spermatozoa tracked for a minimum of 15 frames with the CellSoft System. (A) high AALH; (B) low AALH.

et al (1979) and Johnson et al (1981), episodes of circular (type I) or erratic (type II) motion alternated with the progressive (type III) pattern of motion (Fig. 4). The duration of each phase was often several seconds. The motility characteristics of the same sperm during different episodes in the progressive phase was quite variable, and frequently the motility parameters and characteristics were typical of nonhyperactivated sperm (Fig. 4).

Classification of Sperm Motility Patterns

Motility Parameter Classification Potential—Many nonhyperactivated sperm (Fig. 5) were mistakenly identified as hyperactivated when criteria suggested for classification of hyperactivated human sperm (Robertson et al, 1988; Burkman, 1991) were applied to rabbit sperm populations incubated for 1 and 2 hours in DM. The more restrictive threshold values for VCL and LIN (Robertson et al, 1988) were used, and, because of differences in the AALH sample means of human and rabbit sperm, a value

of AALH that was 1.5–2 times the AALH sample mean was used for classification. A similar result was obtained when the motility parameters used to classify sperm from a mouse carrying the t complex were used. The misclassification suggests that the motility parameters LIN, VCL, and AALH, or the threshold values subjectively determined for them for sperm from human and mouse carrying the t complex, are not applicable to rabbit sperm. In order to objectively establish criteria for the classification of rabbit sperm motility patterns, motility parameters were measured with the CellSoft system for 322 sperm incubated for 16–18 hours under capacitating conditions (Brackett and Oliphant, 1975; Brackett et al, 1982), and 899 nonhyperactivated sperm.

Summary statistics for each of the 10 motion parameters are given in Table 1. For all motion parameters except LIN, the standard deviation differs between classes; in particular note VCL, MALH, AALH, AALH/LIN, and VCL*AALH, where the standard deviation of the parameters for cells whose motions mimicked hyperac-

A

VCL   112 µm/sec
LIN   0.26
WOB   0.67
AALH   5.8 µm

B

VCL   107 µm/sec
LIN   0.08
WOB   0.48
AALH   5.3 µm

x

y

C

VCL   128 µm/sec
LIN   0.21
WOB   0.41
AALH   5.0 µm

D

VCL   71.3 µm/sec
LIN   0.36
WOB   0.64
AALH   3.1 µm

E

VCL   47.6 µm/sec
LIN   0.8
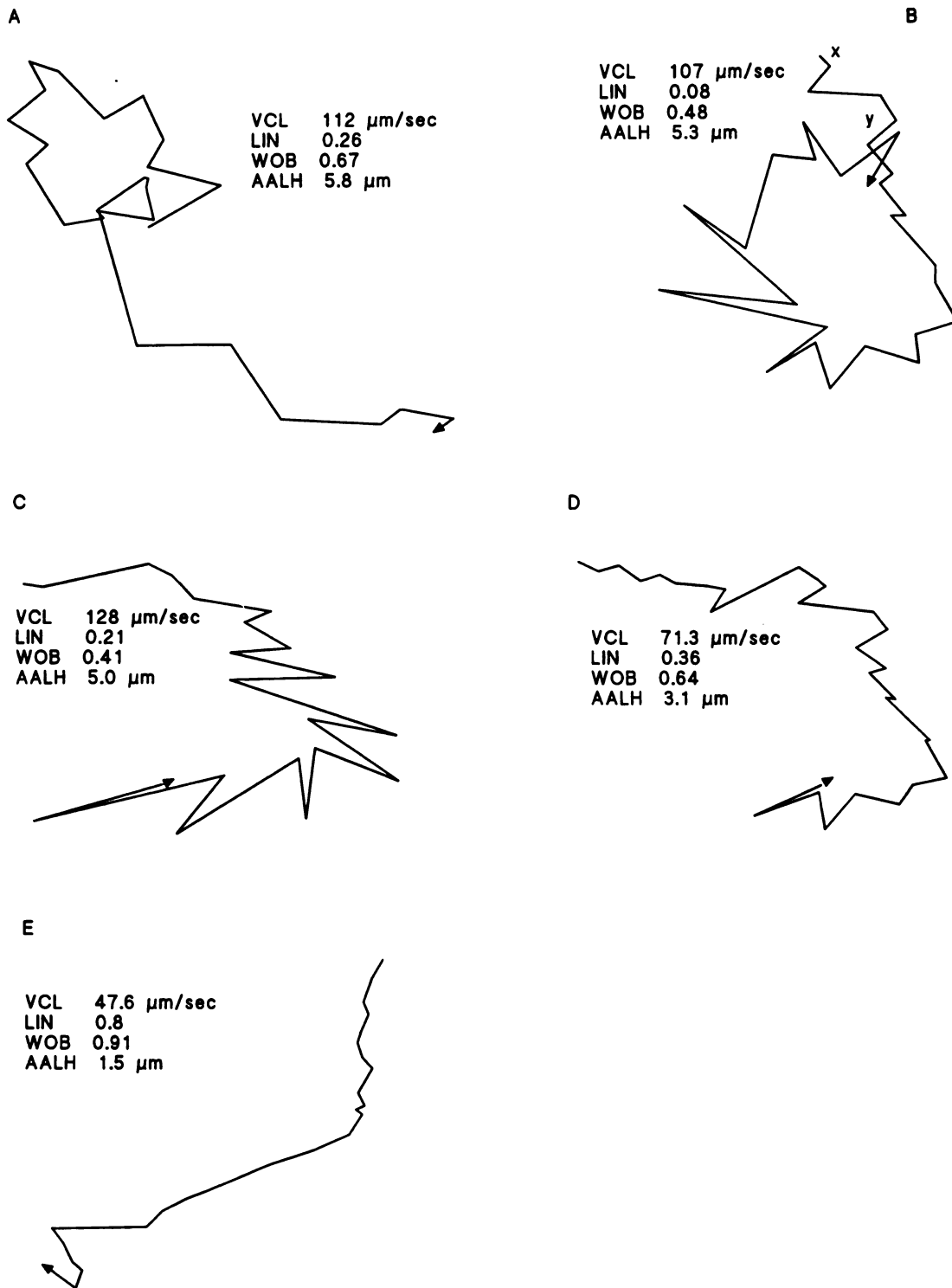WOB   0.91
AALH   1.5 µm

FIG. 4. Type IV biphasic motility patterns of hyperactivated rabbit spermatozoa obtained with the CellSoft Research Module. (A) Biphasic trajectory. (B–E) Trajectories of one sperm in different phases of the biphasic pattern. (B) random phase—the section x,y is the end of the progressive phase before entry into the random phase; (C) progressive phase immediately after exit from random phase; (D) hyperactivated progressive phase; and (E) Nonhyperactivated progressive phase.

VCL 123 μm/sec A
LIN 0.2
WOB 0.92
AALH 3.8 μm

B

VCL 106 μm/sec
LIN 0.21
WOB 0.9
AALH 3.3 μm
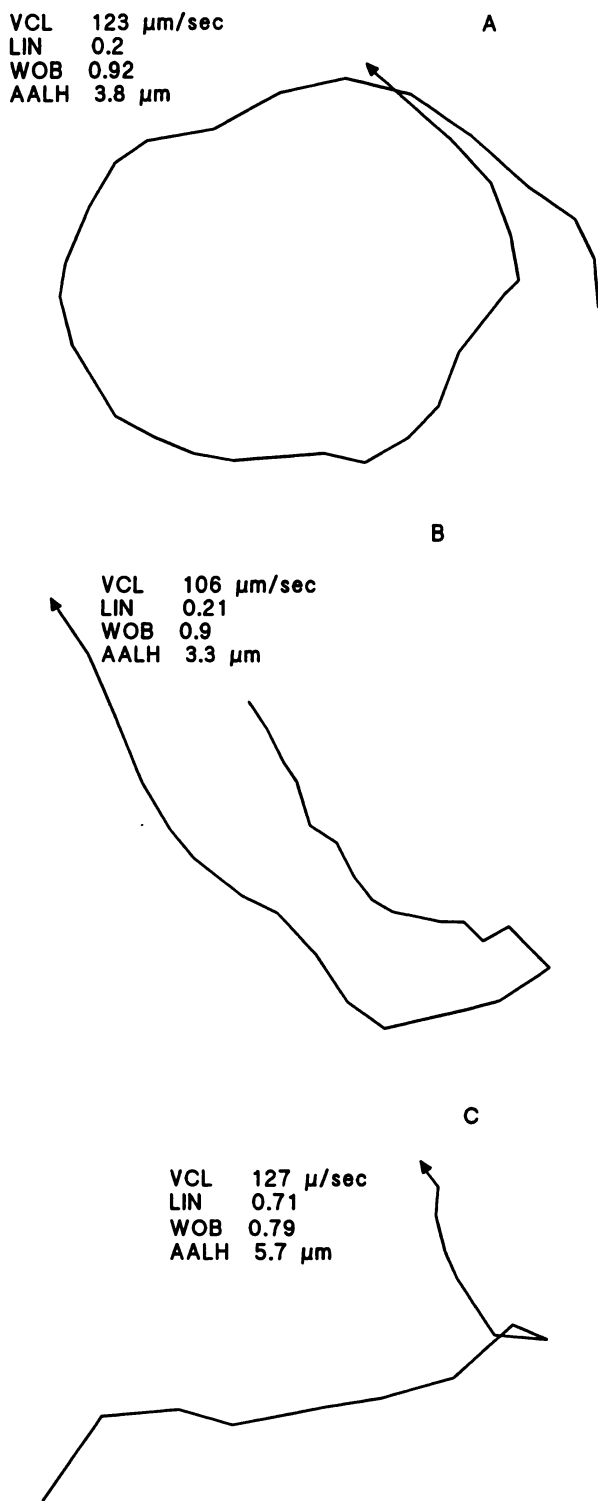
C

VCL 127 μ/sec
LIN 0.71
WOB 0.79
AALH 5.7 μm

FIG. 5. Motility patterns of nonhyperactivated rabbit spermatozoa classified as hyperactivated based on motility parameters VCL, LIN, and AALH. (A) circular trajectory; (B) hairpin trajectory; and (C) loop trajectory.

tivated motility far exceeded those for nonhyperactivated cells. All differences were statistically significant according to the Squared Ranks Test ($P < 0.01$) (Conover, 1980). Right-skewness of the hyperactivated distributions, and several unusually large values for the motility parameters of hyperactivated cells, contributed to the difference. Hyperactivated cells generally showed smaller values for VSL, LIN, BCF, STR, and WOB. Differences in location were supported by the Mann-Whitney Test ($P < 0.01$) (Conover, 1980). Although the differences noted are apparent from graphical inspection, the reported statistical significance should be taken only in an exploratory data analysis context because the "differing only in location" premise for the Mann-Whitney Test is not satisfied, and for each test the sample sizes are so large that the tests will be extremely sensitive to alternatives from the "no difference" hypothesis.

Classification potential was assessed graphically by comparing the motility parameter distributions for hyperactivated and nonhyperactivated cells. Figure 6 shows stacked boxplots of the hyperactivated (H) and nonhyperactivated (N) class distributions for each motility parameter. Data were normalized by subtracting the mean of the combined hyperactivated and nonhyperactivated classes and dividing by its standard deviation (Fig. 6) to support examination of the relative classification potential among motility parameters. For LIN and WOB, at most 25% of the nonhyperactivated cells show values in the same range as those of the hyperactivated class, indicating that both have strong potential for classification. Based on the degree of separation of the boxes representing the inner 50% of the data, it is likely that AALH, MALH, and VCL*AALH will also be reasonable classifiers. Note that a classification rule based on VCL alone does not appear to be as promising, and BCF has limited classifying potential.

The relative frequency distributions for LIN, VCL, AALH, and WOB are given for each motility class in Figure 7. LIN, VCL, and AALH were selected for display because of their suggested use in classifying hyperactivated human sperm (Robertson et al, 1988; Mortimer and Mortimer, 1990; Burkman, 1991; Murad et al, 1992), and WOB was selected for its importance in this study. Hyperactivated sperm were absent in the range of 0.8–1.0 for both LIN and WOB, and conversely high percentages of nonhyperactivated cells, LIN, 75.5% and WOB, 94.3%, were found over this range. This strongly suggests good classifying potential for each. AALH showed only minimal distribution overlap; VCL had considerably more. The individual concomitants of hyperactivation suggested by Mortimer and Mortimer (1990) for human sperm are consistent with these results despite the fact that rabbit sperm values are reported here.

Table 1. *Summary statistics for the motility parameters determined by CellSoft for both hyperactivated and nonhyperactivated cells*

Parameter	Hyperactivated		Nonhyperactivated	
	Mean ± SD	(Range)	Mean ± SD	(Range)
VCL (μm/second)	137.6 ± 52.0	(51.0–344.8)	83.1 ± 35.7	(23.2–191.3)
VSL (μm/second)	30.4 ± 21.1	(0.1–104.4)	71.0 ± 35.5	(0.7–175.5)
LIN	0.24 ± 0.18	(0.01–0.74)	0.85 ± 0.18	(0.02–0.99)
MALH (μm)	9.9 ± 4.9	(0.2–27.8)	2.3 ± 1.3	(0.6–10.9)
AALH (μm)	7.1 ± 3.3	(1.1–20.1)	1.6 ± 0.9	(0.4–10.9)
BCF (Hz)	12.2 ± 5.5	(1.2–25.9)	15.0 ± 3.8	(1.2–27.2)
STR	0.58 ± 0.30	(0.02–0.98)	0.90 ± 0.14	(0.04–0.99)
WOB	0.40 ± 0.15	(0.01–0.79)	0.94 ± 0.08	(0.23–1.00)
AALH/LIN (μm)	92.5 ± 192.2	(2.2–2,008.0)	2.4 ± 3.7	(0.4–53.5)
VCL·AALH (μm²/second)	1,124.3 ± 984.3	(96.4–6,459.7)	144.9 ± 128.3	(16.4–722.9)

Linear Classification Models

Combinations of the motility parameters VCL, LIN, and AALH or MALH have been advocated as a means for the classification of sperm hyperactivation status (Robertson et al, 1988; Mortimer and Mortimer, 1990; Burkman, 1991). Figure 8 illustrates in the two-parameter case why a combined rule for classification might be desirable. Ordered pairs for four-parameter combinations are shown in scatterplots, with the hyperactivation class indicated for the data values. Also shown is a quadrant within which hyperactivation would be predicted. This quadrant is formed by the intersection of half planes generated by partitioning the individual parameters according to hyperactivation. For Figure 8A–C and E, the partitions are formed using rules established by Mortimer and Mortimer (1990), and the partitioning for Figure 8D is achieved in a manner to be addressed later. The quadrant where hyperactivation is predicted is reasonably pure, i.e., contains few nonhyperactivated cells. Thus greater purity is attained using two motility parameters instead of one supporting a combined interpretation approach. However, for all but the plot involving VCL and WOB, hyperactivated cells are also plentiful in other quadrants, suggesting that classification rules based on these partitions would be adequate to guarantee a cell as hyperactivated, but it would not be adequate to correctly classify cells in a mixed population as hyperactivated or nonhyperactivated. In Figure 8E all three parameters defined by Mortimer and Mortimer (1990) are shown in relation to hyperactivation. Among the hyperactivated cells incorrectly classified (according to LIN and AALH) as nonhyperactivated, there is a mix of VCL values. This creates doubt that improvement in purity is possible through inclusion of that parameter. Furthermore, several sperm correctly classified as hyperactivated do not meet that criterion for VCL.

Table 2 summarizes the performance of a subset of 24 models considered. Models 12–14 were based on the combination of motility parameters suggested by earlier work-
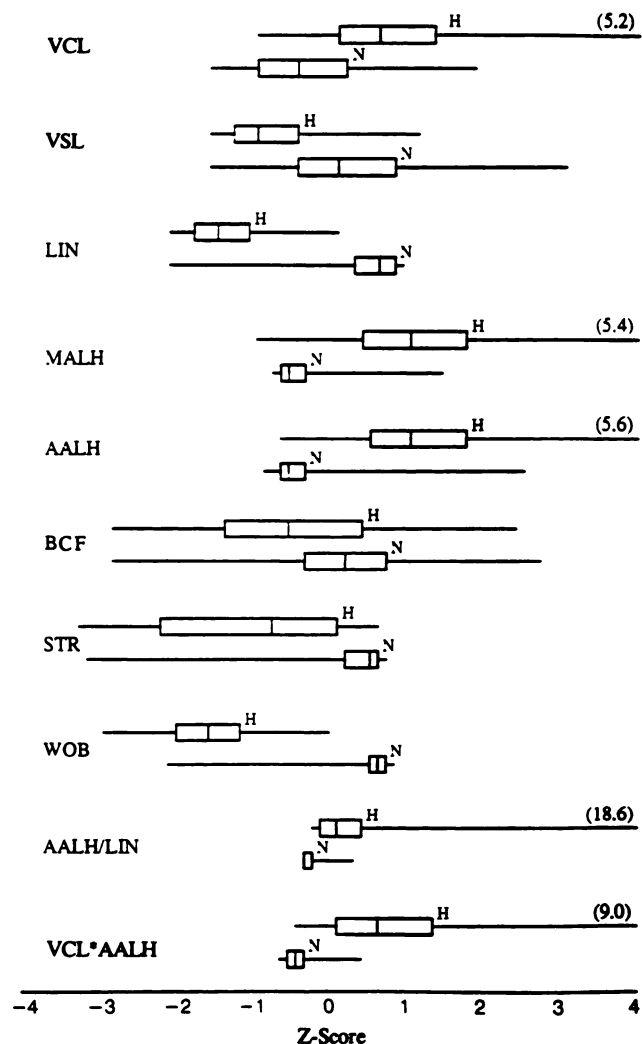

FIG. 8. Boxplots of motility parameter distributions of hyperactivated (H) and nonhyperactivated (N) motility. For each motility parameter, a mean and standard deviation were computed with motility classes combined. Data were normalized by subtracting the mean and dividing by the standard deviation for the purpose of allowing relative comparisons among parameters. The standard five-number summary was used for boxplot construction, with the right whisker truncated for five of the plots. The maximum value is listed in parentheses in those five cases.
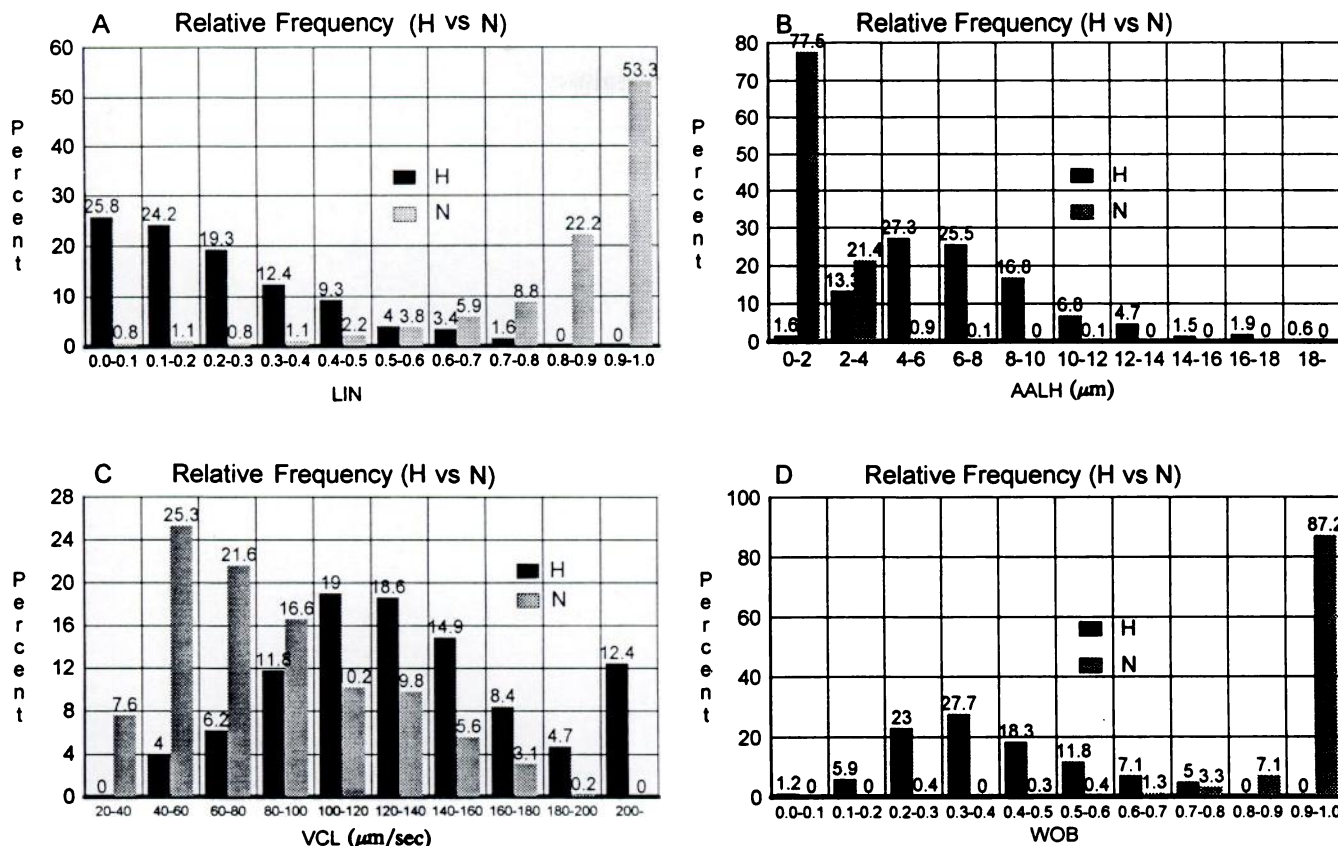
A   Relative Frequency (H vs N)

B   Relative Frequency (H vs N)

C   Relative Frequency (H vs N)

D   Relative Frequency (H vs N)

FIG. 7. Histogram of the relative frequency distributions of motility parameters for hyperactivated (H) and nonhyperactivated (N) motility.

ers (Robertson et al, 1988; Mortimer and Mortimer, 1990; Burkman, 1991). All of the motility parameters used individually to model hyperactivation are listed except BCF. Surprising efficiency was seen for several models despite the fact that in this study efficiency is defined in terms of the percentage of cells (hyperactivated and nonhyperactivated) correctly classified. For example, the discriminant analysis model using WOB misclassified only 22 hyperactivated cells and 19 nonhyperactivated cells, for an efficiency of 96.64%. The r^2 value of 0.838 indicates that 83.8% of the variation was explained in the (0,1) codes for hyperactivated and nonhyperactivated cells, respectively. Models based on LIN, AALH, and MALH also perform well, consistent with expectations created by Figure 6. Model performance is not as impressive for the remaining parameters, e.g., VSL, which reports markedly different misclassification performance for the discriminant model as compared to the regression model, an explained variation of only 23.5%, and an efficiency of only 71–81%. The conclusion is that VSL is not a good classifier for hyperactivation. The remaining models 10–14 each show good classification ability, but models 12–14 are inferior to the two models involving WOB, or even to the model based on WOB alone in supporting a regression or

discriminant model. WOB was the only motility parameter always included in the "best" two-, three-, and four-variable models chosen by the BMDP software for all possible subsets regression.

The large number of models considered that reasonably could be used to predict hyperactivated motility were culled based on parsimony and collinearity to arrive at a best few models. From the perspective of parsimony, a model with as high efficiency as possible and with as few terms as possible is desirable. Thus three- and four-variable models that did not perform as well as two-variable models were eliminated. Models comprised of collinear terms were avoided, e.g., VCL*AALH and AALH had a correlation of 0.933, meaning that AALH is capable of explaining 87% of the variation in VCL*AALH. The detrimental effect of including collinear terms in a regression model is that the standard error of prediction is inflated, making the model less reliable, particularly for data sets other than the one upon which the model was formed. This concern also prevents WOB and LIN from both being included in a model, their correlation being 0.904. Extensive evaluation of the best models produced in light of these two concerns left just three: models 1, 10, and 11, using WOB, WOB and AALH, or WOB and VCL,
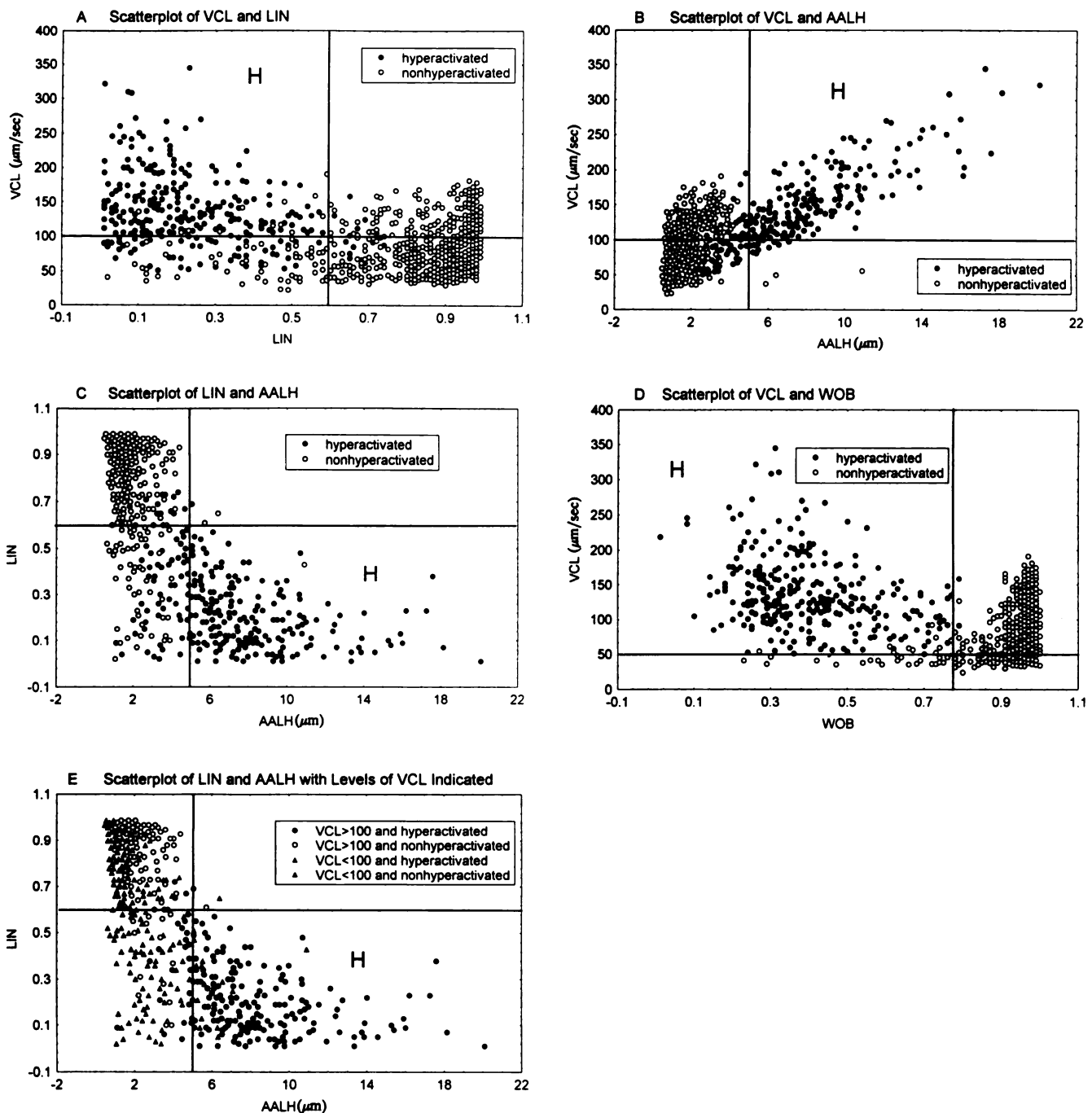
FIG. 8. Scatterplots of motion parameters with class identifiers for cells displaying hyperactivated and nonhyperactivated motility. The quadrant predicted to hold hyperactivated cells is denoted by H. In A–C, and E, the decision criteria are based on Mortimer and Mortimer (1990), and in D the criteria are based on a CART approach.

respectively. The two-variable models were chosen based on the slight improvement in discriminant model efficiency. Though not really sufficiently large to create a collinearity problem, the correlation between WOB and AALH was −0.812, whereas the correlation between WOB and VCL was only −0.468. All else being equal, the dis-

criminant model based on WOB and VCL that classifies a cell as hyperactivated if WOB < 0.60 + 0.0007 VCL is the model of choice. This model was 97.1% efficient for the data set examined. The regression model differed only in intercept, 0.57 instead of 0.60, with slightly less efficiency, 96.6%.

Table 2. *Summary of best models using discriminant (D)/regression (R) analysis based on 322 hyperactivated (H) and 899 nonhyperactivated (N) cells*

Model	Variables	Misclassified (No.)		Efficiency (%)	
		H (D/R)	N (D/R)	D/R	r^2
1	WOB	22/31	19/15	96.64/96.23	0.838
2	LIN	21/34	63/46	93.12/93.45	0.702
3	AALH	59/91	5/4	94.76/92.22	0.639
4	MALH	63/109	16/9	93.53/90.34	0.600
5	VCL·AALH	113/188	4/0	90.42/84.60	0.411
6	STR	132/171	78/36	82.80/83.05	0.356
7	VCL	108/209	205/56	74.37/78.30	0.261
8	VSL	56/210	301/26	70.76/80.67	0.235
9	AALH/LIN	190/283	1/0	84.36/76.82	0.140
10	WOB, AALH	21/32	16/10	96.97/96.56	0.856
11	WOB, VCL	23/30	12/11	97.13/96.64	0.847
12	VCL, LIN, AALH	23/34	34/29	95.33/94.84	0.757
13	VCL, LIN, MALH	24/38	40/30	94.76/94.43	0.746
14	VCL, LIN, VCL·AALH	24/40	50/36	93.78/93.78	0.729

CART

An approach distinct from the regression and discriminant analyses above is given by tree-structured classification. The principal software used was CART, with FACT software used for corroboration. Only the CART results are reported. In running CART, all the motility parameters considered earlier as possible predictors were included. Only WOB and VCL were chosen, with the rule: classify as hyperactivated if WOB < 0.78 with VCL > 51 μm/second.

Of the 1,221 cases examined, only 12 nonhyperactivated cells and 2 hyperactivated cells were misclassified, for an efficiency of 98.9%. This efficiency is higher than for any of the previous models discussed. Despite the low value for VCL, compared to the values suggested by Mortimer and Mortimer (1990), Burkman (1991), Olds-Clarke (1989), and Olds-Clarke and Johnson (1993), this rule has
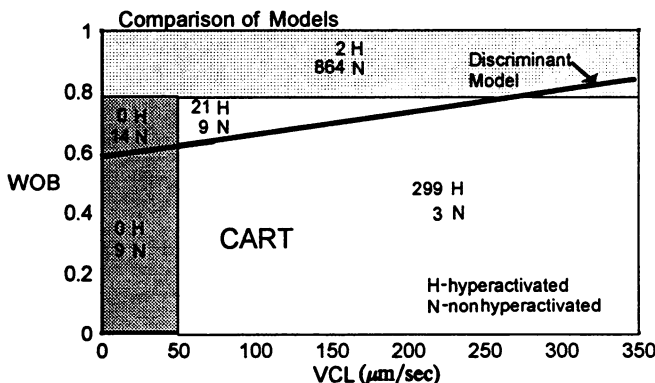

FIG. 9. A schematic of the discriminant and CART decision criteria. The unshaded region represents the set of ordered pairs based on WOB and VCL that predict hyperactivated motility. The points falling below the bold line would indicate hyperactivated motility according to the discriminant model. The numbers of cells in each class found in those regions are indicated.

great appeal in consideration of the data in Figure 8D. There, the incidence of nonhyperactivated cells with low WOB and low VCL is high enough to cast doubt on a model based on WOB alone. VCL in this use is merely refining a classification rule based primarily on WOB.

The use of LIN, AALH, and VCL was also investigated. CART did not choose VCL. The tree was slightly more complex, having five nodes instead of three as above. The classification efficiency was 96.5%. When a model based on WOB and AALH was attempted, CART did not choose to use AALH, opting instead for a rule based only on WOB, for an efficiency of 97.0%. Other runs using linear combinations of variables were attempted but resulted in more complex decision trees.

Of all of the CART models examined, one of the simplest to implement was also the best. The CART model based on WOB and VCL performed most efficiently in classifying hyperactivated and nonhyperactivated cells with the least penalty in model complexity.

Comparison of Models

Figure 9 illustrates decision criteria delivered by the discriminant and CART models using VCL and WOB. To understand the model differences, the region WOB × VCL, where WOB ranges from 0.0 to 1.0 and VCL ranges from 0 to 350 μm/second, has been partitioned according to the hyperactivity decision rules for each model. A cell whose WOB and VCL values locate it in a shaded region would be classified nonhyperactivated by CART, and a cell in the unshaded region would be classified as hyperactivated. The bold line represents the discriminant model. Points falling below that line would be classified as hyperactivated and above that line classified as nonhyperactivated. The true number of hyperactivated and nonhyperactivated cells present is indicated within each region.

Table 3. *Summary statistics for the motility parameters determined by CellTrak for both hyperactivated and nonhyperactivated cells*

Parameter	Hyperactivated		Nonhyperactivated	
	Mean ± SD	(Range)	Mean ± SD	(Range)
VCL (μm/second)	136.9 ± 51.1	(50.4–383.5)	77.9 ± 37.0	(25.0–186.0)
VSL (μm/second)	28.5 ± 18.3	(0.0–109.3)	57.9 ± 38.8	(0.0–174.0)
LIN	0.24 ± 0.17	(0.01–0.82)	0.73 ± 0.26	(0.01–0.99)
AALH (μm)	14.3 ± 6.5	(1.5–63.0)	3.9 ± 2.1	(1.3–13.0)
STR	0.53 ± 0.27	(0.0–0.99)	0.84 ± 0.22	(0.0–0.99)
WOB	0.42 ± 0.15	(0.14–0.90)	0.84 ± 0.21	(0.12–0.99)
AALH/LIN (μm)	122.1 ± 156.7	(4.4–1,575.8)	11.3 ± 31.9	(1.4–390.0)
VCL·AALH (μm²/second)	2,153.2 ± 1,708.3	(223.4–12,434.4)	327.5 ± 290.1	(38.0–2,275.0)

Consider first the rectangular region within which CART would classify cells as hyperactivated. Below the discriminant model there were 299 hyperactivated cells correctly classified and 3 nonhyperactivated cells incorrectly classified. In the same region, but above the discriminant model, there were 21 hyperactivated cells correctly classified by CART and 9 nonhyperactivated cells incorrectly classified. Note that the discriminant model would have incorrectly classified the 21 hyperactivated cells. CART is 12 cells more accurate than the discriminant model in this region. The other region where a real difference is observed is the shaded region corresponding to low values of WOB and VCL. There the discriminant model would incorrectly classify 9 nonhyperactivated cells, bringing the CART advantage in performance to 21 cells.

The role of VCL is clearly seen in Figure 9. Using VCL to establish a lower threshold improves classification over using WOB alone. Twenty-three cells would have been incorrectly classified using a WOB criterion without taking into consideration VCL. The conclusion is that WOB is a stable measure and good classifier except for very slow-moving cells, when WOB will sometimes errantly indicate hyperactivity.

In making a recommendation for general use, the choice would be the CART model for WOB and VCL. The claimed efficiency is at worst 98%, based on a cross validation estimate of efficiency that repeatedly excludes different subsets of the data from the analysis and then gauges efficiency of the model as it is applied to the excluded subset.

Analysis with the CellTrak System

The videotapes analyzed by the CellSoft system were reanalyzed by the CellTrak system, and motion parameters for 1,119 hyperactivated and nonhyperactivated sperm were collected. Table 3 shows the summary statistics for the motility parameters. The mean values for the motility parameters determined by the CellTrak and CellSoft (Table 1) systems are different, most notably the value for AALH. Modeling efforts were more favorable with the CellTrak data. Graphical analysis of the data

revealed a structure between the motility parameters and the true class of hyperactivation that was similar to the structure found in the analysis of the CellSoft data. Application of the CART decision rule, based on the CellSoft data for WOB and VCL, to the data produced by CellTrak, resulted in a misclassification of only 40 cells, for an efficiency of 96.4%. When the CART software was used to analyze the CellTrak system data, the following model was produced: WOB < 0.69 with VCL > 55 μm/second. This new model has an efficiency of 97.0% as determined using cross validation. These results show that the motility parameters WOB and VCL in combination can efficiently classify rabbit sperm into hyperactivated and nonhyperactivated motility classes. For maximum classification efficiency the model utilized should be calibrated by CART analysis of hyperactivated and nonhyperactivated motility parameters measured by the same motion analysis system.

Discussion

The decline in rabbit sperm motility with increasing duration of incubation and the variation in this behavior with sperm from different bucks have been observed previously (Brackett and Oliphant, 1975; Hosoi et al, 1981; Brackett et al, 1982; Kim et al, 1989; Young et al, 1992). The appearance of substantial numbers of rabbit sperm with motions that are characteristic of hyperactivated motility after incubation for 16–20 hours, when 12 hours earlier they appeared quiescent or possessed poor motility, was unexpected. This result in retrospect may not be so surprising, because an incubation period of 17–22 hours is optimal for *in vitro* capacitation of rabbit sperm in DM containing BSA (Brackett et al, 1982). Of interest are the inability of sperm from some rabbits to develop motion patterns mimicking hyperactivated motility and the difference among rabbits in the degree to which their sperm acquired these motion patterns during incubation. This diversity in the behavior of sperm from rabbits appears not to be confined to motility, and it may be a characteristic of rabbit sperm (Akruk et al, 1979; Hosoi et al,

1981; Brackett et al, 1982; Chen et al, 1989; Kim et al, 1989).

Several hyperactivated motility patterns have been observed during incubation of human sperm under capacitation conditions (Burkman, 1991). In contrast, sperm from hamster, mouse, guinea pig, sheep, pig, and rhesus monkey apparently acquire only one or two hyperactivated motility patterns when capacitated *in vivo* or incubated under capacitation conditions (Yanagimachi, 1970; Yanagimachi and Usui, 1974; Fraser, 1977; Cummins, 1982; Suarez et al, 1992; Boatman and Bavister, 1984; Olds-Clarke, 1986; Suarez, 1988). Species differences in hyperactivated motility may account for the disparity in the numbers of motility patterns reported, but the dissimilar protocols used in the studies and in the tracking of sperm may also be a reason. The pattern of tracks traced by the head of a hyperactivated spermatozoon will vary with the orientation of the dominant plane of movement of the spermatozoon to the plane of observation. Automated motion analysis systems track only the sperm head, and analysis of hyperactivated sperm motion by such systems will likely produce a diversity of hyperactivated motion patterns. A critical factor is likely to be the sampling conditions, such as the frame rate and the analysis interval in relationship to the velocity and frequency of oscillation of the sperm (Owen and Katz, 1993).

The kinetics of the development by rabbit sperm of the motion patterns mimicking hyperactivation are likely to vary with sperm from different rabbits. It would also be doubtful that sperm within one ejaculate develop these motion patterns synchronously and maintain these movements for the same period. The population of hyperactivated sperm would probably be heterogeneous with respect to the hyperactivation state. Some of the different motion patterns mimicking hyperactivated motility observed during incubation under capacitation conditions may be expressions of the development and/or decline of hyperactivated motility, particularly when hyperactivation motion patterns are combined from sperm of several males.

The objectives of the statistical analysis were to provide decision criteria for the classification of motility classes for rabbit sperm and to suggest better methods for determining such criteria for hyperactivated sperm from other species. With regard to methods, several comments can be made. When comparing the motility parameter distributions for hyperactivated and nonhyperactivated motility, it should be recognized that a difference in means only suggests a potential for discriminating capability. Further examination of a distribution is appropriate with the goal of gauging the likely misclassification. To accomplish this objectively, wider use of formal statistical methods such as discriminant analysis, regression, or CART

is recommended because subjective partitioning of a motility parameter range into hyperactivated and nonhyperactivated classes, an approach frequently used (Neil and Olds-Clarke, 1987; Robertson et al, 1988; Olds-Clarke, 1989; Mortimer and Mortimer 1990; Burkman, 1991; Olds-Clarke and Johnson, 1993), will likely lead to inadequate decision rules. Further, misclassification and efficiency should be calculated in terms of all cells misclassified, not just nonhyperactivated cells misclassified as hyperactivated. Reporting should take into account that any decision rule established is dependent on the data set upon which it was based. The present results were given in terms of cross classification efficiency to emphasize the applicability of the model to other data sets. Finally, great care must be given when combining the information from several motility parameters to establish decision criteria. Extensive use of multidimensional graphics and the analysis techniques listed above will improve this process.

For this study simple decision criteria were established using the above suggestions. The cross classification efficiency reported was 98%, the worst of several cross validation attempts made for the CellSoft CART model. The important motility parameters were consistently WOB and VCL, chosen by three different analytical approaches for data produced by two motion analysis systems. Because of the different algorithms used by the CellSoft and CellTrak systems to compute the average path velocity, and reflected in the large difference in values of AALH computed by the two systems, the threshold values for WOB in the models produced by the two systems are different. However, the efficiency of classification was reduced by <1% when the CellSoft model was applied to data produced by the CellTrak system. Because the performance of the models for data sets produced by other motion analysis systems is unknown, a model specifically calibrated by CART for the motion system employed should be utilized for analysis of hyperactivated motility.

The subjectively determined high threshold value for VCL and AALH suggested in classification schemes for human and mouse sperm will give a low estimate of the extent of hyperactivation development because many hyperactivated sperm with the biphasic motion pattern, as well as sperm in transition to, or in decline from the hyperactivated state, when VCL would be lower, would be classified as nonhyperactivated. Fewer such sperm will be eliminated by the models proposed (Figs. 2–4). WOB is the primary classifier, with VCL serving to refine the classification for slow-moving sperm where WOB most often errantly indicates hyperactivity. The models should provide a closer approximation to the extent of hyperactivation development, and they will facilitate the use of hyperactivation not only as a tool for the clinical as-

sessment of fertility, but also for toxicological studies (Williams et al, 1990; Young et al, 1992).

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