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The Automated Analysis of Rat Sperm Motility Following Subchronic Epichlorohydrin Administration: Methodologic and Statistical Considerations

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The automated analysis of sperm motion endpoints is potentially useful in identifying male reproductive toxicants and ultimately in predicting fertility in humans. The present study was designed to evaluate the automated analysis of rat sperm motility characteristics following subchronic administration of epichlorohydrin. This type of validation is a prerequisite for inclusion of sperm motion measurements in the process of reproductive risk assessment. In the present studies videotapes were made of cauda epididymal spermatozoa from Long-Evans rats, both untreated and treated with epichlorohydrin. From analysis of videotapes of control epididymal spermatozoa, the relationship of various sperm motion endpoints and settings of the CellSoft computer-assisted sperm motion analysis system (Cryo Resources, Ltd., New York, NY) is described. Optimal settings of the system for analysis of rat spermatozoa are detailed. Employing data from both control and epichlorohydrin-treated animals, a statistical methodology is described that evaluates: (1) the distributions of CellSoft generated sperm motion endpoints, (2) the correlations between these endpoints, and (3) techniques for detection of dose-related effects.

Key words: Rat sperm, motility, videomicrography, epichlorohydrin.

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Progressive motility is a characteristic needed by spermatozoa to attain their full reproductive potential. Technologies have therefore been developed for the objective measurement of sperm movement components using image analysis computer software. These computer-assisted sperm motion analysis (CASA) systems are generally set up to measure sperm concentration, percentage of motile spermatozoa, curvilinear velocity (frame-to-frame), straight line velocity (beginning-to-end

frame), and the linearity of sperm motion. Depending on the system, additional motion endpoints can be measured, including the amplitude of lateral head displacement, sperm head beat/cross frequency, percentage of circularly swimming spermatozoa, and change in the direction of travel. Several recent reports have demonstrated the use of CASA systems in the basic, clinical, and toxicologic research environments. Some of these studies (Mathur et al, 1986; Katz and Davis, 1987; Knuth et al, 1987; Working and Hurtt, 1987; Budworth et al, 1988; Ginsburg et al, 1988; Mack et al, 1988; Stephens et al, 1988) demonstrated the accuracy of some motion measurements and compared the variability of certain motion parameters when measured with automated systems and the results obtained using other methods. While most of these studies have focused on human spermatozoa, only one has focused on automated motion analysis of rat cauda epididymal spermatozoa (Working and Hurtt, 1987). However, in this study the influence of available program changes in image analysis settings, on the measurement of sperm motion endpoints was not described. Clearly, inappropriate settings may produce data that lack precision and accuracy, an effect that could complicate the detection of true treatment-related effects.

The use of CASA in the toxicologic setting requires certain considerations. First, which settings of the image analysis software will allow: (a) accurate estimation of the percent motile spermatozoa (eg, setting the videoscreen contrast for the digitized sperm image and the minimum number of video frames to be analyzed) and (b) accurate estimation of motion endpoints (eg, setting the maximum allowable frame-to-frame (instantaneous) velocity and the minimum track length for calculation of velocity)? Second, what are the appropriate statistical methods for analyzing the distributions of the resulting motion endpoints? Intrinsic to this latter consideration are the selection of informative motion endpoint parameters (eg, means versus other distribution characteristics) and the evaluation of statistical methods for testing dose-related effects on these parameters (eg, univariate versus multivariate analysis).

These considerations are addressed in the present report. We have measured the effects of different image analysis software settings of one CASA system (CellSoft, Cryo Resources, New York, NY) on the motion endpoints of cauda epididymal spermatozoa from both untreated Long-Evans rats and from animals treated with a model male reproductive toxicant-epichlorohydrin. Epichlorohydrin decreases sperm function in rodents, as manifested by infertility and changes in motion parameters (Toth et al, 1989). We investigate and make recommendations for the optimal setting of the CellSoft system for analysis of rat spermatozoa. Furthermore, we examine the effects of epichlorohydrin, a model male reproductive toxin, on sperm motion endpoints and recommend statistical methods for analyzing appropriate motion response parameters.

Materials and Methods

Animal Sample Preparation

Dosing Protocol: Male Long-Evans rats were received from Charles River Breeding Laboratories (Portage, MI). Upon arrival (100 days old), the animals were fed Purina Lab Chow 5001 and filter-purified tap water *ad libitum*. Animal rooms were maintained at approximately 25 C and 55% humidity, with a 12:12 light-dark cycle, light commencing at 0600 hours. Animals were housed singly in plastic shoebox cages for 4–5 weeks before beginning treatment. They were randomly assigned to treatment groups to produce equivalent mean body weights across dose groups (n = 20 per dose group).

Epichlorohydrin (99+%) (Aldrich Chemical Company, Milwaukee, WI) was diluted with corn oil (Sigma Chemical Company, St. Louis, MO) and administered by gavage for 21 consecutive days at doses of 12.5, 25, and 50 mg/kg/day (n = 20/group). Control animals received corn oil in a 1-ml per kg body weight solution. Dosing solutions were made fresh weekly and stored in the refrigerator. Animals were killed by asphyxiation on the morning of the second day following administration of the final dose (experimental day 23).

Tissue Preparation: The right cauda epididymidis was excised, nicked several times with a razor blade, and subsequently incubated for 3 min in a covered Petri dish at 37 C in 10 ml Dulbecco's phosphate-buffered saline (with Ca^{++} and Mg^{++}), pH 7.2 with 10 mg/ml bovine serum albumin (Fraction V, Sigma Chemical Co.). Sperm motion was videotaped within 5 min of excision of the cauda. The concentration was approximately 4–10 × 10^6 spermatozoa/ml. An aliquot of this mixture was diluted 10–20-fold in the above medium and 10 μl were placed on a Petroff Hausser chamber (20 μm depth) (Hausser Scientific, Philadelphia, PA). From digitized image analysis, the approximate dimensions of the sperm head were: length, 12 μm; width, 7 μm. To maintain the temperature of the chamber, a Fryer (Fryer Co. Inc., IL) stage warmer apparatus was set to 37 C. A special steel template fitted to the microscope stage and containing a narrow slit for the light path conducted heat from the stage warmer to the Petroff Hausser chamber.

Videomicrography

Instrument Setup: Movement of spermatozoa in the Petroff Hauser chamber was videotaped for subsequent computer analysis with the CellSoft (CRYO Resources, NY) computer-assisted sperm motion analysis system. Videotapes were made with a Panasonic VHS video cassette recorder (Model AG 6300) and Panasonic video camera (Model WV 1410) mounted on an Olympus BH-2 microscope (Olympus MTV-3 adapter) set up to give a pseudo dark field image [12.5X : 4X SPlan objective, 2.5X photoocular; 1.25X Nomarski differential interference contrast (D.I.C.) intermediate tube (D.I.C. not used); phase contrast condenser set at 40]. At least 20 consecutive microscope fields per sperm sample were videotaped following a prearranged side to side grid pattern. A Panasonic time/date generator (Model WJ 810) was used to allow reanalysis of various fields from samples at approximately the same tape location as in previous analyses.

CellSoft: Computer analysis of sperm motion parameters was done using the basic CellSoft program and the CellSoft Research Module, which allows frame by frame analysis of individual sperm tracks using "track files" generated during motion analysis using the basic CellSoft program. These "track files" contain the location of centroids for each sperm cell for each video frame. CellSoft system-generated measurements include the following motion endpoints: percent motile cells (number of spermatozoa exceeding threshold minimum velocity/total number of spermatozoa); curvilinear velocity (mean frame-to-frame velocity); straight line velocity [velocity between centroids (computer-calculated centers of sperm mass) in first and last frames tracked]; linearity (ratio of straight line velocity/curvilinear velocity × 10); amplitude of lateral head displacement (ALH) (displacement of the centroid from a computer-calculated mean trajectory); beat/cross frequency (frequency of centroid crossing this mean trajectory); and change in direction (mean of absolute value of frame-to-frame turning angles (changes from previous direction of movement).

The basic CellSoft program stored sperm motion data for individual cells in an ASCII file format. These files were electronically transferred to a mainframe computer where programs to vary the minimum sample for motility and minimum sample for velocity settings were developed. This subsequently allowed us to use variable system settings in the motion analysis of a single sample without reanalyzing the sample with the basic CellSoft program.

Tables 1 and 2 contain lists of the operator-set CellSoft parameters and their settings. They also indicate whether they were set according to literature recommendations or varied and evaluated to determine their effect on sperm motion analysis. All setting evaluations were done using samples from untreated control animals. Stated below are more detailed descriptions of some image analysis procedures and software settings than those listed in Tables 1 and 2.

For initial evaluation, system settings for the minimum number of frames sampled for motility and velocity measurements were set to include the greatest number of cells without producing artifacts: minimum sample for motility = 2; minimum sample for velocity = 3. At a minimum sample for motility = 2 setting, cells were required to appear in the first two video frames of an analysis to be counted as motile. At a minimum sample for velocity = 2 setting, the linearity measurements for cells tracked for only two frames are all equal to the maximum value of 10. Inclusion of two-frame sperm tracks for velocity and linearity measurement, therefore, would be of little use.

TABLE 1. CellSoft Parameter Settings Used in the Sperm Motion Analyses of Long-Evans Rats*

Parameter	Setting
Set According to Literature	
Number of frames per field	15
Number of frames per second	30
Threshold velocity	20 μm/sec
Cell color	white
Pixel scale	3.26 μm
Dilution factor	0.5
Cell size range	20–50 pixels
ALH Endpoints	
Minimum number of frames†	11
Minimum velocity	20 μm/sec
Minimum linearity	3.5
Circular Motion Endpoints	
Minimum number of frames	10
Minimum velocity	20 μm/sec
Maximum radius	100 μm

*Set according to literature.
†Set in subsequent data processing program.

At least 200 cells were analyzed per animal. Since some fields were less crowded than others (mean = 20 cells, SD = 9), 20–22 fields were analyzed for all animals to keep constant the time spent in the Petroff Hausser chamber. The number of spermatozoa analyzed for velocity as a percentage of total cells analyzed was independent of the number of spermatozoa per field in the cell density range stated above.

The pixel (videoscreen picture element) scale was calibrated using an optical micrometer on the microscope under conditions identical to those used for sperm videotaping and the Pixel Scale Calibration option of the

TABLE 2. CellSoft Parameters Varied and Evaluated

Parameter	Range Evaluated	Recommended Setting
Threshold grey level	Extreme settings	Midpoint of extreme settings
Minimum sampling for motility	2–15 frames	2 frames
Maximum velocity	300–2700 μm/sec	1500 μm/sec
Minimum sampling for velocity	3–20 frames	3 frames
Minimum sampling for linearity*	3–20 frames	11 frames

*Set in subsequent data processing program

CellSoft program. Our pixel scale was measured to be 3.26 μm/pixel. This differs slightly from the scale used by Working and Hurtt (1987) (3.4 μm/pixel) using the same instruments, since the height of our video camera was adjusted slightly to give a monitor image that was parfocal with the ocular image of the microscope.

The maximum number of frames analyzed per field (15), the threshold velocity (20 μm/sec), and the cell size range (20–50 pixels) were those described previously by Working and Hurtt (1987).

The measurement of ALH and beat/cross frequency included only those spermatozoa with linearity values greater than 3.5 (Budworth et al, 1988). For the endpoint percent circularly swimming cells, the minimum number of points was 10 and the minimum radius was set at 100 μm. These settings were not optimized in our studies but were consistent with those used in studies of the motion of spermatozoa from other species (Budworth et al, 1988; Mack et al, 1988).

The following settings were optimized by repeated analysis of videotapes: (1) the digitized videoscreen sperm image (threshold grey level), (2) the maximum allowable frame-to-frame (instantaneous) velocity; by repeated analysis of individual cell files with our auxiliary computer program: (1) the minimum sample used to calculate percent motile spermatozoa; and by repeated analysis of track files (1) the minimum sample (track length) used for calculation of velocity and linearity.

Manual estimates for percent motile spermatozoa were determined by visually counting the number of nonmotile sperm cells and the total number of sperm cells during a playback of videotapes of epididymal spermatozoa from 10 control animals. A minimum of 250 sperm cells were counted in each sample.

Sperm Motion Response Parameters and Methods of Statistical Analysis

The endpoints evaluated included percent motile spermatozoa, percent circularly swimming spermatozoa out of motile cells, beat/cross frequency, curvilinear velocity, linearity, straight line velocity, lateral head displacement, and change in direction. Where appropriate, the distributions of the response endpoints within, as well as among the animals, were examined. The distributions were tested for normality using Kolmogorov's D statistic (Siegel, 1956) and examined graphically for symmetry. A list of potential response parameters of interest was compiled to examine the dose-related effect of epichlorohydrin on sperm motion. Response parameters are variables used to characterize sperm motion endpoint data collected using the CASA system. Because the mean and standard deviation may not be adequate summary statistics for distributions that are not symmetrical, and with the "suspicion" that doses could affect not only the central tendency (mean) of motion endpoints but the shape of the distribution as well, the following distributional parameters were examined: mean, 5% and 10% trimmed means (excludes lower and upper 5 and 10% of values, respectively) median, minimum, maximum, percentiles (1st, 5th, 10th, 25th, 75th, 90th, 95th, and 99th), standard deviation, range and interquartile range. The distributions of these response measures were examined and the parameters were analyzed with the methods described below to test for a dose-related response.

Parameters of motion endpoints were analyzed by three univariate methods to address the three questions: (1) are there any differences among the dose groups?, (2) is there a dose-related response?, and (3) what is the lowest observed effect level? If the data, raw or transformed, satisfied normality and homogeneity requirements, the three methods (Winer, 1971) were, respectively, one-way analysis of variance (ANOVA), linear regression, and t-tests between the control and the treated groups with the modified Bonferroni correction on the p-values (Simes, 1986). The data for percent motile spermatozoa and percent circularly swimming spermatozoa were transformed using the arc sine transformation prior to any analysis. For variables that did not meet the above requirements, the tests (Lehmann, 1975) were, respectively, the Kruskal-Wallis test, Jonckheere's test, and Wilcoxon tests between the control and the treated groups with the modified Bonferroni correction. All tests were evaluated at a significance level of 0.05.

In addition to the univariate analyses described above, multivariate analysis of variance procedures (MANOVA) (Winer, 1971) were used to test distributional parameters simultaneously. Because the MANOVA test considers characteristics of the entire response distribution simultaneously, it may provide a more sensitive test for distributional "shifts" than the individual univariate tests. The MANOVA procedures used tested for an overall dose-related effect and pairwise compared each dose level to the controls.

To evaluate the relative sensitivity of the various sperm motion endpoints, several "size of effect" measures, as described by Cohen (1977), were calculated. For an overall effect measure, the proportion of variance (PV) associated with the dose groups was calculated. The PV, also called the coefficient of determination (r-square), is a measure of the dispersion of the treatment means and equals the proportion of the total variance of all groups combined, accounted for by the variance in treatment means. The size of the effect based on the linear regression analysis was calculated as the ratio of the absolute value of the slope to its standard error and is a function of the Pearson's correlation coefficient and the sample size. For each endpoint, the control data were used to estimate the minimum detectable significant difference for a comparison between the control and a treated group. The estimates were based on the z-score statistic and calculated for several sample sizes and power levels. Additionally, the relationships between response endpoints were examined using correlation analysis.

Results

CellSoft Image Analysis System Optimization

For rat spermatozoa, the shape of the digitized sperm image (and therefore, the calculated center of mass for the head/midpiece combination) is dependent upon the videoscreen contrast (or

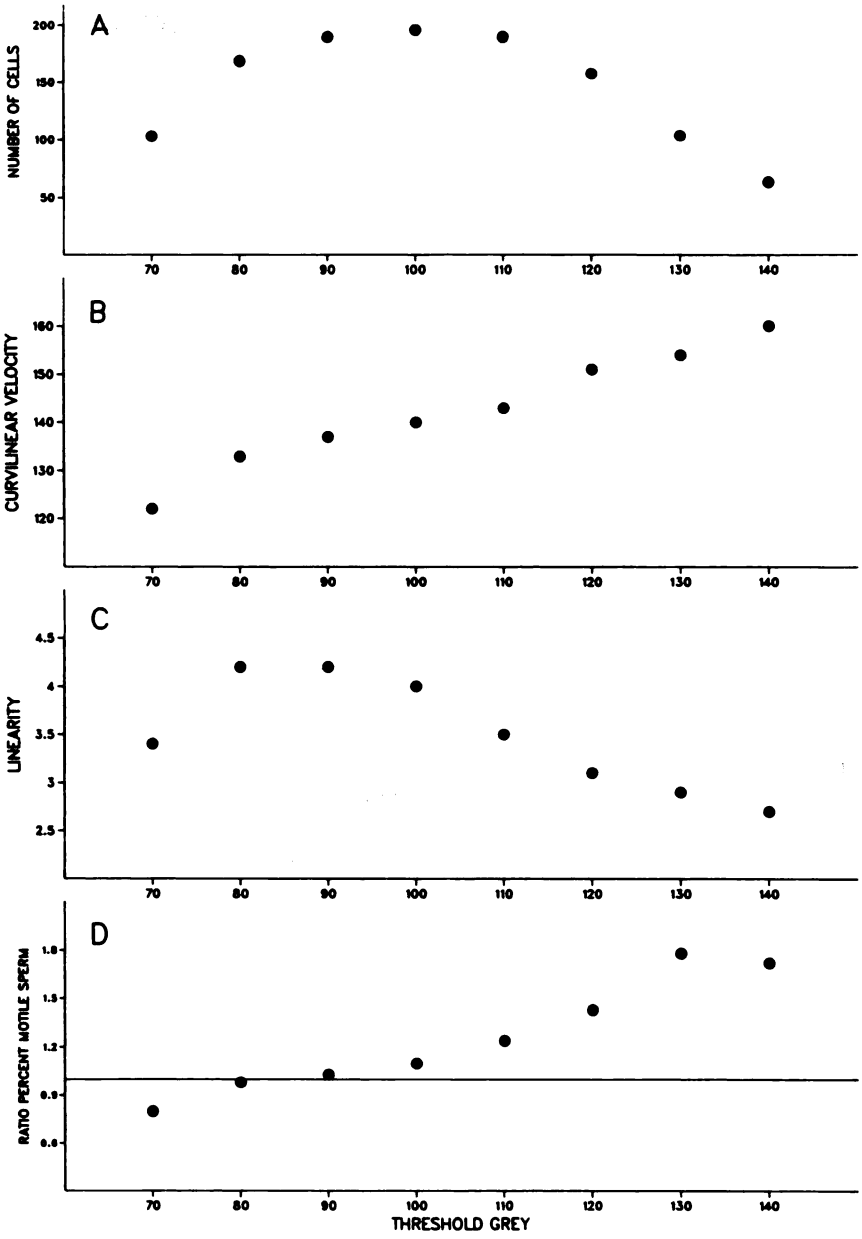

Fig. 1. The total number of cells analyzed (A), curvilinear velocity (B), linearity (C), and ratio (CellSoft/manual) of percent motile spermatozoa (D) at different threshold gray level settings. Points are means of three replicate analyses from one control rat.

threshold grey level). We examined the influence of varying the threshold grey level on the following: (1) the number of spermatozoa analyzed, (2) mean curvilinear velocity, (3) mean linearity, and (4) percent motile spermatozoa for sets of 10 video fields from each of three control animals (Figure 1). Two extreme conditions of videoscreen contrast can be identified over the range of relative threshold grey level values: (1) the point at which any spermatozoa lose intactness and (2) the point at which the sperm image can become no larger because of interference. For the sample in Fig. 1, these extremes were at 125 and 50 threshold grey units, respectively. We determined the optimal (within 10% of a manual estimate of percent motile cells) threshold grey level range to be near the midpoint of the two extreme settings (approximately 88 units). Subsequent analyses employed this approach for setting the grey level. (This evaluation was carried out at the most inclusive minimum sample for motility setting of two frames.)

Comparison of manual estimates for percent motile spermatozoa from 10 control animals to CellSoft estimates using the paired t-test ($\alpha = 0.05$)

TABLE 3. Comparison of Manual and CellSoft Estimates of Percent Motile Spermatozoa*

Minimum Number of Frames	CellSoft % Motile	Two-sided p-Value
2	53.4	0.60
3	52.3	0.91
4	51.3	0.52
5	51.2	0.49
6	51.1	0.46
7	50.7	0.39
8	50.4	0.33
9	50.3	0.29
10	49.9	0.23
11	49.7	0.19
12	49.4	0.16
13	49.0	0.13
14	48.6	0.09
15	48.4	0.08

*Mean manual % motile = 52.5, n = 10

showed that use of the "midpoint" grey level for each control set of fields and a minimum sample for motility setting of two frames (Table 3) resulted in accurate CellSoft estimates for percent motile spermatozoa. At a minimum sample for motility setting of 15 frames, the difference between the manual and CellSoft-generated percent motile cell estimates approached significance. We concluded that the minimum sample for motility should be set at two.

TABLE 4. Effect of Changes in Maximum Velocity Setting on Selected Sperm Endpoints*

Maximum Velocity Setting (μm/sec)	Mean Number of Cells Analyzed for Velocity	Percent of Cells Tracked for Maximum Length (15 Frames)	Mean Curvilinear Velocity (μm/sec)
300	116 ± 7.6†	10	131.4 ± 0.75‡
600	179 ± 2.1†	55	165.6 ± 4.00‡
900	193 ± 7.5	70	173.1 ± 3.15
1000	197 ± 3.1	74	171.5 ± 2.68‡
1100	198 ± 7.8	76	172.6 ± 3.18
1200	196 ± 5.5	76	175.2 ± 2.18
1500	196 ± 6.6	74	175.1 ± 2.40
1800	185 ± 3.1†	72	176.8 ± 1.97
2100	177 ± 4.6†	73	177.0 ± 3.58
2400	163 ± 2.1†	70	171.0 ± 7.91
2700	146 ± 6.0†	64	177.9 ± 5.09

*Values represent means of triplicate analyses of 10 video fields from one control animal.

†Significantly different from number of cells analyzed at maximum velocity = 1500.

‡Significantly different from mean velocity at maximum velocity = 1500.

The maximum velocity setting in the CellSoft system sets a limiting radius around each sperm cell, within which in the subsequent frame, either (1) absence of the spermatozoa or (2) the presence of additional spermatozoa, stops sperm tracking and uses the motion measurements only up to that previous frame. In setting the maximum velocity for the CellSoft system, our goal was to include the fastest cells for the longest times in our analyses without eliminating cells or cutting short track lengths. After analyzing a number of control samples at a maximum velocity setting of 300 μm/sec (as suggested by Working and Hurtt, 1987), the sample with the highest curvilinear velocity was used to examine the effects of changing the CellSoft setting. We studied the effect of changing the maximum velocity on the percentage of cells tracked for the maximum track length (15 frames). We also evaluated the effect of setting changes on the number of cells analyzed for velocity and mean curvilinear velocity. A range of maximum velocity settings were compared pair-wise by t-test (α = 0.05). In the control sample with the highest curvilinear velocity, only 10% of the cells analyzed for velocity were tracked for the maximum time when the maximum velocity was set at 300 μm/sec, while 74–76% of the cells were included at settings of 1000–1500 μm/sec (Table 4). At any maximum velocity setting, less than 12% of the cells had maximum track lengths between 11 and 14 frames (data not shown). Examination of the data indicated that a plateau was reached for both cells analyzed and percent cells tracked for a maximum length between settings of 1000 and 1500 μm/sec. Mean curvilinear velocities were then tested for a significant difference from that obtained at 1500 μm/sec. Table 4 shows that no significant difference in the curvilinear velocity of these spermatozoa occurred after the maximum velocity was increased over 1000 μm/sec. Thus, a setting of 1500 μm/sec was subsequently employed in the sperm motion analyses of epichlorohydrin-treated animals.

The minimum number of track points (frames analyzed) needed to describe curvilinear velocity reliably was evaluated by analyzing spermatozoa from the same set of control fields with both the minimum sample for velocity and the maximum number of frames analyzed, set at the same value (eg, at a setting of 10, only sperm tracks of exactly 10 frames were analyzed). Ten fields were analyzed at each minimum frame setting (5–17 frames). The maximum velocity was set at 1500 μm/sec; other

settings were as previously described. The mean curvilinear velocity was inversely related to track length (Table 5). These velocity means were independent of track length setting only if 11 frames or more were analyzed. To determine whether a minimum of 11 frames was required to describe reliably the motion of the spermatozoa or if the observed association was due to the elimination of faster swimming cells that crossed paths and were subsequently eliminated from further analysis, we further analyzed the same 10 fields with the basic CellSoft program (20 frame maximum analysis) and saved the "track files" for individual cells. Subsequently, the track files for those motile cells that had been tracked for exactly 20 frames were processed using the CellSoft Research Module. The output was the instantaneous velocities for each of the 19 intervals between the 20 frames for each cell track. These instantaneous velocities were cumulatively averaged (ie, intervals 1 and 2, then intervals 1, 2, and 3,...) for each cell. These cumulative averages (for specific accumulated track lengths) for each cell were subsequently averaged across all cells. As can be seen in Fig. 2, the average cumulative velocity for intervals 1–19 is the same as that for the average of intervals 1 and 2 ($p > 0.05$). It was judged that sperm tracks of greater than or equal to three frames could be included to estimate reliably the curvilinear velocity endpoints of rat sperm samples.

TABLE 5. Mean Curvilinear Velocity (± SD) versus Track Length

Track Length (Frames)	Mean Curvilinear Velocity (μ/sec)
5	194.3 ± 76.2†
6	194.2 ± 65.0†
7	183.6 ± 56.7†
8	187.6 ± 57.7†
9	183.7 ± 53.7†
10	181.1 ± 53.0†
11	176.3 ± 54.3
12	173.8 ± 52.6
13	174.6 ± 53.0
14	170.6 ± 48.2
15	172.2 ± 48.8
17	167.0 ± 51.9

*Values represent duplicate analyses of 10 video fields from one control animal.

Track length (frames) = Minimum Sample Velocity = Maximum number of frames analyzed.

†Differs significantly from velocity analyzed over 17 frames.

This approach of using cumulative averages for successive intervals of sperm tracks of exactly 20 frames was also applied to evaluate the minimum track length required for reliable estimates of linearity. Figure 3 shows the cumulative linearities of sperm cells tracked over exactly the same motion

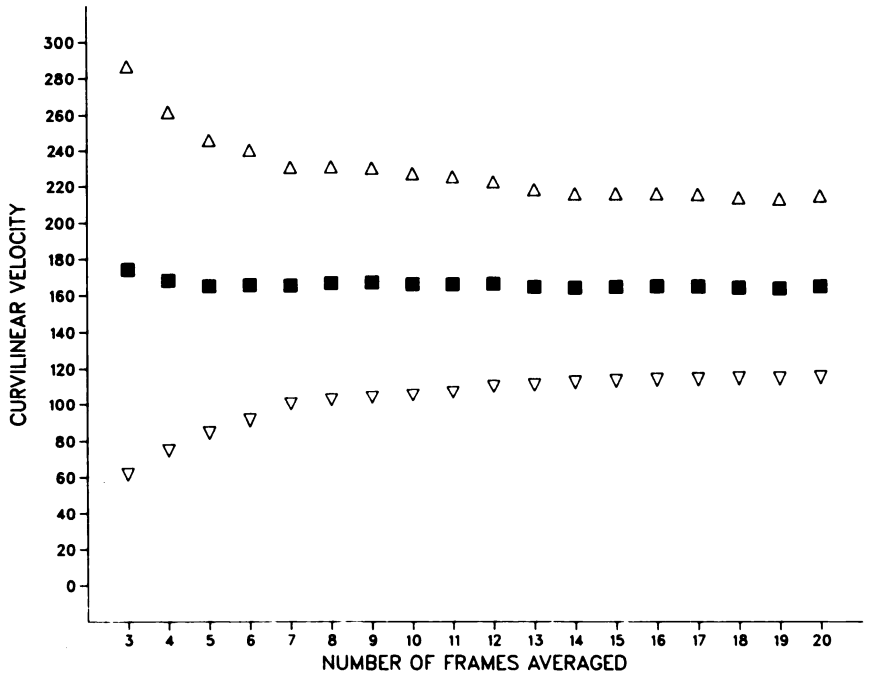

Fig. 2. Mean (■) cumulative curvilinear velocity (± SD [Δ]) at different track lengths for one control rat (n = 189 cells).

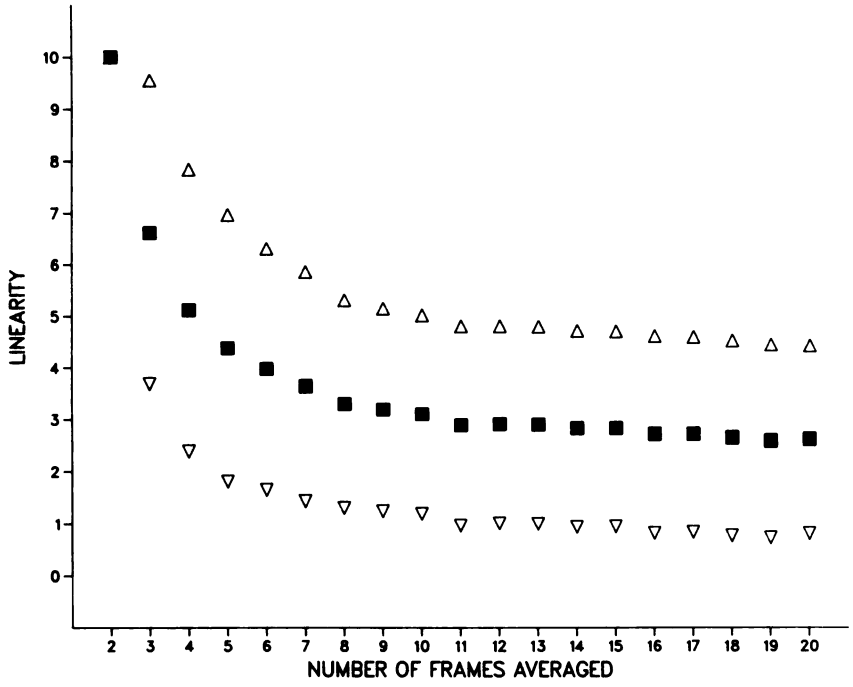

Fig. 3. Mean (■) cumulative linearity (± SD [Δ]) at different track lengths for one control rat (n = 297 cells).

intervals. The values for linearity are significantly different from those obtained at minimum sample for linearity of 20 frames until at least 11 frames have been analyzed ($p < 0.05$). Thus, the "minimum sample for linearity" was set at 11 frames in our auxiliary computer program. Since linearity is derived from the ratio of straight line velocity to curvilinear velocity with three frames being adequate to describe curvilinear velocity (curvilinear velocity showing a complete independence from track length) and 11 frames required to describe linearity, 11 frames were used to estimate straight line velocity. In the present study, these settings resulted in an average of 96% of the motile cells being included in curvilinear velocity estimates, 84% in linearity and straight-line velocity estimates, and 49% in ALH and beat/cross frequency estimates.

Statistical Analyses of the Distributions of Sperm Motion Endpoints

Analyses of the distributions of sperm motion endpoints within both control and treated animals generally revealed that the distributions were not normal or symmetrical. All distributions were unimodal and in most cases were positively skewed (longer tail extending toward higher values). This indicates that, depending on the degree of skewness and the total number of cells analyzed, the mean value could be a biased summary statistic for the individual sample response distribution and that other distribution characteristics measuring shape and location should be considered in the evaluation of a toxic effect.

Some of the distribution parameters (minimum, 1st, 5th, 95th, and 99th percentiles, maximum and range) were subject to large variability due to the influence of a few very extreme cells. These variables were, therefore, not used to test for a dose-related effect. The trimmed (5% and 10%) means and the overall mean or median value were equivalent for all parameters, probably due to the large number of cells analyzed per sample (> 200). Because of the redundancy, the trimmed means were also deleted from the hypothesis testing.

For each treatment group, the mean of each distribution parameter was calculated from the individual animal values. Figure 4 graphically displays the means of the distribution parameters for curvilinear velocity, linearity, straight line velocity, the amplitude of lateral head displacement and change in direction. The distribution of beat/cross frequency data was discrete in nature due to the method of calculation and thus, was not included in the distributional analysis. Shifts in the response distributions could be seen for each sperm motion endpoint. The average mean and median distributional values were very close in all the sperm motion endpoints except for change in direction at the higher

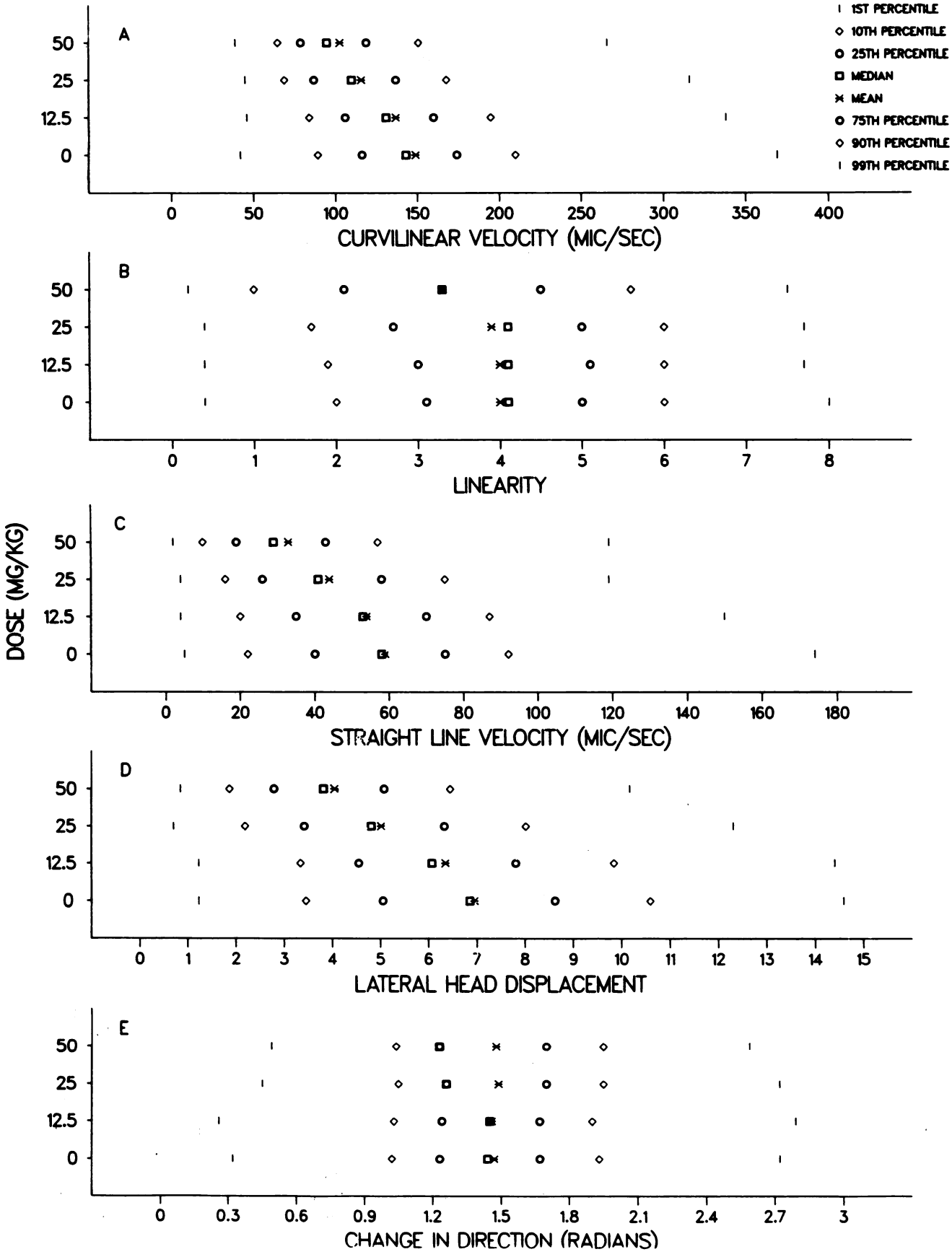

Fig. 4. The average distributions of curvilinear velocity (A), linearity (B), straight line velocity (C), amplitude of lateral head displacement (D), and change in direction (E) for untreated and epichlorohydrin-treated rats. Points are means of distribution parameters from 18, 16, 18, and 15 rats in the 0, 12.5, 25, and 50 mg/kg dose groups, respectively.

dose levels. Table 6 lists the summary statistics for each response parameter and the results of the statistical analyses. With the exception of percent motile spermatozoa, percent circularly swimming spermatozoa, and change in direction, there was a significant dose effect of epichlorohydrin on each of the sperm motion endpoints. In all cases where the overall test for differences among the dose groups was significant, a highly significant linear trend was also observed with all p-values being less than 0.03 and most less than 0.0001. Mean beat/cross frequency and linearity variation statistics (standard deviation and interquartile range) increased with increasing dose. All other significant response parameters decreased with increasing dose.

The multivariate analysis of variance tested jointly the 10th, 25th, 50th, 75th and 90th percentiles of the response distributions of the sperm motion endpoints. The overall MANOVA test for differences among the dose groups found a significant shift in the response distributions for linearity, curvilinear velocity, straight line velocity, and lateral head displacement ($p < 0.0001$). The MANOVA pairwise test between the control group and other dose levels found a significant effect on each of these endpoints at the 25 mg/kg and 50 mg/kg dose levels and additionally, on lateral head displacement at the 12.5 mg/kg dose level.

The consistency and strength of the evidence for a significant dose effect at a specific level can be evaluated by examining the results of the univariate analyses. If the results of the multivariate test were statistically significant, the univariate tests would indicate which percentile parameters were contributing significantly to the MANOVA result. In the univariate analyses of the individual response parameters, the lowest dose at which a significant effect was found as detected by pairwise comparison to the control group, differed among the sperm motion endpoints. The entire response distribution for ALH and curvilinear velocity shifted significantly lower at each dose level with the exception of the 10th percentile at the 12.5 mg/kg dose. This result for curvilinear velocity contrasts with the analysis by MANOVA, which did not reveal an effect at the 12.5 mg/kg dose. Comparison of the differences in mean percentile values between the 12.5 mg/kg dose group and the controls across the response distribution shows that for curvilinear velocity the difference increased with increasing percentile points. However, this increase was not large enough for a significant result in the multivariate analysis or a significant difference in the univariate analysis of variability parameters. For ALH, the statistically significant MANOVA result for the 12.5 mg/kg dose group was due to significant shifts in location at and below the 50th percentile. No significant difference in the variability was observed at this dose level for ALH. For both curvilinear velocity and ALH, there were significant shifts in the response distributions at the 25 mg/kg and the 50 mg/kg dose levels that increased with increasing percentile points. In addition, the variability of these endpoints within an animal, as defined by the interquartile range and the standard deviation, was significantly different from the controls.

The effect on linearity at the 25 mg/kg dose level in the multivariate analysis was due primarily to a shift of the 25th percentile point that also resulted in a significant effect on the interquartile range statistic. At this dose level, the linearity response distribution was not significantly affected above the 25th percentile point by epichlorohydrin. At the 50 mg/kg dose level, the linearity response distribution was significantly different from the control distribution at every percentile point except the 90th, but the difference between the mean values in the experimental and the control groups decreased with increasing percentile points (the only significant sperm motion endpoint for which this type of effect was observed).

The mean, 25th, and 75th percentile of the straight line velocity differed from the controls at the 12.5 mg/kg dose level. For all other parameters, 25 mg/kg was the lowest dose at which a significant effect was found, as in the MANOVA test. The significant effect at the 12.5 mg/kg dose level was due mainly to a shift at the 25th percentile. At the 25 mg/kg dose level, the differences in mean values between the experimental and control groups were primarily at and below the 50th percentile, while at the 50 mg/kg dose the differences increased across the entire response distribution.

Various size of effect measures were calculated to evaluate the relative sensitivity of the various response endpoints and their parameters (Table 7). Each effect size measure was listed for one parameter of central tendency (mean) and one parameter of variability (interquartile range) for the endpoints whose distributional characteristics were analyzed. A comparison of the relative values indicated that lateral head displacement and the velocity endpoints were the most sensitive in showing an effect of epichlorohydrin on sperm

TABLE 6. Means (± Standard Deviation) of Sperm Motion Parameters and Statistical Analysis Results*

Variable	Dose (mg/kg) 0 (n = 18)	12.5 (n = 16)	25 (n = 18)	50 (n = 15)
Percent motile sperm†	58.7 (14.8)	57.5 (13.1)	55.6 (9.8)	51.8 (10.8)
Percent circularly† swimming sperm	17.3 (4.3)	18.1 (3.9)	17.9 (4.7)	18.0 (3.9)
Beat/cross frequency	10.2 (0.8)	10.7‡ (0.4)	10.8‡ (0.5)	11.0‡ (0.5)
Curvilinear velocity (μm/sec)				
MANOVA (10, 25, 50, 75, 90)		[0.2067]§	[0.0001]	[0.0001]
10th percentile	89.2 (8.9)	83.9 (11.9)	69.5‡ (6.8)	64.6‡ (7.7)
25th percentile	116.1 (11.0)	105.6‡ (13.5)	87.1‡ (6.9)	79.0‡ (8.6)
Mean	148.7 (14.5)	137.6‡ (14.8)	116.9‡ (10.1)	103.3‡ (11.7)
Median	143.0 (13.3)	130.7‡ (15.1)	110.5‡ (10.0)	95.4‡ (10.7)
75th percentile	173.5 (16.8)	160.1‡ (14.6)	136.6‡ (11.7)	119.4‡ (14.3)
90th percentile	209.7 (24.1)	195.4‡ (18.4)	168.2‡ (15.8)	151.1‡ (26.1)
Standard deviation¶	57.6 (22.7)	52.3 (17.5)	47.1‡ (14.5)	40.6‡ (11.2)
Interquartile range	57.4 (9.1)	54.5 (7.5)	49.5‡ (7.8)	40.4‡ (7.8)
Linearity ($[V_{s1}/V_{c1}] \times 10$)				
MANOVA (10, 25, 50, 75, 90)		[0.6166]	[0.0153]	[0.0001]
10th percentile	2.0 (0.49)	1.9 (0.52)	1.7 (0.44)	1.0‡ (0.38)
25th percentile	3.1 (0.35)	3.0 (0.54)	2.7‡ (0.40)	2.1‡ (0.49)
Mean	4.0 (0.35)	4.0 (0.34)	3.9 (0.34)	3.3‡ (0.38)
Median	4.1 (0.34)	4.1 (0.37)	4.0 (0.36)	3.3‡ (0.50)
75th percentile	5.0 (0.34)	5.1 (0.34)	5.0 (0.35)	4.5‡ (0.50)
90th percentile	6.0 (0.43)	6.1 (0.32)	6.0 (0.34)	5.6 (0.51)
Standard deviation‖	1.5 (0.18)	1.6 (0.19)	1.6 (0.15)	1.7‡ (0.25)
Interquartile range	2.0 (0.21)	2.1 (0.34)	2.3‡ (0.25)	2.5‡ (0.41)
Straight line velocity (μm/sec)				
MANOVA (10, 25, 50, 75, 90)		[0.3959]	[0.0001]	[0.0001]
10th percentile	22.4 (6.2)	20.5 (7.0)	15.9‡ (4.4)	10.2‡ (2.7)
25th percentile	39.5 (6.4)	34.9‡ (8.5)	26.3‡ (4.9)	19.1‡ (3.9)
Mean	59.0 (8.8)	54.0‡ (7.2)	44.0‡ (5.8)	33.3‡ (5.6)
Median	57.8 (7.8)	52.9 (9.5)	41.2‡ (6.2)	29.4‡ (4.8)
75th percentile	74.8 (8.7)	69.8‡ (8.2)	57.8‡ (7.3)	42.7‡ (7.7)
90th percentile	91.9 (11.8)	86.6 (7.5)	75.0‡ (9.1)	56.7‡ (8.7)
Standard deviation¶	30.9 (11.3)	28.2 (6.6)	24.5‡ (5.6)	23.2‡ (15.1)
Interquartile range	35.2 (5.2)	34.9 (4.5)	31.5‡ (5.5)	23.6‡ (5.3)
Lateral head displacement (μm)				
MANOVA (10, 25, 50, 75, 90)		[0.0105]	[0.0001]	[0.0001]
10th percentile	3.5 (0.64)	3.3 (0.80)	2.2‡ (0.38)	1.9‡ (0.32)
25th percentile	5.1 (0.66)	4.6‡ (0.91)	3.4‡ (0.54)	2.8‡ (0.38)
Mean	7.0 (0.75)	6.4‡ (0.87)	5.0‡ (0.56)	4.1‡ (0.44)
Median	6.9 (0.76)	6.1‡ (0.97)	4.8‡ (0.58)	3.8‡ (0.46)
75th percentile	8.6 (0.92)	7.8‡ (1.02)	6.3‡ (0.72)	5.1‡ (0.57)
90th percentile	10.6 (1.22)	9.8‡ (1.02)	8.0‡ (0.87)	6.5‡ (1.02)
Standard deviation	2.8 (0.48)	2.6 (0.30)	2.3‡ (0.34)	1.9‡ (0.44)
Interquartile range	3.6 (0.59)	3.2 (0.52)	2.9‡ (0.46)	2.3‡ (0.43)
Change in direction† (radians)				
MANOVA (10, 25, 50, 75, 90)		[0.9178]	[0.2481]	[0.6200]
10th percentile¶	1.02 (0.116)	1.03 (0.061)	1.05 (0.057)	1.04 (0.094)
25th percentile¶	1.23 (0.054)	1.24 (0.066)	1.26 (0.047)	1.23 (0.089)
Mean	1.47 (0.051)	1.46 (0.066)	1.49 (0.043)	1.48 (0.091)
Median	1.44 (0.044)	1.45 (0.065)	1.48‡ (0.034)	1.46 (0.092)
75th percentile¶	1.67 (0.056)	1.67 (0.080)	1.70 (0.046)	1.70 (0.117)
90th percentile¶	1.93 (0.100)	1.90 (0.116)	1.95 (0.080)	1.95 (0.126)
Standard deviation	0.40 (0.057)	0.40 (0.044)	0.38 (0.043)	0.38 (0.046)
Interquartile range	0.44 (0.043)	0.44 (0.052)	0.44 (0.053)	0.47 (0.076)

*Except as noted, all tests for overall dose-related effects and linear trends were significant, $p < 0.03$.
†Overall test, $p > 0.05$; linear trend, $p > 0.05$.
‡Differs significantly from the control.
§p-value for Wilks' Statistic.
‖Overall test, $p > 0.05$; linear trend, $p < 0.01$.
¶Analyzed with Kruskal-Wallis, Jonckheere's and Wilcoxon tests rather than ANOVA, regression and t-tests.

TABLE 7. Size of Effect Measures for Response Endpoints and Parameters

Parameter	Overall Test*	Linear Regression†	Minimum Detectable Difference‡ Power = 0.70 Sample Size 10	15	20	Power = 0.90 Sample Size 10	15	20
Percent motile spermatozoa	0.04	1.7	20	16	14	26	21	18
Percent circularly swimming spermatozoa	0.01	0.4	15	12	11	20	16	14
Beat/cross frequency	0.21	3.7	8	7	6	11	9	8
Curvilinear velocity								
Mean	0.66	10.4	11	9	8	14	12	10
Interquartile range	0.39	6.4	18	14	12	23	19	16
Linearity								
Mean	0.40	6.0	10	8	7	13	10	9
Interquartile range	0.26	4.7	12	10	9	15	12	11
Straight line velocity								
Mean	0.67	11.2	17	13	12	22	18	15
Interquartile range	0.45	6.9	16	13	12	21	17	15
Lateral head displacement								
Mean	0.74	12.7	12	10	8	16	13	11
Interquartile range	0.47	7.6	18	15	13	24	19	17
Change in direction								
Mean	0.05	0.9	4	3	3	5	4	4
Interquartile range	0.06	1.7	11	9	7	14	12	10

*Proportion Variance
†|B|/SE(B): Slope/Standard Error of Slope
‡Percentage

motion. For these endpoints, the size of effect measured as either the proportion of total variance or slope to standard error ratio, was of the same magnitude. Except for change in direction, for which no dose-related effect was observed, the effect size of the mean value was greater than the effect size of the interquartile range. This indicates that the effect of epichlorohydrin on the shape of the endpoint distribution as measured by the interquartile range, although significant, was less than the effect on the central tendency.

For each sperm motion endpoint, the minimum detectable difference as a percentage change between two groups was estimated. The values were based on the variability estimates from the control group, the normal distribution z-score statistic and a two-tailed test at a significance level of 0.05. Table 7 lists the estimates for various animal sample sizes and power levels. The minimum detectable percentage change in the mean value and in the interquartile range statistic were of the same magnitude for linearity and straight line velocity. For curvilinear velocity, lateral head displacement, and change in direction, smaller percentage changes in the mean value as compared with the interquartile range would be considered statistically significant. This difference is a reflection of their respective coefficients of variation, which indicate that there is greater variability in the shape of the distributions within animals than in the central tendency. The smallest coefficient of variation was observed for the mean change in direction.

The correlation coefficients for the response endpoints were estimated separately using the mean values from the control animals and the mean values from the animals exposed to epichlorohydrin (Table 8). For each correlation estimate, the means for each endpoint were calculated for the same subset of cells, eg, mean curvilinear velocity and mean linearity for a sample were estimated for cells tracked for 11 or more frames. As expected, there was a significant correlation in the control data and in the treated animal data between straight line velocity and both curvilinear velocity and linearity, the latter being derived from the ratio of the two velocities. Lateral head displacement was also significantly correlated with curvilinear velocity and straight line velocity in both the control and treated animals. For some of the parameters, the estimated coefficients and their statistical significance differed between the two data sets. This is probably due in part to the difference in sample sizes in the two groups, but

also could be a reflection of the differential dose effect of epichlorohydrin. In all but one of these correlations, the association was significant in the treated animals but not in the controls, even though no significant dose-related effect was observed in several of the endpoints (eg, percent motile spermatozoa and percent circularly swimming spermatozoa). The strength of the associations in these cases was low and the maximum percent variability in one parameter explained by the second parameter never exceeded 36%.

Discussion

The present work is focused on optimization of videomicrographic system settings for use of the CellSoft CASA system on rat spermatozoa and the subsequent estimation and analysis of motion response parameters. The results presented could provide guidance for: (1) the many instrumental settings that must be evaluated and specified for a given instrument to reduce sampling bias, (2) selection of the appropriate response parameters, including measures of central tendency and variability, for each endpoint, and (3) the statistical approaches to analyzing such data.

An objective technique for the adjustment of the threshold grey level setting of the digitized sperm image has not been previously approached. Most references suggest reduction of video contrast to decrease the background of cellular debris. In the rat, epididymal sperm suspensions are relatively free of debris. We have shown that a "window" of threshold grey settings exists wherein percent motile sperm measures are considered accurate. It appeared that the variation of motion endpoints was reduced within this "window" while the number of cells included in the analyses is maximized in the midst of these grey level values. Ideally, this range should be identified for each sample set of fields. Mack et al (1988) refer to a plateau region within the CellSoft threshold grey level settings within which the measure of percent motile cells is "at least 75% of maximum." Since no mention is made of the accuracy of these counts, and since we have observed artificially inflated values for percent motile cells at high threshold grey settings, we recommend identification of the midrange of extreme grey level settings as an objective technique that should be used for each set of fields (one chamber).

For the maximum velocity setting, we found that below an average sperm density of 30 spermatozoa field (analyzing for 0.5 sec) the maximum velocity setting could be raised substantially (> 1500 μm/sec) before a significant reduction was observed in

TABLE 8. Pearson's Correlation Coefficients

Variable	Mean Linearity	Mean Straight Line Velocity	Mean Change in Direction	Mean Lateral Head Displacement	Beat/ Cross Frequency	Percent Motile Sperm	Percent Circularly Swimming Sperm
Mean curvilinear velocity	0.44*‡	0.87‡	−0.15	0.94‡	−0.20	0.49‡	0.02
	0.15†	0.77‡	−0.05	0.83‡	−0.07	0.36	−0.12
Mean linearity		0.80‡	−0.27	0.03	0.32‡	−0.08	0.20
		0.73‡	−0.63‡	−0.13	0.79‡	−0.004	0.007
Mean straight line velocity			−0.20	0.79‡	−0.04	0.23	0.11
			−0.30	0.62‡	0.27	0.32	−0.04
Mean change in direction				−0.11	0.04	−0.01	−0.42‡
				−0.06	0.12	0.19	−0.21
Mean lateral head displacement					−0.30‡	0.35‡	0.08
					−0.42	0.25	0.04
Beat/cross frequency						−0.09	−0.03
						0.24	0.11
Percent motile sperm							0.007
							0.32

*Coefficient based on treated animals only (n = 49).
†Coefficient based on control animals only (n = 18).
‡Significantly different from 0 ($p < 0.05$).

the number of cells analyzed for velocity and in the number of cells tracked for the maximum track length (15 frames). Comparison of our values (obtained at maximum velocity settings of 1100 to 1500 μm/sec) with those obtained at a maximum velocity setting of 300 μm/sec (as reported by Working and Hurtt, 1987) shows that a large increase was seen in the number of cells analyzed for velocity, the number of cells tracked for the full analysis time, and in the mean curvilinear velocity.

Minimum track lengths necessary for analysis of percent motile spermatozoa, curvilinear velocity and linearity (and straight line velocity, the numerator for the linearity calculation) were also evaluated. Access to auxiliary computer programs allowed us to reanalyze CellSoft individual cell files and frame-to-frame track files by either: (1) excluding cells of various track lengths for examination of effects on percent motile spermatozoa or (2) cumulatively averaging frame-to-frame velocities and linearities. In addition, once optimal CellSoft settings were determined, these auxiliary programs allowed us to set varying minimum track lengths for different motion endpoints. The values listed in Tables 1 and 2 are those equal to or above which the respective motion endpoints were independent of track length. Compared with the recommendations of maximum velocity setting for human sperm analysis ($\geq$ 7 frames: Mack et al, 1988; Ginsburg et al, 1988), our value for minimum sample for velocity ($\geq$ 3 frames) is rather low and allows for inclusion of the fastest cells, which are also the cells most likely to cross with other spermatozoa or swim off the screen, resulting in short track lengths. We also found that a minimum track length of 11 frames were required to estimate linearity adequately for rat sperm cells. Values of linearity for sperm cells tracked for less than 11 frames were significantly higher than when those same cells were tracked for 11 or more frames. Although Budworth et al (1988) reported an independence of the sperm linearity measurement and track length when analyzing bovine sperm cells, the results of Knuth et al (1987) and of Mack et al (1988) showed evidence of a dependence on track length for human spermatozoa.

The use of a computer-assisted sperm motion analysis system to investigate the effect of a known reproductive toxin was evaluated. Various response parameters measuring characteristics of sperm motion were evaluated in terms of a dose-related effect of epichlorohydrin and sensitivity as effect measures for toxicologic research. In general, we found that the distributions of the response endpoints within control and treated animals were unimodal but not normal or symmetrical and in most cases positively skewed. As a result, distributional parameters should be used in conjunction with the mean to test adequately for a toxic effect. We recommend parameters that describe the shape as well as the location of distributional points. These include percentile points and measures of variability within an animal.

Multivariate, as well as univariate statistical procedures, are recommended for the analysis of sperm motion data. Multivariate hypothesis testing procedures have an advantage in that more than one response parameter or endpoint can be tested simultaneously. Using these methods, the entire distribution of a sperm motion endpoint within an animal can be tested for a toxic effect. It is expected that MANOVA analyses may provide a more sensitive test for shifts in the distribution of sperm motion endpoints. Additionally, the multivariate procedures aid in controlling the false positive rate that would be inflated in the univariate analyses as a result of the numerous non-independent hypotheses tests. Since this possibility exists, we recommend the application of the modified Bonferroni correction or another similar correction to the pairwise test procedure when determination of the lowest or no effect level (LOEL or NOEL) is the primary objective. In all cases, the data should be examined for consistency of an effect across the various distributional parameters of the sperm motion endpoints. If the objective of the study is not to investigate a lowest effect level, but rather to compare all dose levels, we recommend a multiple comparisons procedure such as Tukey's in which all pairwise testing is performed at a known overall significance level.

In the current study of epichlorohydrin, a significant effect was found on curvilinear velocity, linearity, straight line velocity, lateral head displacement and beat/cross frequency. Except for beat/cross frequency, these sperm motion parameters decreased with increasing dose. The mean value for beat/cross frequency was significantly greater than the control at each dose level tested. For the sperm motion endpoints whose distributions were significantly affected by exposure to epichlorohydrin, the greatest shifts were generally observed in the lower half of the distributions, and the higher the dose level, the larger the shift across the entire distribution. Evaluation of the numerous response

parameters and the results of their statistical analyses indicate that the evidence was consistent for a dose-related effect for curvilinear velocity, straight line velocity, and lateral head displacement at both the 25 mg/kg and 50 mg/kg levels of epichlorohydrin, with suggestive evidence for an effect at the 12.5 mg/kg dose. For linearity, a consistent effect was found only at the 50 mg/kg dose level.

Epichlorohydrin had the greatest effect on the velocity endpoints and lateral head displacement as measured by the proportion of variance statistics. For these endpoints, significant shifts in the location but not in the shape of the response distributions were observed at the 12.5 mg/kg dose level. At greater dose levels, significant shifts in both location and shape were observed, which would indicate a dose effect throughout the distribution of the endpoint. The estimated minimum detectable differences were consistent with those reported by Working and Hurtt (1987) using the Fischer 344 rat. Except for the associations found between the velocity endpoints, linearity, and lateral head displacement, little if any association was observed between the values of the other endpoints. These endpoint correlation results were not inconsistent with those reported by Budworth et al (1988) in the analyses of bull spermatozoa. The correlation analyses were performed using the mean value of endpoints whose distributions typically are dissimilar. This could account for some of the discrepancies noted. A more sensitive analysis would relate directly the motion endpoints of a cell, rather than relating summary statistics for endpoints whose distributions may differ in shape. This type of analysis is recommended for future studies.

The results of this study support the use of a computer-assisted sperm motion analysis system to test for a reproductive toxic effect. The system can reliably provide evidence for characterizing the effect of a toxicant. Further studies are currently underway employing a battery of model male reproductive toxicants known to affect sperm motion. Application of the statistical methodology described in this study to test for changes in motion endpoints could help to identify which statistical approaches to motion analysis are the most incisive. Investigations that include animal fertility studies could also help to identify patterns of motion changes that can predict infertility.

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