Computer-Assisted Human Semen Analysis
Sampling Errors and Reproducibility

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Videomicrographic computer-automated semen analysis systems allow quantitative description of sperm motility, velocity, progression, and head movement amplitude and frequency with unprecedented ease. The minimum number of spermatozoa needed for stable results, the variability of measurements and optimum methods of sampling the ejaculate were determined for one such system (CellSoft, CRYO Resources, New York, NY). Sampling a minimum of 225 spermatozoa yields stable measurements, and analyzing four microscope fields in triplicate provides data with the lowest coefficient of variation. The variability attributable to the instrument itself was acceptable for all measurements (6.2% to 15.4%) except mean amplitude of lateral head displacement. Limitations of these results and the potential utility of videomicrographic sperm movement analysis are discussed.

Key words: computer automated semen analysis, sperm movement analysis.

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Recent studies have suggested that sperm movement analysis may have predictive value for assessing male fertility (Aitken et al, 1982a, 1982b), although data obtained from routine semen analysis contain little for predicting a male's fertilizing potential (Smith et al, 1977). In this context, computer-assisted semen analysis has emerged as a powerful tool for acquiring such data with unprecedented speed and accuracy. These analyses were available in the past only with microcinematographic (David et al, 1981), time-lapse (Overstreet et al, 1979), multiple-exposure (Makler, 1978) or computerized image analysis (Dott, 1975; Liu and Warme, 1977; Katz and Overstreet, 1981) techniques, and were generally too cumbersome for application in other than a research setting. Commercial systems capable of performing fine movement analysis on sperm populations are now available and have been installed for use in both clinical and research applications, but as yet there are no standardized procedures to minimize errors associated with such automated systems.

Data regarding the source and magnitude of errors inherent in this technique are essential for intra- and

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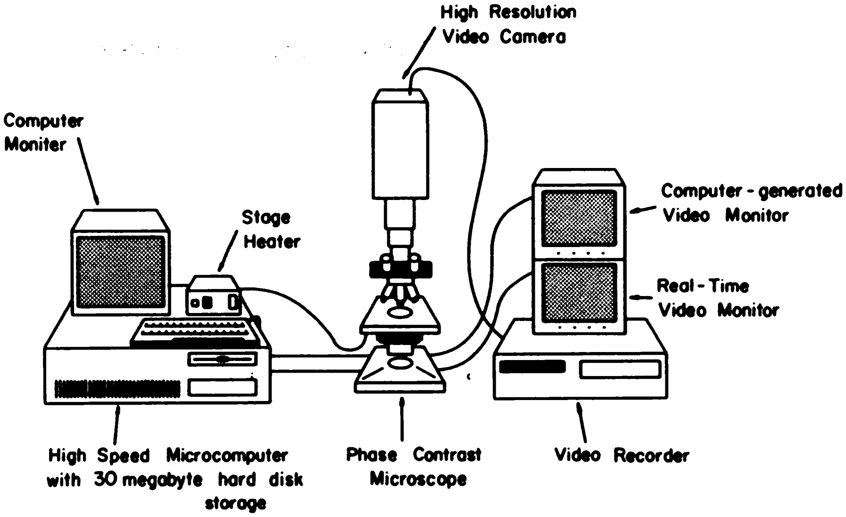

Fig. 1. Hardware configuration of the CellSoft Automated Semen Analyzer.

interlaboratory comparison. In this study, we report the variability and reproducibility of data for movement measurements obtained on freshly ejaculated human spermatozoa using the CRYO Resources CellSoft Computer Automated Semen Analyzer.

Materials and Methods

Hardware comprising the CellSoft Automated Semen Analyzer system is shown in Fig. 1. An Olympus BH-S microscope (Olympus Optical Co., Ltd., Tokyo, Japan) equipped with phase contrast condenser and 10 × achromatic S-plan objective, 6.7 × photo eyepiece and a high resolution video camera (Panasonic WV-1410, Matsushita Electric Industries, Secaucus, NJ) feeds the video signal to a video recorder, from which analysis can be done in real time or the image stored for later processing. Two Panasonic TR-930 high resolution video monitors allow viewing of both the actual video image and computer-generated image of sperm tracks. The real-time or stored video image is then digitized and the digital image is directed to the microcomputer (Leading Edge Model MH, Canton, MA) operating at 8 MHz, which contains proprietary hardware and software (CellSoft, CRYO Resources, New York, NY) allowing frame-by-frame analysis of sperm tracks. A description of the settings under which these analyses were completed (as suggested by the manufacturer) are shown in Table 1, and data obtained during CellSoft computer-automated analysis are indicated in Table 2. A threshold curvilinear velocity of 10 microns/sec was used to define a motile cell, and the grey scale was set subjectively by the user for each ejaculate to exclude non-sperm objects whose luminosity differed from that of a sperm head under phase contrast optics. The system allows storage of data obtained from analysis of a single microscope field, which is averaged with data obtained from analysis of one or more subsequent fields. In this way, a composite analysis is created from a number of areas under the microscope; cumulative data can be reported at any point. Debris and cellular elements other than spermatozoa are effectively excluded from analysis by virtue of their size or luminosity characteristics. The software also allows manual modification of the number of nonmotile objects counted as a sperm head for each field if the operator determines that an object viewed on the video screen is incorrectly included in the sperm analysis.

Ten ejaculates were obtained from healthy donors from our AID program after at least 3 days of abstinence by masturbation into sterile plastic collection jars. Each donor was known to have established at least one pregnancy in the past year and was therefore presumed fertile at the time of study. Samples were maintained at 37 C from collection through microscopic processing with a stage

TABLE 1. General and Lateral Head Displacement Parameters used for Computerized Semen Analysis

Parameter	Value
Sampling frequency, frames/second	30
Number of frames to analyze	20
Maximum allowable velocity, microns/second	120
Minimum number of points for motility	4
Minimum number of points for velocity	7
Pixel scale, microns/pixel	0.688
Minimum size to exclude an object from analysis, pixels	5
Maximum size to exclude an object from analysis, pixels	25
Minimum number of points for calculation of lateral head displacement measurements	7
Minimum velocity for calculation of lateral head displacement measurements, microns	18
Minimum linearity for calculation of lateral head displacement measurement	3.5

TABLE 2. Measurements made with the CellSoft Computer Automated Semen Analyzer System

Measurement	Explanation
Concentration	Number of spermatozoa/ml semen
Motility	Percent of spermatozoa meeting minimum critieria for movement
Velocity	Average progressive head velocity of all motile spermatozoa
Linearity	Ratio of straight distance to actual distance traveled, averaged over all spermatozoa meeting minimum criteria
Maximum amplitude of lateral head displacement	Largest deviation of the sperm head from the mean path averaged over all spermatozoa*
Mean amplitude of lateral head displacement	Mean deviation of the sperm head from the mean path, averaged over all spermatozoa*
Head beat cross frequency	Number of times the sperm head crosses the mean path/second, averaged over all spermatozoa

*These lateral head displacement measurements represent head deviations from the curval mean path. The overall amplitude of displacement is thus double the measured value.

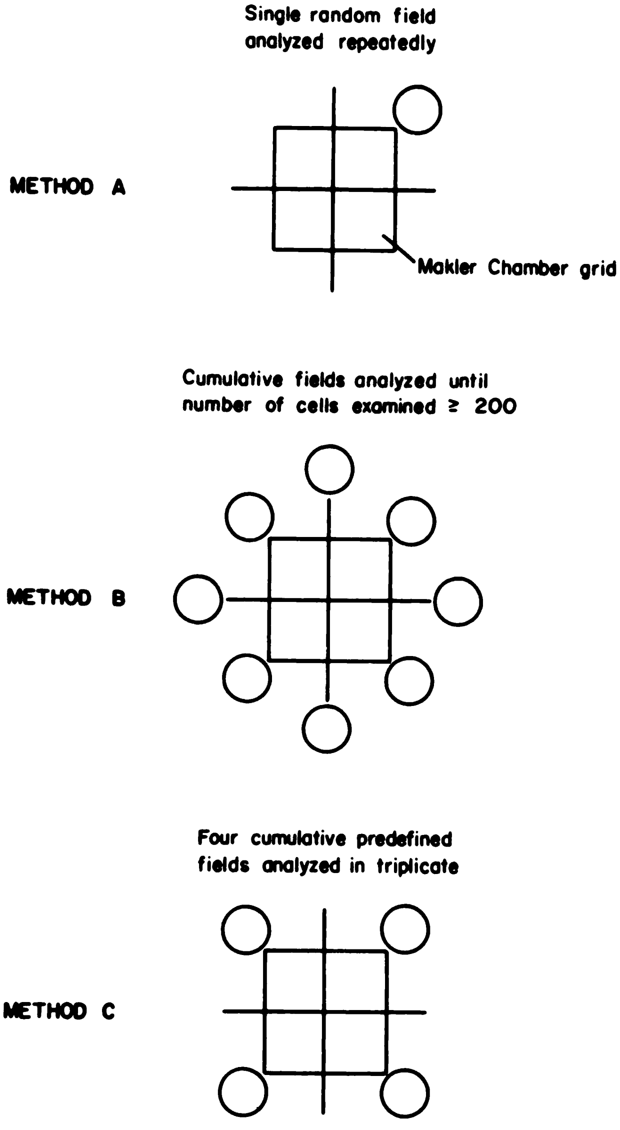

Fig. 2. Schematic diagram of the three sampling methods used in Experiment 2. Each circle represents a single 10 × microscope field located outside the Makler Chamber grid.

warmer, and processed within 30 minutes of liquefaction. After thorough mixing by up and down pipetting, a 10-μl sample of each ejaculate was placed on a warmed Makler chamber (Sefi Medical Industries, Haifa, Israel) and analyzed as described below.

Experiment 1

Samples were analyzed repeatedly with cumulative reporting of results as the number of cells analyzed increased, thus allowing study of the stability of measurements as a function of the number of cells analyzed for each of the 10 ejaculates. Fields were chosen at random around the Makler chamber grid moving in a clockwise direction. Because there was no way to control the exact number of cells examined at each point, the numbers of spermatozoa analyzed were grouped into intervals of $<$ 50, 50 to 100, 101 to 150, 151 to 200, 201 to 250, 251 to 300, 301 to 400, 401 to 500, $>$ 500. Since we were interested in the trend in the measurements as a function of the number of cells analyzed and not their absolute value, the ratio of each subsequent measurement to the first was computed at each interval. A ratio of 1.0 therefore indicated that measurements were not changing, while a value less than or greater than 1.0 indicated they were falling or rising, respectively. Again, because each specimen did not necessarily have an analysis at each measurement interval, these intervals had an unequal N. With missing data, an analysis of variance (ANOVA) or linear trend analysis could not be used to study within-subject variability. Data were analyzed by determining the average ratio ± standard error of the mean (SEM) over all 10 subjects for each interval. Regression analysis was used to test the hypothesis that each measurement ratio as a function of cell number had no trend, ie, a zero slope.

Experiment 2

Measurement error associated with examining sperm populations under the microscope was determined by comparing three sampling methods (Fig. 2). In method A, a single field under the microscope was arbitrarily chosen and, for each ejaculate, five repeated analyses of the same field were obtained. In method B, a sufficient number of different fields under the microscope were examined until

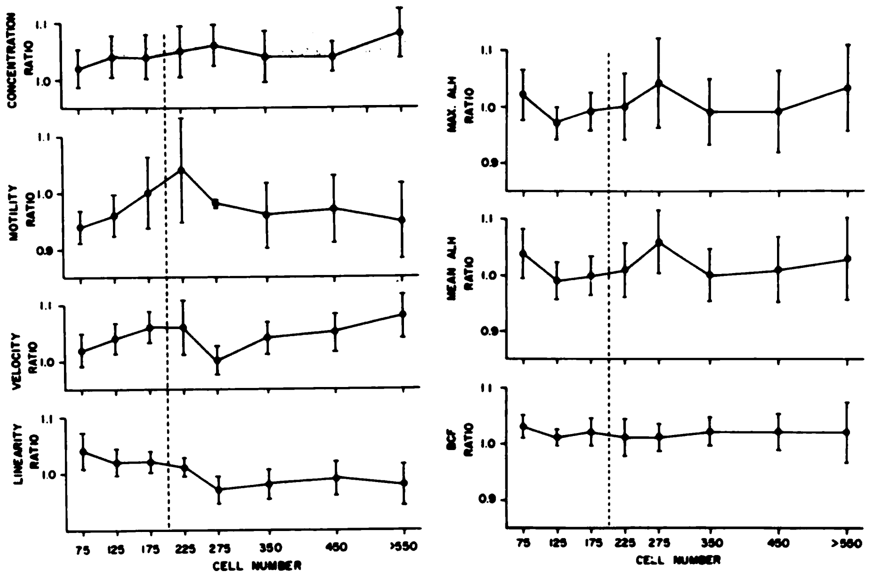

Fig. 3. Ratio of sperm measurements (Mean ± SEM) as a function of the number of spermatozoa examined. The points on the abscissa represent midpoints of intervals for grouping the number of spermatozoa examined, while the ratios along the ordinates were computed by dividing the measurement at each interval by the measurement at the initial interval.

a composite with at least 225 spermatozoa were viewed, again with five separate repetitions for each ejaculate. In method C, four predefined fields were examined giving a composite analysis that was repeated three times for each ejaculate, reloading the Makler chamber after each analysis; analysis of this composite of 12 fields was also repeated a total of five times. The mean ± SEM of the five replications of each method was then determined for each ejaculate, and the three protocols were compared using repeated measures multivariate ANOVA. Within-sample coefficients of variation were determined and averaged over the 10 ejaculates for each measurement, and compared using the student's *t* test.

Experiment 3

Reproducibility of measurements was determined by analyzing a videotaped representative sample of each ejaculate 10 times, each time analyzing the same portion of the videotape using the digital counter of the recorder. The average within-sample coefficient of variation was then computed for each of the seven measurements under study.

Statistical analyses were carried out on a Digital Equipment Corporation VAX 11/780 computer using BMDP software package (Dixon et al, 1985).

Results

The average values and ranges of each of the seven measurements for the 10 subjects are shown in Table 3.

The data from Experiment 1 regarding the stability of measurements as a function of the number of cells examined are presented in Fig. 3, showing the mean (± SEM) ratios for each of the seven measures. The standard errors depicted, while seemingly large, were at most only 8.6% of the mean. If one examines only the initial portion of each regression line through 175 cells, a significant nonzero slope is obtained only for the motility and linearity measurements. Beyond 225 cells, no slopes were significantly different from zero ($P > 0.05$). Therefore, for each of the seven measurements studied, the data does not change once a minimum of 225 spermatozoa are sampled, the first interval where a zero slope is obtained for all measurements. The average number (and range) of cells studied for each sampling method in Experiment 2 were 50 (16 to 98) for Method A, 243 (233 to 290) for Method B, and 281 (220 to 358) for Method C. Multivariate ANOVA failed to show a significant difference between the three sampling methods of

TABLE 3. Summary Statistics for the Ten Ejaculates Tested, Obtained by Triplicate Analysis (Total of 12 Microscope Fields) of at least 225 Cells

Measurement	Mean ± SEM	Range
Concentration (millions/cc)	96.9 ± 20.6	42-217
Motility (percent)	52.8 ± 8.8	14.8-91.4
Velocity (microns/sec)	48.8 ± 2.3	37.6-62.1
Linearity	6.6 ± 0.2	5.4-7.6
Maximum amplitude of lateral head displacement (microns)	2.4 ± 0.2	1.6-3.3
Mean amplitude of lateral head displacement (microns)	1.9 ± 0.1	1.1-2.8
Beat Cross Frequency (cycles/sec)	15.0 ± 0.2	13.5-46.4

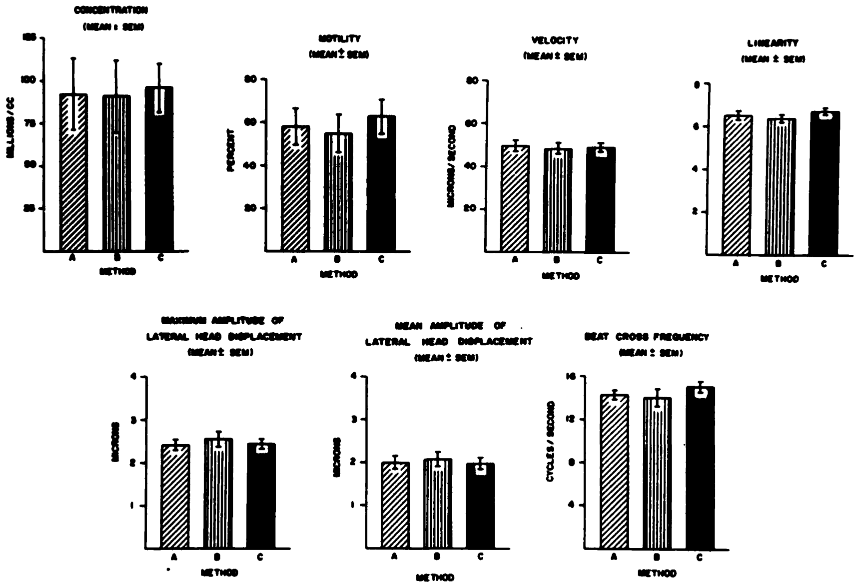

Fig. 4. Repeated measures analysis of variance comparing the Mean ± SEM for each measurement between the three sampling methods.

Experiment 2 for any of the seven measurements (Fig. 4). The average within-sample coefficient of variation for each measurement and sampling protocol was then determined over the 10 ejaculates tested (Fig. 5). Method C resulted in consistently lower coefficients of variation, and for all measurements except motility and velocity it was statistically lower than at least one other method. This indicates less variability in the data using the triplicate analysis method. Such a triplicate analysis, counting at least 225 cells, can be completed in 5 minutes.

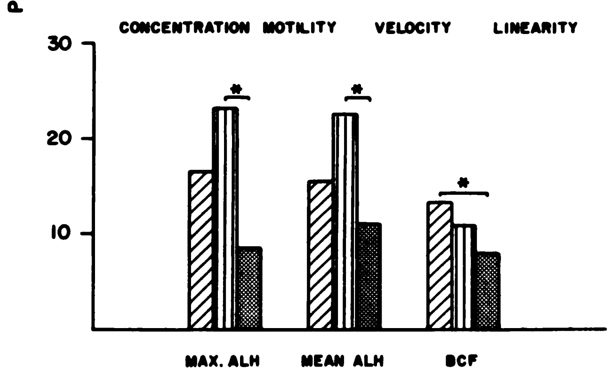

Fig. 5. Average within-sample coefficients of variation for three sampling methods. * = statistically significant difference, $P < 0.05$, Student's *t* test.

Data regarding the reproducibility of repeated measurements using videotaped samples are shown in Fig. 6, comparing the within-sample coefficients of variation averaged over the 10 ejaculates. Computer-automated semen analysis allows acquisition of concentration, motility, velocity and linearity measurements with average coefficients of variation of 6.2%, 8.3%, 10.9%, and 10.4%, respectively, when the analyses were repeated 10 times on each specimen. The variation of data regarding head movement amplitude and frequency are somewhat higher at 13.9%, 15.4%, and 26.1% for beat cross frequency, maximum and mean amplitude of lateral head displacement, respectively.

Discussion

Quantitative sperm motility assessment using some means of image recording and analysis was first reported in 1949 (Rothschild, 1949), and was in use by other investigators several years later (Rikmenspoel and Van Herpen, 1957; Janick and MacLeod, 1970). The CellSoft system represents a combination and refinement of computerized videomicrographic analysis techniques pioneered separately by Dott (1975), Liu and Warme (1977), and Katz and Overstreet (1981), and multiple-exposure or time-lapse methods introduced by Makler (1978, 1980a, 1980b) and Overstreet et al (1979). Maps of the sperm heads within the established pixel field are stored and analyzed, recreating the individual paths of spermatozoa found within the microscope field over the 2/3-second analysis window used in this study. These sperm tracks are then individually analyzed for velocity, linearity, and head displacement amplitude and frequency. Combined with the numbers of objects fitting the luminosity and size characteristics of a nonmotile sperm head, a precise movement description of the sperm population can be obtained. Additional fields can be analyzed at the user's option, reporting composite statistics on the total number of spermatozoa examined.

In implementing such bioassays of sperm movement, variations in measurement must be determined before the results can be interpreted. The outcome of such tests may be influenced by small variations in test conditions, sampling differences or errors in the machine itself. The present study was directed at establishing instrument reliability, sampling methods, and minimal cell numbers for analysis.

The CellSoft computer-automated system allows specification of parameters that guide the algorithm in deciding if a spermatozoon is motile, and if so, whether it has been tracked for a sufficient period of time to compute velocity, linearity, amplitude of lateral head displacement, and beat cross frequency measurements and include these in the summary report. Thus, although the number of frames sampled for each ejaculate remains constant (20 frames at a rate of 30 Hz), different numbers of spermatozoa are sampled for each of these measurements as various cells will meet one criteria but not another. For example, a spermatzoon can be tracked for a number of frames and with a velocity sufficient to classify it as motile, while its other movement characteristics are not included in the cumulative values. At present, it is not possible to determine during the analysis the number of cells entering into each cumulative measurement. Rather, this information is obtained from a report of the individual cell data generated after the analysis is complete. Modification of the instrument to allow such reporting during analysis might be useful. In that case, analysis could proceed until, for example, 100 spermatozoa whose amplitude of lateral head displacement and beat cross frequency measurements were calculated and included in the summary statistics.

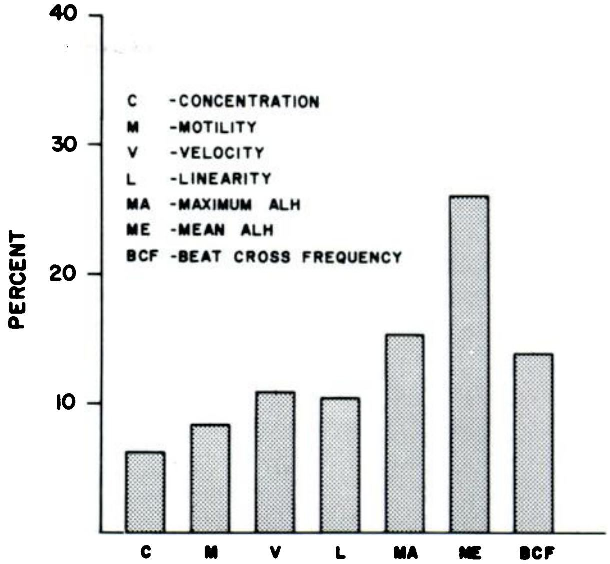

Fig. 6. Average within-sample coefficients of variation obtained by repeated analysis of videotaped specimens.

The number of spermatozoa seen under a single microscope field at any magnification is related to cell concentration and the dispersion of spermatozoa on the slide. In the first experiment we determined how many cells were needed, regardless of the number of microscope fields needed to obtain that number of cells, before the results can be viewed as representative of the population. While such an analysis might tend to obscure some of the within-subject variation, it is clear from our data that there is no significant change in sperm measurements among the ejaculates after 225 cells are analyzed. Although several fields may need to be analyzed to reach this number, depending on cell concentration and dispersion, this minimum number of cells is required for reliability of data.

However, one important result of the progressive selecting-out of cells included in the analysis (see above) is that relatively asthenospermic or oligozoospermic ejaculates will have few spermatozoa contributing to the linearity, velocity and head yawing amplitude and frequency measurements. As a result, the summary data might not be robust since too few spermatozoa have contributed to the average values. In this situation, the minimum number of spermatozoa to be analyzed may need to be increased, although that recommendation is not supported by the data presented here. For ejaculates with higher motilities, on the other hand, it seems unlikely that the added time would yield much of a statistical improvement in the results since the data are already stable.

The second experiment compared sampling from only a single field vs. different fields. Although analysis of variance failed to show significant differences between the three sampling methods, the differences in the coefficients of variation support recommending one of the methods over the other two. Method C, in which four predefined areas of the slide were examined in triplicate with averaging of the results, was considered the least biased sampling method, and was compared with the other two. As expected, the within-sample coefficients of variation, ranging from 4.8 to 14.0%, were lowest. In all cases, method B, which analyzed sufficient different fields to examine 225 cells, resulted in higher coefficients of variation than the least biased method. Although the coefficients of variation with method A were slightly lower than with method B, they too tended to be higher than those obtained with the averaging method. Method C appears to give data with the least variability, and would therefore be expected to provide the most consistent and reproducible results. Conceivably, small temperature differences, light, drying of the sample, changes in the depth of the preparation, or some other factor, may account for these differences in measurements from field to field. Their effect appears to be negated, however, when a sufficiently large number of fields are analyzed. It should also be emphasized that all specimens in this study allowed analysis of at least 225 cells by the completion of the triplicate run. In oligozoospermic ejaculates, even more fields may need to be analyzed to reach this minimum number.

Reliability of the instrument was examined by repeated analysis of the same field for each of the 10 ejaculates. We found within-sample coefficients of variation for concentration, motility, linearity, velocity, beat cross frequency, and maximum amplitude of lateral head displacement to average less than 15.0%, which is considered acceptable for such a biologic test system. The relatively low coefficients of variation will allow detection of true changes in these measurements when greater than 15%, and the system should thus be sensitive enough to detect relatively small changes in most sperm movement characteristics. These coefficients of variation are in close agreement with those published by Schieber et al (1986), who found coefficients of variation of 8.7% for motility and 5.3% for velocity using a manual analysis system, and are near the intraassay coefficient of variation for sperm velocity of 13.1% reported by Aitkin et al (1982a). On the other hand, much larger changes in mean lateral head displacement (greater than 26%) are needed before one could attribute them to actual changes within the sperm population rather than instrument error. These measurements are phase-dependent, and therefore our method of replaying the videotape might have introduced excessive variation if analysis did not begin at exactly the same portion of the videotape with the cells in the same phase of movement. Indeed, when the two extreme values that contributed to this large error were removed, the mean coefficient of variation for amplitude of lateral head displacement was in the range of 15%. It is still important to realize, however, that potentially the variation can be this high when interpreting amplitude of lateral head displacement results. Periodic determinations of variation while using the instrument will allow each experiment or set of clinical analyses to be interpreted in light of this variability.

These machine reproducibility data can be used with the data from method C in Experiment 2 to compare the variability inherent in the technology with the field-to-field variability of sperm movement. It is evident from Figs. 5 and 6 that, for all measurements except percent motility, the average within-sample coefficient of variation due to the instrument is greater than that due to the field effect. It thus appears that the limiting factor in the variance of all movement measurements except percent motility is the instrument itself. For percent motility, the field-to-field variance is more significant, substantiating the need to average many microscope fields in obtaining some of these measurements.

An issue of paramount importance in investigating and accepting a new measurement technique relates to its validation. While these data address the issue of consistency and reproducibility of computerized semen analysis, they do not necessarily indicate that any of the measurements obtained are the true values. However, using established World Health Organization procedures (Belsey et al, 1980), we have shown a high correlation between CellSoft computer-automated semen analysis and manual determinations of sperm concentration, overall motility and progression/linearity (Ginsburg, 1987).

One should also realize that these reported limits of variability apply only to the instrument when configured as shown in Table 1. Since different specimens such as infertile seminal or washed spermatozoa or spermatozoa in cervical mucus will undoubtedly be analyzed under different optimum machine settings, the variation of the instrument under these different operating conditions will have to be defined separately. Indeed, the report by Knuth et al (1987) emphasizes the importance of parameter settings on the variation of results. Standardization of such settings is necessary before data can be compared from one laboratory to the next. We are presently investigating the optimum settings to analyze washed spermatozoa in media and spermatozoa within cervical mucus.

Conventional methods of semen analysis rely on simple bulk measurements of sperm quality that often do not correlate well with fertility. Although sperm density shows a significant correlation with infertility at concentrations below 60×10^6/cc (David et al, 1979), even the predictive value of this measurement has been questioned since approximately 50% of men with a sperm density less than 10×10^6/cc can initiate a pregnancy when their female partner is fertile (Smith et al, 1977). Clearly, other tests of sperm fertility and fertilizing capacity are required to assess adequately male infertility.

Analysis of sperm movement may allow greater discrimination of fertile from infertile semen specimens (Albertsen et al, 1983) than is possible with routine semen analysis. Aitken et al (1982a, 1982b, 1985), using a time-exposure photomicrographic system, have studied the correlation of sperm movement with mucus penetration and zona-free hamster egg penetration ability, and have reported that velocity and amplitude of lateral head displacement are indeed important in predicting successful sperm penetration into cervical mucus and hamster oocytes. The mean amplitude of lateral head displacement measurements reported in this study (range of 1.1 to 2.8 microns) are one-half the total amplitude of displacement, since they represent deviations of the head centroid from the curval mean path of the sperm head. The overall amplitude of lateral head displacement is therefore in the range of 2.2 to 5.6 microns, which agrees with the values reported by others (Aitkin et al, 1985). Similar results were reported independently by Amit et al (1982) regarding mucus penetration and by Jeulin et al (1986) regarding human *in vitro* fertilization. The data obtained manually by these investigators can now be obtained rapidly and easily using the CellSoft system.

Thus, while the CellSoft automated semen analyzer system allows acquisition of data regarding concentration, motility, velocity, trajectory, and head yawing amplitude and frequency with high reproducibility, the clinical utility of the measurements made with this instrument in separating fertile from infertile sperm populations is only beginning to be explored. We are presently examining this discriminating ability using cervical mucus penetration, hamster egg penetration and human egg fertilization as end points.

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Funding Available for Contraceptive Research

The Contraceptive Research and Development Program (CONRAD), in a Cooperative Agreement with the United States Agency for International Development (U.S.A.I.D.) seeks to improve or develop methods of fertility regulation for developing countries through worldwide projects at universities, research institutions, private companies and the like. CONRAD focuses on the early stages of contraceptive research and development, from targeted or applied basic research and progressing through the first two phases of clinical trials. As CONRAD support is Federal, no research on methods that act after fertilization will be considered.

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