COMPUTER AUTOMATED ANALYSIS OF RHINOCEROS SPERM MOTILITY

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INTRODUCTION

With the increasing destruction of the world's natural habitats, it has become obvious that zoos may provide the last refuge to species faced with extinction. This has placed an increasing demand on successful reproduction of these animals in captivity. However, the limited number of founder animals distributed among several institutions has led to a significant amount of inbreeding which may threaten the future viability of these captive populations. This is especially true of the megavertebrates, such as the rhinoceroses, in which space limitations restrict the number of individuals which can be feasibly maintained at a single institution. The transport of adult animals between institutions for breeding purposes is not only cost prohibitive but also risky and stressful to the animals and therefore not a practical solution to this problem.

The shipment of genetic material (ie. spermatozoa) from captive or semi-wild stocks for artificial insemination of captive females could provide the key for successful genetic management of captive populations of endangered species. One only needs to look as far as the domestic cattle industry in the United States to realize the benefits derived from artificial insemination in the genetic management of animals. Since Polge and Rowson's (1952) initial studies on the freezability of bovine spermatozoa, there have been remarkable advances in the development and applications of sperm freezing techniques, such that today nearly all cows are artificially inseminated with frozen-stored semen. Unfortunately, techniques that have proven successful in domestic cattle have met with little success in other species of animals. Therefore it may be necessary to specifically design semen preservation techniques for each species.

The development of alternative semen preservation techniques necessitates a quick and reliable method to determine semen quality following cryopreservation. While visual estimation of the percentage of progressively motile cells is the most common laboratory test of semen quality in use today, these estimates have not been shown to serve as a good predictor of fertility - possibly due to the subjective nature of this assay. Freund and Oliveira (1987) studied the subjective nature of visual estimates of human sperm motility using sixty trained technicians from thirty independent laboratories and found that, on average, percent motility was overestimated by twenty to thirty percentage units. Furthermore, they demonstrated that experience did not improve these estimates - relatively new technicians did not estimate the percentage of motile cells any worse than veteran technicians. Deibel et al. (1976) studied the same phenomenon for bull semen and reported a standard deviation of twenty percentage units. Obviously, more accurate and precise methods that are free from observer bias are essential for research.

Several objective methods for the determination of the percentage of motile cells have been developed (See Jequier and Crich, 1986 for review). These methods have been demonstrated to be both accurate and precise and, at least in the bull (Budworth et al., 1987), have recently been correlated with relative fertility. However, these objective methods have not been adopted by most laboratories due either to their expense, the limited information provided or the amount of time required to analyze each semen sample.

We have recently adapted the objective motility analysis techniques of time-lapse photomicrography and videomicrography that have been successfully used to examine semen quality in other species, to the analysis of rhinoceros semen. Although both techniques produced accurate and precise results (unpublished data), in practice, they proved to be too tedious and time consuming to be productive - the average analysis time for a single sample was 1.5 hours and 3.0 hours for time-lapse photography and videomicrography, respectively.

The recent, rapid development of inexpensive yet powerful personal computers has made it economically feasible to produce fully automated semen analysis systems that are not only accurate and precise but also extremely fast. There are currently four commercially available motion analysis systems that have been specifically designed for the analysis of human sperm motility (Expert Vision, Motion Analysis Corp., Santa Rosa, Calif.; Labscan VI, T.S. Scientific, Perkasie, Penn.; Hamilton-Thorn, Hamilton-Thorn Research, Danvers, Mass.; and CellSoft, Cryo Resources, Ltd., New York, NY). One of these systems, CellSoft^{TM}, has recently been adapted for the analysis of rat and bull sperm motility (Working and Hurtt, 1987, and Budworth et al., 1988, respectively). Therefore the purpose of this study was to determine if this computer automated semen analysis system could also be adapted for the rapid analysis of rhinoceros sperm motility.

MATERIALS AND METHODS

Sperm Sample Preparation

The semen used in all experiments was collected in 1986 from a 25-30 year old Indian rhinoceros *(Rhinoceros unicornis)* housed at the Milwaukee County Zoo. The semen was diluted 1:1 with extender, frozen in 250µl pellets on dry ice, and stored in liquid nitrogen until used earlier this year. Each pellet was thawed in 1.5 ml of a modified Tyrode's medium (TALP: Bavister, 1989) at 37°C producing a sperm concentration of approximately 10 x 10^6/ml. An aliquant of this sample was killed by freeze-thawing (three times) to produce a sperm sample with zero percent motility. This killed sample and the remaining portion of the original sample were then used to produce a series of four sub-samples with varying percent motility by mixing aliquots of the original and killed samples in the ratios of 1:0, 1:1, 1:3, and 0:1 (original:killed), hereafter referred to as sub-samples A,B,C, and D, respectively. All samples were maintained at 37°C until used for videomicrography.

Videomicrography

Specialized motility chambers were prepared by pressing four dry transfer dots (McCanorma symbols #CS220, McCanorma, LePerray-en-Yvelins, France) onto cleaned and siliconized (Bavister, 1989) standard microscope slides to form a 20x20 mm square. The slides were pre-warmed to 37°C and within 30 minutes post-thaw, 7µl of each of the four sub-samples were deposited on separate slides and a siliconized 22x22 mm No.1 coverslip was applied to each. The coverslips were held above the slides by the four dots thereby producing motility chambers with a depth of approximately 25-30µm. Three to seven fields at predetermined locations on the motility chamber of each sample were videotaped (30 frames/sec) for 30 seconds each using an Olympus BH-2 phase contrast microscope (Olympus Optical Co., Tokyo, Japan) equipped with an IF-550 interference filter, a 10X S-Plan negative phase objective, a 3.3X photo-ocular, and a Panasonic WV-1410 high resolution video camera (Matsushita Electric Industries, Secaucus, NJ) connected to a Panasonic TR-930 high resolution video monitor and a Sony VO-5600 3/4" videocassette recorder (Sony Corp., Tokyo, Japan). Each motility chamber was maintained at 37°C during videomicrography using a FHK slide warmer (FHK Industries, Tokyo, Japan) and the elapsed time was recorded on the video tape with a VTG-33 FOR-A time generator (FOR-A Company, Ltd., Tokyo, Japan). In order to calibrate the final magnification on the video monitor, a 0.01mm Olympus micrometer was video taped using the same equipment used to video tape the sperm samples.

Manual Frame-by-Frame Analysis from Video Tape

Manual motility analysis of each sperm sample was performed from previously recorded video tapes by a method similar to that first described by Katz and Overstreet (1981) for the analysis of human and bull sperm. A 48x63 cm sheet of transparent acetate was overlaid onto the screen of a 27 inch television monitor. The recorded video image of the micrometer was traced onto the transparency and used to calibrate the final magnification on the video monitor, which was calculated to be 720X (1μm = 0.72mm). A 29x40 cm rectangle was drawn onto the transparency and the percentage of motile cells for each sample and the average velocity were determined by plotting the movement of each sperm cell that appeared within the rectangle. Only sperm that remained within the rectangle were used for analysis, sperm that entered or left the rectangle during the observation period were ignored. For each field of each sample, the video tape was paused and the location of each sperm was recorded on the transparency with a water soluble marker. The video tape was advanced one frame at a time (ie. 1/30 sec/frame) and the new location of each sperm was plotted on the transparency until the locations of each sperm in 16 consecutive video frames (ie. 0.5 sec.) were plotted. Any sperm that had moved a distance of greater than 7mm on the video screen in the 16 video frames (ie. 19.44 μm/sec) were considered motile. If the motility status (ie. motile vs. non-motile) of a specific sperm cell was questionable due to movement caused by drift or collision with another sperm, the video tape was reviewed at normal speed to observe the beat of the flagellum and thereby verify its motility status. The percent motility for each sample was calculated by dividing the total number of motile sperm by the total number of sperm observed in all fields of the sample. The velocity (μm/sec) for each motile sperm was determined by measuring the distance traveled (mm) in the 16 consecutive video frames (ie. 0.5 sec) and dividing by a magnification correction factor (0.72mm/μm). The average velocity for all samples was then calculated from the velocity values determined for each motile sperm.

Computer Automated Analysis from Video Tape

Computer automated analysis of each sperm sample was performed using the same video tapes used for manual frame-by-frame analysis. The motion analysis system used in this study consisted of a Sony VO-5600 3/4" videocassette recorder, CellSoft™ digitized image analysis hardware and software (Cryo Resources, Ltd., New York, NY), two Panasonic TR-930 video monitors and an IBM PC-AT microcomputer (International Business Machines Corp., Boca Raton, FL). The operation and algorithms used for motion analysis have been described elsewhere (Working and Hurtt, 1977; Budworth et al., 1988) and will not be described here; however, the software provides eight user defined parameter settings which were optimized specifically for the analysis of rhinoceros sperm motility - these settings appear in Table 1. Computer automated analysis of each sperm sample was repeated five times using essentially the same video frames that were analyzed by the manual frame-by-frame method. The motion analysis system used in this study produces a summary report for each sample that includes sperm concentration, percent motility, average velocity, average linearity as well as velocity and linearity distributions, however only the percent motility and average velocity values were used for comparison with the values obtained by the manual analysis method.

RESULTS AND DISCUSSION

Katz and Overstreet (1981) concluded that an accurate estimation of percent motility and average velocity of a semen sample can be obtained with a high degree of precision using manual videomicrographic frame-by-frame analysis. This technique introduces little ambiguity into the analysis since the analyzer views the sperm movement directly. Sperm can be easily distinguished from debris by their head morphology and the presence of a flagellum. Motile sperm can be readily distinguished from non-motile sperm by reviewing the tape at normal speed to observe the beat of the flagellum. Therefore, in this study, the results obtained by manual videomicrographic frame-

by-frame analysis were assumed to be the standard against which the results obtained by computer automated analysis were compared. Since essentially the same video frames, as determined from the video time code, were used for the motility analysis by both manual and computer methods, any measurable differences in the results obtained by the two methods should be attributable to the analysis technique rather than sampling error.

The accuracy of the computer automated analysis system was examined over a range of percent motility values. Since there is no perfect standard for percent motility, a standardized set of sub-samples were prepared with varying percent motility. By expressing the percent motility of each sub-sample as a relative motility, using the equation:

$$\text{Relative motility} = \frac{\text{Percent motility of sub-sample}}{\text{Percent motility of original semen sample}}$$

and if the sub-samples were prepared accurately, one would expect the prepared sub-samples to have relative motility values equal to the proportion of the original semen sample in each sub-sample (ie. 1.00, 0.50, 0.25, and 0.00 for sub-samples A,B,C, and D, respectively). The percentage of motile cells for each sub-sample, as determined by manual videomicrographic frame-by frame analysis, was 24.62, 12.79, 6.72, and 0.00 (A,B,C, and D, respectively). As illustrated in Figure 1, these percent motility values (expressed as relative motility values) and the percentage of killed sperm in each sub-sample exhibit a linear relationship with an R^2 value of 0.999. This indicates that the sub-samples were prepared properly and substantiates the accuracy of the manual analysis method, since by linear regression analysis, 99.9% of the variation between sub-samples can be explained by the percentage of killed sperm in each sample.

The percentage of motile cells in sub-samples A,B,C, and D, as determined by CellSoft™ computer automated analysis, was 24.54 ± 0.51, 13.22 ± 1.32, 7.13 ± 0.51, and 0.62 ± 0.25, respectively (mean ±SD, n=5). As indicated in Figure 2, the percent motility values for each sub-sample and the percentage of killed sperm in each sub-sample exhibit a linear relationship with an R^2 value of 0.993 thereby attesting to the accuracy of this method over the range of percent motility values examined. As illustrated in Figure 2, the percent motility values for sub-samples A,B, and C are not significantly different ($p > 0.05$) from the percent motility values obtained by manual videomicrographic frame-by-frame analysis; however, the percent motility values for sub-sample D (0.00% and 0.62%, manual and computer automated methods, respectively) are statistically different. The statistical difference for sub-sample D could be attributed to a minimal amount of background noise in the computer automated system that was not statistically significant at other percent motility levels and is probably within the accuracy limits required by most researchers. No attempt was made to examine the accuracy of the computer automated analysis system over a range of velocity values; therefore, it was not surprising that the average velocity values for each sub-sample were not significantly different. Furthermore, as indicated in Table 2, there is good agreement in the range and average velocity values as determined by the two methods. These data suggest that the CellSoft™ computer automated semen analysis system can accurately (± 1.5 percentage units) estimate the percentage of motile sperm in rhinoceros semen - at least within the range examined (0% - 25%) and provide a reasonable estimate of average velocity. However, it should be emphasized that similar results may not be obtained at other sperm concentrations or with semen samples containing excessive quantities of debris particles that are within the size range of the sperm cells - additional experiments will be necessary to examine these variables.

CONCLUSIONS

The CellSoft[TM] computer automated semen analysis system (Cryo Resources, Ltd., New York, NY.) can be used to analyze the motility characteristics of rhinoceros spermatozoa using the general parameter setting listed in Table 1. This analysis method is fast (requiring less than five minutes for the analysis of each sample), accurate (at least within the percent motility range examined in this study), and precise (± 1.5 percentage units). The validation of the CellSoft[TM] automated semen analysis system for the analysis of human, rat, and bull semen by previous investigators and the validation of the same system for the analysis of rhinoceros semen in this study suggests that this system can be adapted for the semen analysis of many different species. The cost of this system ($20,000 - $40,000) may be cost prohibitive for most zoos; however, analyses can be performed from previously recorded video tapes. The cost of the necessary equipment to produce these video tapes is less than $2,000, which should be well within the budget of most research programs. By working in cooperation with several zoos, video tapes of semen samples could be produced using these inexpensive satellite systems and analyzed at a central location - thereby distributing the costs of the system across several research institutions. Although a correlation between sperm motility characteristics and relative fertility has only been demonstrated in the domestic bull, the use of computer automated semen analysis systems may provide an important tool for the development of semen preservation techniques for many endangered species.

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REFERENCES

Bavister, B.D. (1989). A consistently successful procedure for *in vitro* fertilization of golden hamster eggs. Gamete Research (accepted).

Budworth, P.R., R.P. Amann, and R.H. Hammerstedt (1987). A microcomputer-photographic method for evaluation of motility and velocity of bull sperm. Journal of Dairy Science 70:1927-1936.

Budworth, P.R., R.P. Amann, and P.L. Chapman (1988). Relationship between computerized measurements of frozen-thawed bull spermatozoa and fertility. J. of Andrology 9:41-54.

Deibel, F.C., Jr., J.E. Smith, B.G. Crabo, and E.F. Graham (1976). Evaluation of six assays of sperm quality by means of their accuracy, precision, and sensitivity in separating known induced levels of damage. Proceedings of the 8[th] International Congress on Animal Reproduction and Artificial Insemination 4:888-891.

Freund, M. and N. Oliveira (1987). Visual versus cinemicrographic evaluations of human sperm motility and morphology. Archives of Andrology 19:25-32.

Jequier, A. and J. Crich (1986). Assessment of sperm movement. In: *Semen Analysis: a Practical Guide*. Blackwell Scientific Publications, Oxford, pp. 56-62.

Polge, C. and L.E.A. Rowson (1952). The fertilizing capacity of bull spermatozoa after freezing at -79°C. Nature, London 169:626-627.

Katz, D.F. and J.W. Overstreet (1981). Sperm motility assessment by videomicrography. Fertility and Sterility 35:188-193.

Working, P.K. and M.E. Hurtt (1987). Computerized videomicrographic analysis of rat sperm motility. Journal of Andrology 8:330-337.

Sampling frequency (frames/sec)	30
Number of frames to analyze	15
Minimum number of frames for motility	4
Minimum number of frames for velocity	8
Threshold velocity (μm/sec)	19
Maximum velocity (μm/sec)	175
Threshold grey level	147
Pixel scale (μm/pixel)	1.376
Minimum size (pixels)	5
Maximum size (pixels)	20

Table 1. General parameter settings for CellSoft™ computer automated semen analysis of rhinoceros sperm motility using the videomicrographic equipment described in materials and methods.

	Mean	Range
Manual Videomicrographic Frame-by-frame Analysis	54.8	(20 - 190)
CellSoft™ Computer Automated Semen Analysis	57.9	(19 - 155)

Table 2. Average and range of velocity values (μm/sec) determined by manual videomicrographic frame-by-frame analysis and CellSoft™ computer automated semen analysis.

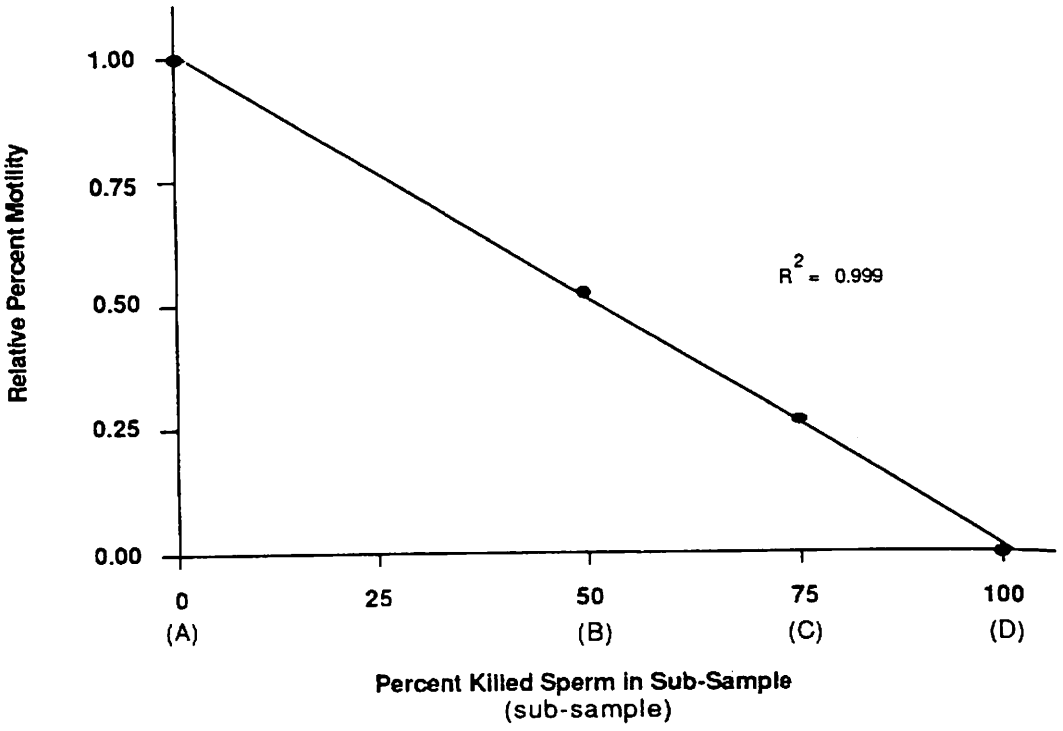

Figure 1. Relationship between the relative percent motility, as determined by manual videomicrographic frame-by-frame analysis, and the percent killed sperm in a sample.

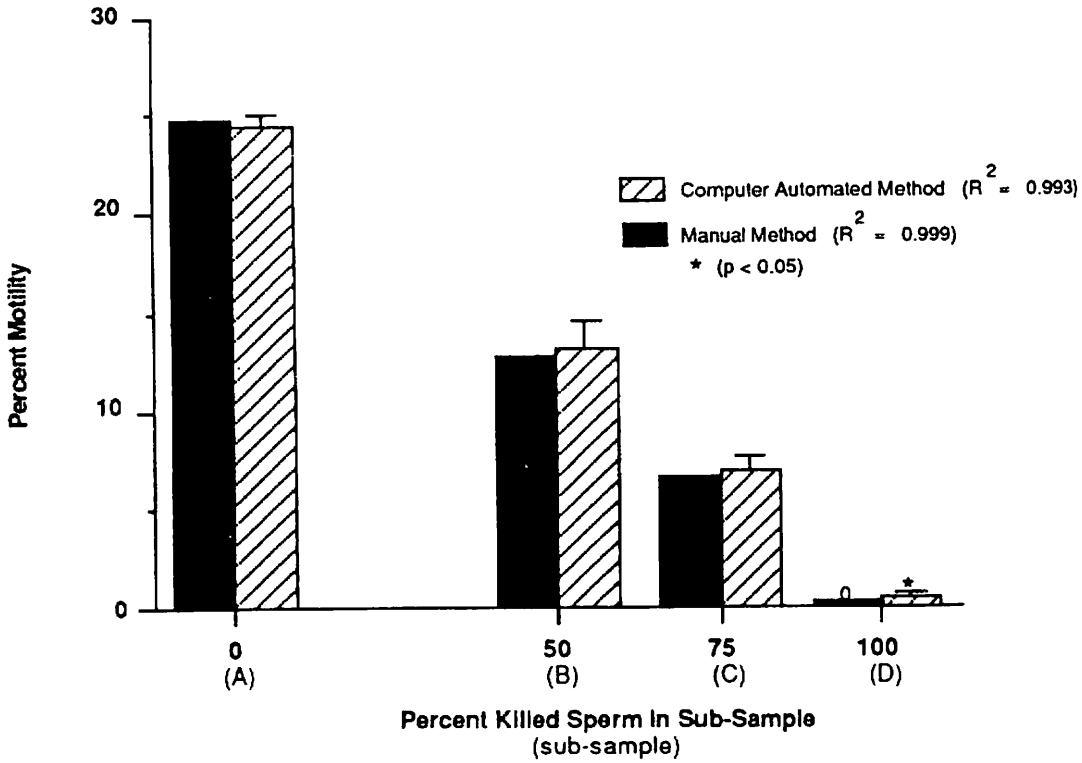

Figure 2. Comparison of the percent motility values determined by manual videomicrographic frame-by-frame analysis and CellSoft computer automated semen analysis.