FLOW CYTOMETRY EVALUATION OF BULL SPERM CHROMATIN STRUCTURE

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Introduction

Our contribution to the evaluation of bull semen samples utilized in a heterospermic insemination study was to correlate data derived by flow cytometric analysis of sperm chromatin structure (3,6,8) with other measures of semen quality and the heterospermic competitive index, which were previously determined by Saacke et al. (11,12,13). Flow cytometry is a rapidly developing field that will certainly contribute to the evaluation of semen quality.

Flow Cytometry

Figure 1 is a schematic of the principles of flow cytometry (15). Cell samples in liquid suspension are typically stained with a specific fluorescent dye and then placed into a sample chamber where positive air pressure forces the sample into a laminar flowing liquid stream passing through a quartz flow chamber. This sample stream is intersected by a laser beam. The laser beam excites the fluorescent

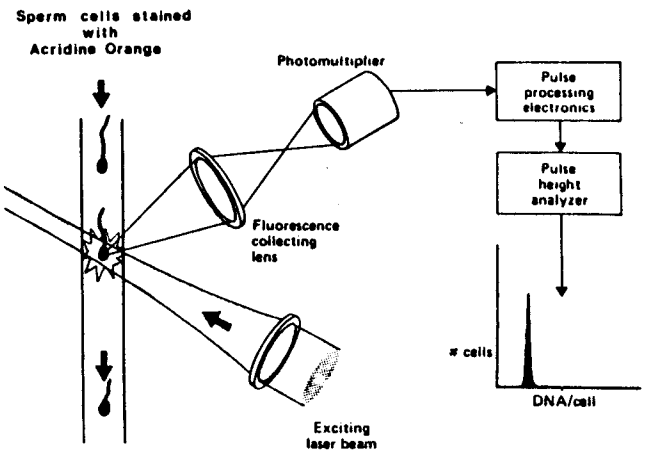

Figure 1. Schematic of flow cytometry adapted from Van Dilla and Mendelsohn (14).

dye, in this case acridine orange complexed with DNA; the resulting fluorescent signal is detected for each cell and converted to an electrical signal which passes through a multichannel analyzer quantitating the amount of fluorescence per cell. A distinct advantage of flow cytometry is the very rapid rate of single cell analysis (see 14 for a review on flow cytometry).

Sperm Chromatin Structure Assay

The nuclear material in sperm consists of DNA molecules complexed with protamine, a basic protein unique to sperm (1). The DNA-protein is highly condensed, almost crystalline in nature, due to the presence of disulfide bonds, and this presumably protects the genetic material from environmental damage. This complex of nuclear material is chromatin. We have studied the structural integrity of sperm chromatin using the sperm chromatin structure assay, defined as the susceptibility of DNA within the cell to acid or heat induced denaturation (7,8,9). DNA denaturation results in the separation of the normally double-stranded DNA molecule into single-stranded molecules. Due to the very high level of nuclear condensation, DNA in sperm with normal chromatin structure does not denature at low pH or high heat. DNA in sperm with an abnormal chromatin structure has increased susceptibility to denaturation.

To measure the relative amounts of double- to single-stranded DNA in individual cells, we use a metachromatic DNA dye, acridine orange (4). Laser beam excitation of this dye intercalated into native, double-stranded DNA produces green fluorescence, while excitation of the dye associated with single-stranded DNA produces a red fluorescence. The following is an outline of the experimental protocol (3,5).

SCSA PROTOCOL

Fresh or frozen/thawed bull semen is diluted in TNE:

0.01 M tris buffer
0.15 M NaCl
0.001 M EDTA
pH 7.4

Admix 0.2 ml sperm cell suspension with 0.4 ml of:

0.08 N HCl
0.15 N NaCl
0.1%Triton-X 100

After 30 seconds add 1.2 ml of:

6μg/ml acridine orange
0.2 M Na_2PHO_4 - 0.1 M Citric acid buffer (pH 6.0)
0.001 M EDTA
0.15 M NaCl

Allow for three minutes staining

Approximately 200 cells/second are passed through a quartz flow channel of a flow cytometer.

Fluorescence microscopy of sperm, from a subfertile bull, treated to potentially denature the DNA and then stained with acridine orange exhibited heterogeneity of fluorescence with some nuclei being either green or red, and others with a mixture between green and red (8). Evaluation by fluorescence microscopy of heat treated, acridine orange stained sperm is very difficult and not feasible. In contrast, flow cytometry can readily measure the precise amounts of red and green fluorescence in each cell. To numerically quantitate the ratio of red and green fluorescence, we use the expression α_t which is the ratio of red fluorescence/(red + green) fluorescence (5). Figure 2 outlines the principles of

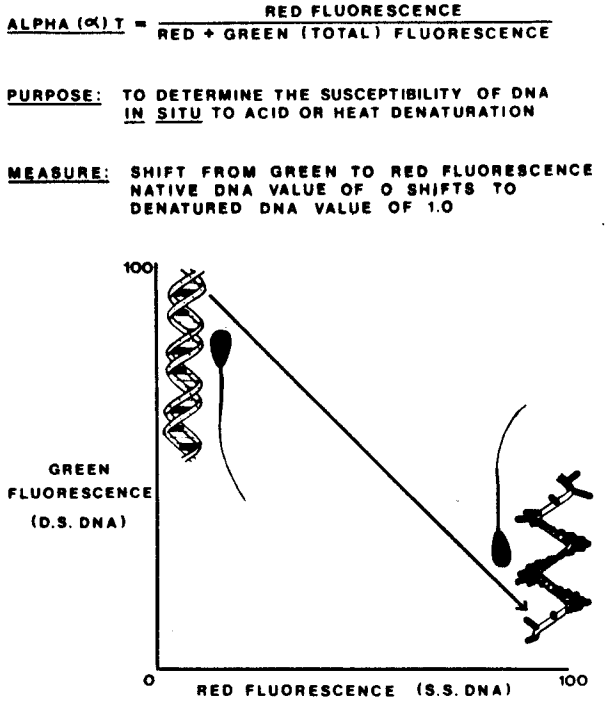

Figure 2. Outline of the principles of the Sperm Chromatin Structure Assay. A color photograph would show the left hand sperm cell with a green nucleus and green fluorescing acridine orange molecules intercalated into the DNA helix; the right hand sperm cell would show a red fluorescing nucleus with red fluorescing acridine orange molecules attached to the single-stranded DNA molecules. The amount of single- and double-stranded DNA is quantitated by the expression α_t which is the ratio of single-stranded DNA to total DNA measured.

this assay. Theoretically, a normal sperm nucleus with 100 percent green fluorescence would have an α_t value of 0 and one with entirely denatured DNA would have an α_t value of 1.0. Since the flow cytometer measures the fluorescence over 1000 increments, we typically express α_t values between 0 and 1000 for easier calculation.

Five thousand sperm cells were measured for each sample. Using a computer interfaced to the flow cytometer, the α_t value was determined for each cell, and the distribution of α_t values determined for each sample. We record the mean of α_t ($X\alpha_t$), coefficient of variation of α_t ($CV\alpha_t$), standard deviation of α_t ($SD\alpha_t$) and $COMP\alpha_t$ which is detailed below. From data gathered on bulls, boars, stallions, humans and mice, the two parameters that appear most valuable are the $SD\alpha_t$ and $COMP\alpha_t$.

Figure 3 shows raw data as viewed on the computer monitor. Panel A was derived from a highly fertile bull and C from a less fertile bull. The elongated pattern of the stained cells (A,C) is due to some unique features of sperm measurement by flow cytometry (10) caused by orientation of the sperm in the flow cell and has no significant effect on the α_t parameters being measured. The elongated cluster of dots is the main population; the dots representing Cells Outside the Main Peak (shown in boxed region in C) are called $COMP\alpha_t$. $COMP\alpha_t$ is the percent of cells that have abnormal

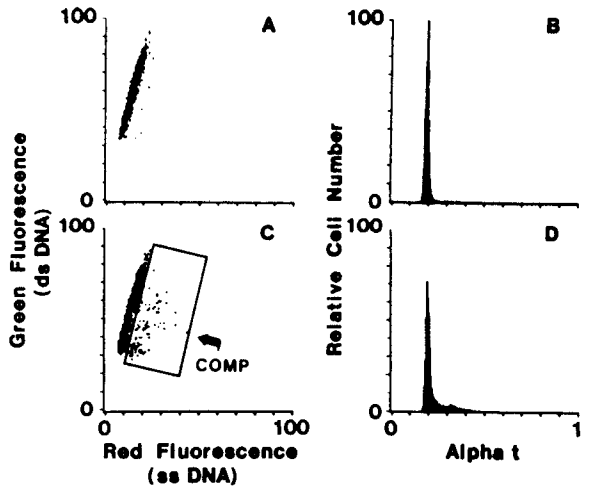

Figure 3. SCSA data on semen obtained from a highly fertile bull (A and B) and one of lesser fertility (C and D). The scattergram in A is relatively homogenous resulting in an α_t frequency histogram that has a narrow distribution and lower standard deviation (B). The scattergram in C is more heterogeneous with a number of cells outside the main population (COMP box) resulting in an α_t frequency histogram with a broader distribution and higher standard deviation (D).

chromatin structure; however, this value does not express the degree of abnormality of these cells. The extent of abnormality is better indicated by the $SD\alpha_t$. A difference in distribution of α_t values is seen between the two frequency histograms (Figure 3, B & D), with higher $SD\alpha_t$ values for histogram D.

To examine how useful this test might be for the breeding industry, repeatability values were estimated. In a previous study (3) conducted on semen samples from Eastern Artificial Insemination Cooperative, $COMP\alpha_t$ was shown to be highly repeatable when measuring straws of the same ejaculate on the same day (0.99) or different days (0.98). Ejaculates from the same bull collected over a several year period and measured on the same day also had a high repeatability (0.70).

$SD\alpha_t$ is a sensitive measurement which was highly repeatable for replicate samples measured on the same day (0.96); however, slight instrument fluctuations over different days contributed to a lower repeatability of $SD\alpha_t$ (0.79) when two straws (same ejaculate) were measured on different days. Procedures have since been improved, which should result in increased repeatability of the $SD\alpha_t$ values. In this particular study (3) of samples collected from 49 bulls at Eastern Artificial Insemination Cooperative over a number of years, the correlation of $SD\alpha_t$ with fertility rating was -0.58 ($p < 0.01$) and the correlation of $COMP\alpha_t$ with fertility rating -0.40 ($p < 0.01$).

In contrast to measuring only a few samples of semen from bulls whose fertility ratings were established over a lifetime of matings, this heterospermic study (2) allowed measurements on the same ejaculates used for insemination. In this study, the percentage of error associated with replicate flow cytometry measurements was relatively low, at nine percent for $SD\alpha_t$, and three percent for $COMP\alpha_t$. The error associated with estimation of abnormal morphology was also low (three percent); however, the percentage errors associated with repeated measurements of motility, photomotility, acrosome integrity, and sephadex filtration were somewhat higher, ranging from 10 percent to 34 percent (12,13).

In a variety of studies related to reproductive toxicology and animal fertility, the measurement of $SD\alpha_t$ has apparently been the most useful determinant of chromatin structure abnormalities (2,3,7,9). For the heterospermic samples, Figure 4 shows the correlation between the $SD\alpha_t$ and competitive index to be -0.94 ($p < 0.01$). Correlations between other measures of semen quality and the competitive index were also high (0.72 to 0.93) (12,13).

Correlations among α_t values and other tests of semen quality were generally high (2). The sperm chromatin structure assay, however, is unique in that cells are killed prior to measurement, and viability of cells at the time of measurement does

not appear to influence the values obtained. Further efforts need to be made to elucidate the defects in sperm chromatin which are reflected by high α_t values, and to clarify the relationships among the α_t values and alternate measures of sperm quality.

In conclusion, we feel the sperm chromatin structure assay has considerable potential for rapid, routine measurement of semen quality and that it provides the opportunity to measure a structural defect in sperm cells which is associated with decreased fertility and which presently cannot be evaluated by any other technique. Lower priced and more user friendly flow cytometers available in the very near future should allow a cost effective use of these instruments in a commercial stud operation.

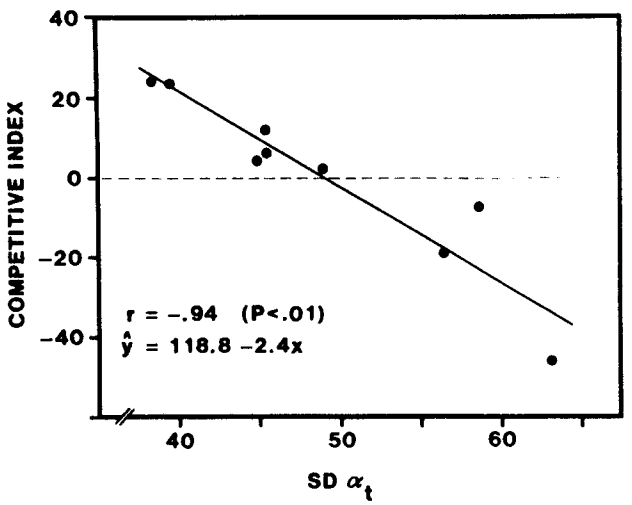

Figure 4. Correlation between competitive index of nine bulls and SDα_t (2).

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References

1. Balhorn, R. 1982. A model for the structure of chromatin in mammalian sperm. J. Cell Biol. 93:298-305.

2. Ballachey, B.E., D.P. Evenson and R.G. Saacke. 1988. The sperm chromatin structure assay relationship with alternate tests of semen quality and heterospermic performance of bulls. J. Androl. Vol 9, 2:109-115.

3. Ballachey, B.E., W.D. Hohenboken and D.P. Evenson. 1987. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. Biol. Reprod. 36:915-925.

4. Darzynkiewicz, Z. 1979. Acridine orange as a molecular probe in studies of nucleic acids *in situ*. In: ***Flow Cytometry and Sorting***, M.R. Melamed, P.F. Mullaney and M.L. Mendelsohn, Eds. New York: John Wiley & Sons, p.285-316.

5. Darzynkiewicz, Z., F. Traganos, T. Sharpless and M.R. Melamed. 1976. Lymphocyte stimulation: a rapid multiparameter analysis. Proc. Natl. Acad. Sci. USA. 73:2881-2884.

6. Evenson, D.P. 1986. Flow cytometry of acridine orange stained sperm is a rapid and practical method for monitoring occupational exposure to genotoxicants. In: ***Monitoring of Occupational Genotoxicants***, M. Sorsa and H. Norppa, Eds. New York: Alan R. Liss Inc., p.121-132.

7. Evenson, D.P., R.K. Baer, L.K. Jost and R.W. Gesch. 1986. Toxicity of thiotepa on mouse spermatogenesis as determined by dual parameter flow cytometry. Toxicol. Appl. Pharmacol. 82:151-163.

8. Evenson, D.P., Z. Darzynkiewicz and M.R. Melamed. 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. Science 240:1131-1133.

9. Evenson, D.P., P.H. Higgins, D. Grueneberg and B.E. Ballachey. 1985. Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry 6:238-253.

10. Gledhill, B.L., S. Lake, L.L. Steinmetz, J.W. Gray, J.R. Crawford, P.N. Dean and M.A. Van Dilla. 1976. Flow microfluorometric analysis of sperm DNA content: Effect of cell shape on the fluorescence distribution. J. Cell Physiol. 87:367-376.

11. Saacke, R.G. 1984. Semen quality, importance of and influencing factors. Proceedings, 10th Technical Conference Artificial Insemination and Reproduction, National Association of Animal Breeders, p.30-36.

12. Saacke, R.G., C.E. Marshall, W.E. Vinson, M.L. O'Conner, J.E. Chandler, K.J. Mullins, R.P. Amann, R.A. Wallace, W.N. Vincel and H.C. Kellgren. 1980. Semen quality and heterospermic insemination in cattle. 9th International Congress on Animal Reproduction and Artificial Insemination (Madrid), 5:75-78.

13. Saacke, R.G., W.E. Vinson, M.L. O'Conner, J.E. Chandler, J. Mullins, R.P. Amann, C.E. Marshall, R.A. Wallace, W.N. Vincel and H.C. Kellgren. 1980. The relationship of semen quality and fertility: a heterospermic study. Proceedings, 8th Technical Conference on Artificial Insemination and Reproduction, National Association of Animal Breeders, p.71-78.

14. Shapiro, H. 1985. ***Practical Flow Cytometry***, New York: Alan R. Liss.

15. Van Dilla, M.A. and M.L. Mendelsohn. 1979. Introduction and resume of flow cytometry and sorting. In: ***Flow Cytometry and Sorting***, M.R. Melamed, P.F. Mullaney and M.L. Mendelsohn, Eds. New York: John Wiley & Sons, p.11-37.