FLOW CYTOMETRY EVALUATION OF TESTICULAR AND SPERM CELLS OBTAINED FROM BULLS IMPLANTED WITH ZERANOL[1]

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ABSTRACT

Flow cytometry of testicular and sperm cells was used to evaluate effects of pre-weaning zeranol implants on spermatogenesis. Forty five Angus-Simmental bulls were randomly assigned to three treatment groups of 15 bulls each: 1) no implant, 2) one implant at 30 d of age and 3) two implants, one at 30 and the second at 120 d of age. Prior to slaughter at approximately 15 mo, semen was collected from 30 bulls, 10 of each group. Following slaughter, testes were weighed, and testicular biopsies and vas deferens sperm obtained from the same 30 bulls. Testicular and sperm cells were stained with acridine orange and measured by flow cytometry. Proportions of testicular haploid, diploid and tetraploid cells were determined by relative amounts of green (DNA) and red (RNA) fluorescence. Treatment of sperm at low pH prior to acridine orange staining potentially induces partial denaturation of DNA, detectable by the metachromatic shift from green (native DNA) to red (single-stranded DNA) fluorescence. The effect of this shift was quantified by alpha-t [α_t = red/(red + green) fluorescence]. Nonimplanted bulls had heavier ($P<.01$) testicular weights than treated bulls. The proportion of haploid cells was greater ($P<.02$) and diploid cells less ($P<.03$) in testes of nonimplanted bulls. Sperm from implanted bulls had altered chromatin structure, indicated by higher ($P<.05$) α_t values. Flow cytometry is an effective means for detecting changes in testicular cell subpopulations and chromatin structure of sperm.

(Key Words: Spermatogenesis, Flow Cytometry, Testes, Bulls, Zeranol, Chromatin.)

Introduction

Flow cytometry (FCM) is a relatively new technology for multiparameter cellular analyses (Melamed et al., 1979). Cells in suspension may be labelled with a specific fluorochrome and passed in a sample stream intersected by a laser beam. The resulting fluorescent signals are detected by photomultiplier tubes and converted to digital analogs for processing. Advantages of FCM include rapid measurement of large numbers of cells, simultaneous measurement of several variables per cell and ease of counting subpopulations of cell types. Studies on male reproductive function and toxicology have utilized FCM to characterize changes in testicular and sperm cells (Evenson et al., 1980a,b; 1984, 1985, 1986; Evenson and Melamed, 1983).

With current interest in feeding intact males, the ability of zeranol, a resorcylic acid lactone, to increase growth has received considerable attention. Improved gains have been reported in some studies (Greathouse et al., 1983; Gregory and Ford, 1983) but not in others (Ralston, 1978; Ford and Gregory, 1983; Unruh et al., 1983; Staigmiller et al., 1985); however, adverse effects of implantation prior to weaning on testicular development, measured by size and weight, have been consistently observed (Ralston, 1978; Greathouse et al., 1983; Juniewicz et al., 1985; Staigmiller et al., 1985).

The purpose of this research was to demonstrate the feasibility of FCM for evaluation of testicular cell populations and spermatogenesis in yearling bulls implanted with zeranol prior to weaning.

Materials and Methods

Animals. Forty-five 3/4 Angus × 1/4 Simmental bull calves averaging 30 d of age were weighed and randomly divided into three treatment groups: control—no implant (0I), one

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zeranol[4] implant (1I) and two zeranol implants (2I). Calves in the treated groups were implanted with 36 mg zeranol under the skin on the lower backside of the ear. Ninety days later calves in the 2I group were given a second 36-mg zeranol implant. After weaning at an average age of 205 d, calves were transported 525 km to a feedlot. A grower ration, consisting of alfalfa hay ad libitum and 2.5 kg corn daily was fed for 90 d; a finishing ration of 84% corn, 10.5% corn silage, 4.8% soybean meal and .7% mineral supplement was fed ad libitum until slaughter. Bulls were slaughtered at an average age of 450 d.

Sample Collection and Preparation. Five days prior to slaughter, semen was collected by electroejaculation of 10 bulls in each group, and immediately placed on ice (4 C). At slaughter, testes and vas deferens were collected and placed on ice. Testes weights were recorded and from bulls that had been electroejaculated, two mid-testicular biopsies weighing approximately 1 g each were surgically excised. One biopsy was placed in neutral buffered 10% formalin for histology; the second in Hanks balanced salt solution[5] (HBSS, $-Ca^{++}$, $-Mg^{++}$). Prior to FCM measurement, the sample in HBSS was minced and filtered to form a cellular suspension. Vas deferens from electroejaculated bulls were stripped to recover sperm cells (vas sperm).

The FCM measurements were made on ejaculated sperm samples the day of collection. Sperm concentrations in 7 of the 30 samples were insufficient for FCM evaluation, and these samples were discarded. Vas sperm and testicular cells were obtained on the same day, but all samples could not be measured by FCM on that day. Thus, vas sperm were mixed with TNE and glycerol, frozen at −20 C for 8 h and then at −100 C for later FCM analyses. Studies in this laboratory have compared fresh vs frozen sperm from humans and mice, and no apparent effects on chromatin structure, measured by FCM after staining with acridine orange (AO) dye, have been noted. Also, bull sperm processed for artificial insemination and frozen in liquid nitrogen are routinely measured in this laboratory, and AO staining patterns are the same as those seen with fresh samples.

Acridine Orange Staining. Testicular cells, ejaculated and vas sperm were stained by the two-step acridine orange (TSAO) staining procedure as described by Darzynkiewicz et al. (1976) and Evenson and Melamed (1983). The first step of this method involves an acid and detergent treatment to permealize cell membranes and partially dissociate basic nuclear proteins, improving uptake of the dye. The second step is addition of AO staining solution.

Acridine orange fluoresces green when intercalated into double-stranded (ds) nucleic acids, and red when associated with single-stranded (ss) nucleic acids (Darzynkiewicz, 1979). In testicular cells, green and red fluorescence levels are indicative of DNA and RNA content, respectively. Haploid (1N), diploid (2N) and tetraploid (4N) cells can be distinguished on the basis of cellular DNA and RNA content. The 1N population can be further resolved into round, elongating and elongated spermatids, based on decreasing levels of green and red fluorescence associated with maturation of the spermatids.

During sperm maturation, RNA is excluded from the cell (Monesi, 1971). The low pH of the TSAO method potentially induces partial denaturation of the DNA in sperm, and differential levels of green and red fluorescence reflect ds and ss DNA content. Susceptibility of sperm DNA to denaturation in situ is quantified by alpha-t [α_t = red/(red + green) fluorescence]. Considerable variation has been observed for α_t values of sperm samples among bulls and also among humans (Evenson et al., 1980a; Ballachey et al., 1985).

Testicular samples contain a low proportion of mature sperm (less than 5%, Evenson and Melamed, 1983), and the decreased DNA stainability of these sperm allows them to be readily distinguished from the spermatids. Mature sperm were not included in computation of proportion of testicular cell types.

Fluorescence Measurements. Fluorescence measurements were made with a Cytofluorograf II[6] interfaced to an Ortho Diagnostics 2150 data handling system. Green (F_{530}) and red ($F_{>600}$) fluorescence[7] emitted by each cell after laser excitation (488 nm, blue line, argon ion laser) were measured on 5,000 cells per

[4] Ralgro: International Minerals and Chemical Corp., Terre Haute, IN.

[5] Gibco Lab., Grand Island, NY.

[6] Ortho Diagnostics, Inc., Westwood, MA.

[7] F_{530} = fluorescence at 530 nm; the band between 515 and 530 nm is measured. $F_{>600}$ = fluorescence greater than 600 nm; the band at 615 to 630 nm is measured.

sample. For ejaculated and vas sperm, α_t was calculated for each cell and the α_t distribution for each sample was determined by computer protocols. A computer program was also used to quantify testicular samples into major populations of 1N, 2N and 4N cells, and 1N subpopulations of round, elongating and elongated spermatids.

Sperm Head Morphology. Slides were made of fresh vas and ejaculated sperm after staining in suspension with .5% Eosin Y. At least 200 cells per bull were scored by light microscopy to estimate percentage of morphologically abnormal sperm heads (ABN).

Statistical Analyses. A one-way analysis of variance with treatment as the main effect was used for the following dependent variables: testicular weight, percentage of 1N, 2N and 4N cells in the testis, percentage of round, elongating and elongated cells within the 1N population, mean and coefficient of variation of the α_t distribution of ejaculated and vas sperm ($\bar{X}\alpha_t$-Ejac, $CV\alpha_t$-Ejac, $\bar{X}\alpha_t$-Vas and $CV\alpha_t$-Vas, respectively) and ABN of ejaculated and vas sperm (ABN-Ejac and ABN-Vas). Linear contrasts were used for comparison of means. Product-moment correlations were obtained among all dependent variables. SAS (1984) procedures were used for statistical analyses.

Results and Discussion

Growth of Bulls. There were no significant differences in beginning weight (body weight at time of first implant), weaning and slaughter weights, gain to weaning and feedlot gain among the three treatment groups. Group averages for testicular weights were 637, 496 and 463 g for 0I, 1I and 2I, respectively. Bulls in the 0I group had heavier ($P<.001$) testicular weights at slaughter than those given one or two implants. The implanted groups did not differ in testicular weight.

Testicular Samples. Proportions of cell types in testicular biopsies determined by FCM are presented in table 1. Bulls in the 1I and 2I groups had an increased ($P<.05$) proportion of 2N and decreased ($P<.05$) proportion of 1N cells relative to 0I bulls. Although proportions of spermatid types within the 1N cell population did not vary significantly with treatment, there was a negative correlation (−.35, $P<.05$) between percentages of 1N cells and round spermatids and a positive correlation (.44, $P<.01$) between percentages of 1N cells and elongated spermatids.

Figure 1 shows one variable frequency histograms (A and D) and two variable cytograms (B, C, E and F) of testicular FCM data on two bulls. The histograms (green fluorescence, i.e., DNA stainability) correspond to the y axis of the cytograms. Red fluorescence (RNA content) is on the x axis of the cytograms. Bull 1 (figure 1; A, B and C) was representative of 0I animals. Bull 2 (figure 1; D, E and F) was selected for comparison because testicular cell proportions differed markedly from controls; he was one of the more adversely affected bulls

TABLE 1. MEAN VALUES OF PROPORTIONS OF BULL TESTICULAR CELL TYPES MEASURED BY FLOW CYTOMETRY[a]

Cell type	Treatment[b]			
	0I	1I	2I	SE
	%			
Diploid	10.7[c]	15.5[d]	20.9[d]	2.61
Tetraploid	11.1	14.1	13.2	1.21
Haploid	78.2[c]	70.4[d]	65.9[d]	2.86
Round[e]	42.9	40.2	48.3	3.19
Elongating[e]	27.0	31.5	26.7	3.07
Elongated[e]	30.1	28.3	25.0	2.78

[a]No. = 10 per group.

[b]0I = no implant, 1I = one zeranol implant, 2I = two zeranol implants.

[c,d]Means in the same row with different superscripts differ ($P<.05$).

[e]As a proportion of total testicular haploid cells.

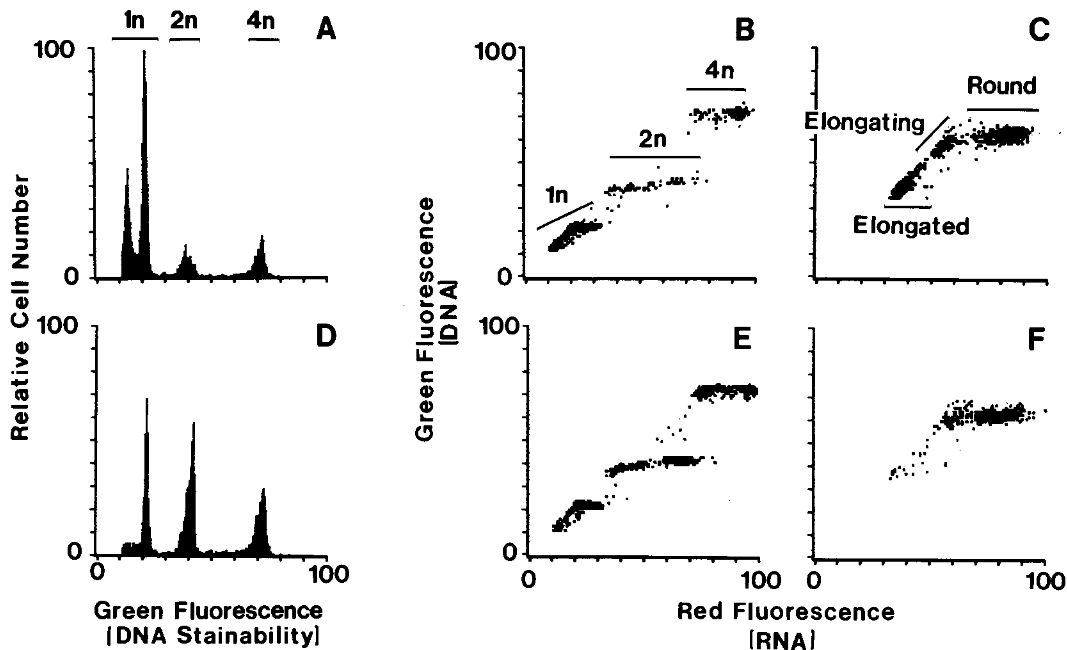

Figure 1. Green fluorescence frequency histograms (A and D) and green and red fluorescence cytograms (B, C, E and F) of testicular cells stained with acridine orange and measured by flow cytometry. The upper row represents cells from a nonimplanted bull (bull 1, 0I; A, B and C); the lower row, cells from a bull receiving two implants (bull 2, 2I; D, E and F). Relative numbers of haploid (1N), diploid (2N) and tetraploid (4N) cells are seen in the frequency histograms, which correspond to the y axis of the cytograms. Cytograms C and F are an enhancement of the 1N populations of cytograms B and E and show greater resolution.

in the 2I group. Individual data for the two bulls are listed in table 2. Note that although bull 1 had a lower slaughter weight and smaller scrotal circumference, his testicular weight was higher than that of bull 2 (table 2).

In figure 1, the histogram peaks from left to right represent green fluorescence from elongated and round spermatids and 2N and 4N cells. 1N, 2N and 4N cells are also readily distinguished in the cytograms (figure 1, B and E). The DNA content and stainability are relatively constant within the 2N and 4N populations, whereas RNA content is more variable. Enhanced cytograms of the 1N cell populations are shown in figure 1, C and F. A comparison of the histograms and cytograms of the two bulls shows a lack of 1N cells (particularly elongated spermatids) for bull 2 vs bull 1 (1N: 49% vs 80%; elongated: 11% vs 36%, table 2).

The FCM analyses of testicular cell proportions were generally confirmed by light microscopic examination of biopsies for bulls 1 and 2 (figure 2; A and B, respectively). A large number of maturing spermatids were present in the seminiferous tubules of bull 1 (A), whereas for bull 2 (B), spermatids were less predominant. However, other regions of the seminiferous tubules of bull 2 did not show this level of deficiency, indicating the difficulty in accurately evaluating 1N cell numbers by microscopic examination.

Sperm Samples. The $\overline{X}\alpha_t$ and $CV\alpha_t$ of ejaculated and vas sperm are listed in table 3. For both sperm samples the $\overline{X}\alpha_t$ was highest ($P<.05$) from 2I bulls. The $CV\alpha_t$-Ejac did not vary significantly with treatment, although the 0I group had the lowest and the 2I group the highest average values. Differences between treatments in $CV\alpha_t$ were significant for vas sperm, with 0I bulls having lower ($P<.05$) average values (18.42) than the 1I (23.49) and 2I (27.14) groups.

Figure 3 shows examples of FCM data obtained on vas sperm from the same two bulls of figures 1 and 2. The signal pattern noted in the cytograms is characteristic of sperm samples measured in a flow cytometer with orthogonal

TABLE 2. INDIVIDUAL VALUES OF WEIGHTS, TESTICULAR AND SPERM MEASUREMENTS ON BULL 1 (0I) AND BULL 2 (2I)[a]

Variable	Bull	
	1	2
Weaning wt, kg	495	690
Slaughter wt, kg	1,160	1,325
Testicular wt, g	571	506
Scrotal circumference, cm	36	39
Testicular cells (%)		
Diploid	9	31
Tetraploid	11	20
Haploid	80	49
Haploid cells (%)[b]		
Round	44	61
Elongating	20	29
Elongated	36	11
Ejaculated sperm		
$\overline{X}\alpha_t$-Ejac[c]	.26	.34
$CV\alpha_t$-Ejac[c]	13.2	15.6
ABN-Ejac[d]	11	35
Vas sperm		
$\overline{X}\alpha_t$-Vas	.22	.39
$CV\alpha_t$-Vas	12.0	32.2
ABN-Vas	26	53

[a]0I = no implant; 2I = two zeranol implants.

[b]As a proportion of total testicular haploid cells.

[c]$\overline{X}\alpha_t$ and $CV\alpha_t$ = the mean and coefficient of variation, respectively, of the α_t distribution for that sample.

[d]ABN = the proportion of morphologically abnormal sperm for that sample, scored by light microscopy.

axes of laser illumination, sample stream flow and fluorescence detection. Because sperm nuclei have an asymmetrical shape and high refractive index, fluorescence detected will depend upon orientation of the nucleus in the flow cell at time of measurement. Varying orientations result in an "elongated" fluorescence signal (Gledhill et al., 1976).

Vas sperm cytograms for the two bulls were markedly different: Bull 1 (figure 3,A) was a "normal" animal with sperm DNA highly resistant to denaturation in situ, whereas sperm from bull 2 were much more heterogeneous, as a high proportion had DNA that was susceptible to denaturation in situ, indicated by increased red fluorescence. The small population of cells represented by the dots extending toward the origin in figure 3, cytogram C, below the major population of sperm cells may be attributed to cellular debris often present in "poorer" samples (low sperm concentration, high α_t values). Differences between the two bulls are reflected in the values, .22 and 12.0 (bull 1), and .39 and 32.2 (bull 2), for $\overline{X}\alpha_t$-Vas and $CV\alpha_t$-Vas, respectively.

The higher α_t values are associated with decreased sperm quality and reduced fertility, as demonstrated in concurrent studies of bull sperm frozen for use in artificial insemination (Ballachey et al., 1985). As discussed by Evenson et al. (1985), alterations of spermatogenesis and chromatin structure contributing to increased denaturation of the DNA in situ have not been delineated. However, a close correspondence is obtained between α_t values of whole sperm vs isolated nuclei from the same sample, indicating involvement of factors which are intrinsic to the nucleus itself.

Percentages of sperm with abnormal morphology are also listed in table 3. Bulls in the 2I group tended to have the highest average ABN-Ejac ($P<.10$). Treatment effects were more pronounced for ABN-Vas, as 0I bulls had a lower ($P<.05$) average than implanted bulls (27.6, 40.7 and 41.7 for 0I, 1I and 2I, respectively).

Positive correlations were noted for ABN-Vas with $CV\alpha_t$-Vas and $\overline{X}\alpha_t$-Vas (.71, $P<.001$ and .48, $P<.01$, respectively; table 4). Correlations of α_t values and ABN-Ejac are also posi-

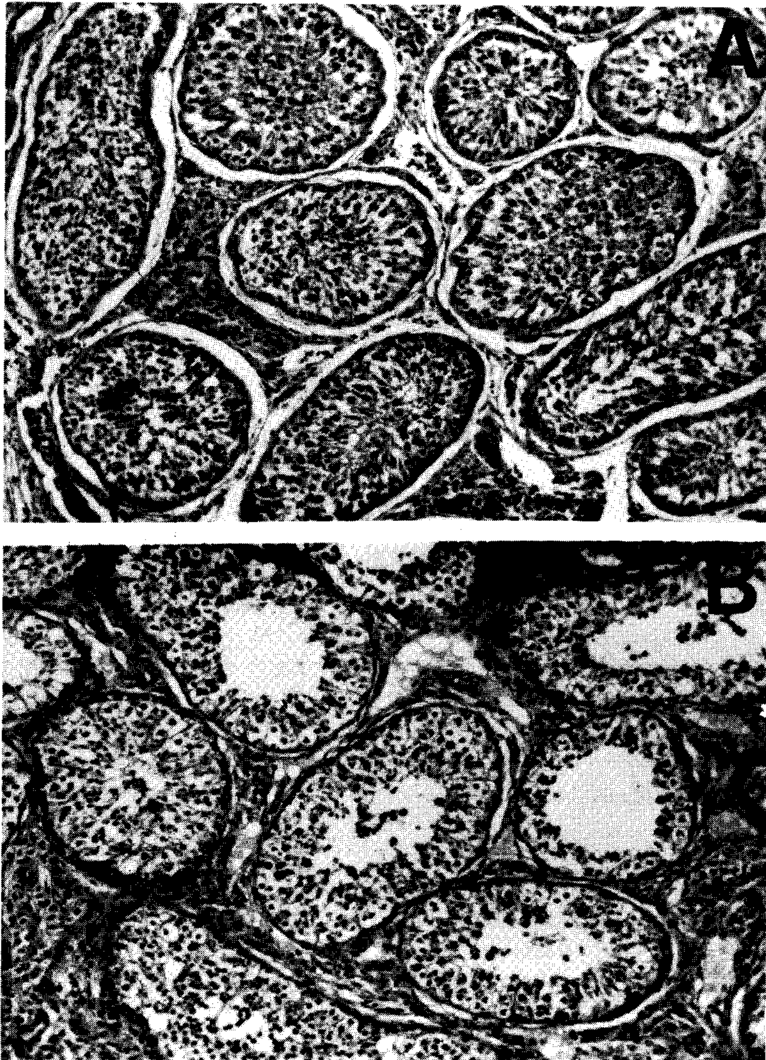

Figure 2. Light micrographs of hematoxylin- and eosin-stained paraffin sections of testicular biopsies from a nonimplanted bull (bull 1, 0I, A) and a bull receiving two zeranol implants (bull 2, 2I, B), showing seminiferous tubules with spermatogenic cells.

tive but not as strong (table 4). Similar associations of α_t values with ABN have been observed for mice (Evenson et al., 1985, 1986). In figure 4, ABN-Ejac (A) and ABN-Vas (B) are plotted against $CV\alpha_t$ values. Note several sperm samples with a high $CV\alpha_t$-Ejac, although morphological abnormalities were scored at lower frequencies. Similar observations have been made in previous work (Evenson et al., 1980a; 1984) and in a study utilizing a variation of AO staining procedures described here (Tejada et al., 1984). Some samples with high α_t values apparently have an increased frequency of chromatin alterations that may be difficult or impossible to evaluate by light microscopy, but are readily detectable by FCM after AO staining.

Correlations among testicular and sperm variables are also listed in table 4. Testis weight was positively correlated (.49, $P<.01$) with proportion of 1N cells and negatively correlated with both $CV\alpha_t$-Ejac and $CV\alpha_t$-Vas (−.41, $P<.05$ and −.46, $P<.01$, respectively). Larger testes (0I bulls) are apparently functioning normally with increased proportions of 1N

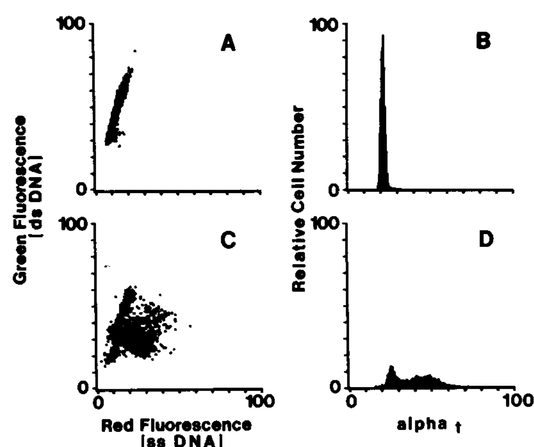

Figure 3. Red and green fluorescence cytograms (A, C) corresponding to α_t frequency histograms (B, D) of sperm cells stained with acridine orange and measured by flow cytometry. Red and green fluorescence correspond to single-stranded (ss) and double-stranded (ds) DNA, respectively. A and B represent cells from a nonimplanted bull (bull 1, 0I); C and D from a bull receiving two zeranol implants (bull 2, 2I).

germ cell populations and production of more homogeneous spermatozoa. Conversely, smaller testes (1I and 2I bulls) have decreased 1N cell populations (which increases the 2N proportion) and greater chromatin heterogeneity among spermatozoa. Although ejaculated and vas sperm samples were generally similar, the r values of .72 (P<.001) between ABN-Ejac and ABN-Vas, and .60 (P<.01) between CVα_t-Ejac and CVα_t-Vas indicate that some differences exist between sperm from the two sources. This may reflect true variation between the two samples, or the variation may result in part from handling differences prior to measurement. Electroejaculation of sperm often results in samples contaminated with dirt and(or) urine. The FCM measurements on ejaculated sperm were made 7 to 12 h after collection and some cellular degeneration may have occurred during that time, depending upon the initial condition of the sample. Vas sperm remained in the vas deferens (attached to the testicles, on ice) until extruded about 6 h after slaughter and were frozen soon after, pending subsequent FCM analyses.

Although zeranol exposure exerted a strong effect on testicular development and spermatogenesis of bulls, considerable variation among bulls within treatments was nevertheless evident. Several bulls of the 0I group had high ABN and α_t values. Bulls of the 2I group were consistently lower in percent 1N cells and CVα_t-Vas than the average of the 0I group. More variability existed among the 1I bulls: some had FCM testicular and sperm analyses in the "normal" range (similar to 0I), whereas others had low percent 1N cells and higher α_t values. This agrees with high variation of testicle size and penile abnormalities noted among implanted bulls by Staigmiller et al. (1985).

TABLE 3. MEAN VALUES OF BULL SPERM VARIABLES

Sperm type	Variable	Treatment[a] 0I	1I	2I	SE
Ejaculated[b]	$\bar{X}\alpha_t$-Ejac[d]	.31[f]	.31[f]	.36[g]	.02
Ejaculated	CVα_t-Ejac[d]	16.2	17.5	19.4	2.06
Ejaculated	ABN-Ejac[e]	24.6	28.6	41.4	5.42
Vas[c]	$\bar{X}\alpha_t$-Vas	.23[f]	.23[f]	.26[g]	.01
Vas	CVα_t-Vas	18.4[f]	23.5[g]	27.1[g]	2.21
Vas	ABN-Vas	27.6[f]	40.7[g]	41.7[g]	5.19

[a]0I = no implant; 1I = one zeranol implant; 2I = two zeranol implants.

[b]No. = 9 for nonimplanted group; No. = 7 for 1I and 2I groups.

[c]No. = 10 per group.

[d]$\bar{X}\alpha_t$ and CVα_t = the mean and coefficient of variation, respectively, of the α_t distribution for that sample.

[e]ABN = the proportion of morphologically abnormal sperm for that sample, scored by light microscopy.

[f,g]Means in the same row with different superscripts differ (P<.05).

TABLE 4. CORRELATIONS AMONG BULL TESTICULAR AND SPERM MEASUREMENTS[a]

Variable	Variable 1	2	3	4	5	6	7	8	9	10
1) Testis wt, g		−.54**	−.04	.49**	−.18	−.41*	−.28	−.17	−.46**	−.25
2) % Diploids			.09	−.92***	.15	.12	.28	.28	.40*	.29
3) % Tetraploids				−.46**	.17	.24	.24	.39*	.10	.40*
4) % Haploids					−.19	−.20	−.34	−.40*	−.39*	−.41*
5) $\bar{X}\alpha_t$-Ejac[b]						.53**	.46*	.39	.31	.46*
6) $Cv\alpha_t$ Ejac[b]							.27	.31	.60**	.48*
7) ABN-Ejac[c]								.41*	.56**	.72**
8) $\bar{X}\alpha_t$-Vas									.58***	.48**
9) $CV\alpha_t$-Vas										.71***
10) ABN-Vas										

[a]For correlations involving epididymal sperm samples, No. = 23; all others, No. = 30.

[b]$\bar{X}\alpha_t$ and $CV\alpha_t$ = the mean and coefficient of variation, respectively, of the α_t distribution for that sample; Ejac = ejaculated sperm; Vas = vas deferens sperm.

[c]ABN = the proportion of morphologically abnormal sperm for that sample, scored by light microscopy.

*P<.05.

**P<.01.

***P<.001.

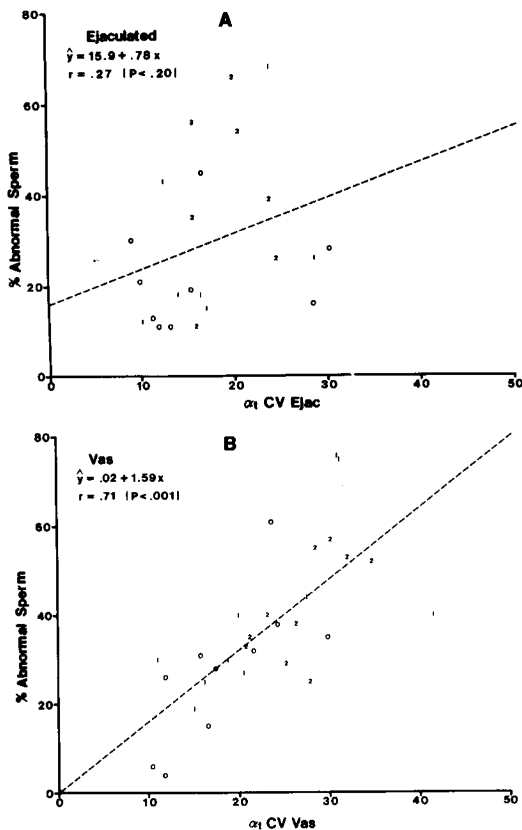

Figure 4. Relationship between abnormal sperm (%) and $CV\alpha_t$ for ejaculated ($CV\alpha_t$-Ejac; A) and vas ($CV\alpha_t$-Vas; B) sperm cells. Each point represents one bull; numbers 0, 1 and 2 indicate nonimplanted, one zeranol implant and two zeranol implant treatment groups, respectively.

Of interest are marked effects of implants at an early age (30 d, 1I; 30 and 120 d, 2I) on bulls approximately 1 yr later. The effects of zeranol on testicular development appear to be permanent. These results contrast with those of Juniewicz et al. (1985), who implanted bulls at 100 d of age and noted recovery of spermatogenic function in bulls approximately 1 yr later.

This research demonstrates the utility and efficiency of FCM for evaluation of male reproductive function. Testicular and sperm cells from bulls implanted with zeranol at an early age were measured approximately 1 yr later. Spermatogenesis in implanted bulls was adversely affected, indicated by alterations in ratios of testicular cell types and increased heterogeneity of chromatin structure in spermatozoa.

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