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# FLOW CYTOMETRY EVALUATION OF TESTIS AND SPERM CELLS AND GROWTH PERFORMANCE OF BULLS IMPLANTED WITH ZERANOL

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## Summary

Effects of preweaning zeranol implants on reproductive function and growth performance were studied in 45 Simmental-Angus bulls. At slaughter, flow cytometry measurements were made on testicular and sperm cells to determine the effects of zeranol on spermatogenesis. There were no differences in weaning or slaughter weights due to implants. Nonimplanted bulls had larger scrotal circumferences and heavier testicular weights than bulls given one or two implants. The testes of implanted bulls had a lower proportion of developing germ cells relative to nonimplanted bulls. The DNA in sperm from implanted bulls was structurally less stable (i.e., more susceptible to denaturation) than DNA in sperm from nonimplanted bulls. Preweaning implantation with zeranol negatively affected testicular function and spermatogenesis, but did not affect growth of yearling bulls.

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

## Introduction

Zeranol, a resorcylic acid lactone, increases growth in steers, and, although the mode of action is not clearly understood, it is widely used. With renewed interest in feeding intact males for increased production efficiency, the ability of growth promotants to improve gains in bulls becomes an important consideration. Effects of zeranol on growth in bulls are not well established and much variation in response has been reported. Detrimental effects of preweaning zeranol implants on testicular size and weight have been consistently observed, but spermatogenic function and semen quality in bulls implanted at an early age have not been well studied. Understanding changes in spermatogenesis caused by zeranol may help to establish the mode of action and overall value of these implants to promote growth in bulls.

Flow cytometry is a relatively new technology with tremendous potential for cellular analyses. Cells in suspension may be labeled with a fluorescent dye specific for the component of interest (e.g., DNA) and passed single file in a sample stream intersected by a laser beam. Upon laser excitation, fluorescence is emitted from the cells. The fluorescence is detected by photomultiplier tubes, converted to a digital analog signal and subsequently analyzed by a computer interfaced to the flow cytometer. Advantages of flow cytometry include rapid measurement of a large number of cells, simultaneous measurement of several variables per cell and ease of classifying and counting subpopulations of cell types. Previous studies (see the preceding paper in this report) have demonstrated the value of flow cytometric analyses to study male reproductive function and reproductive toxicology. The purpose of this research was to

utilize flow cytometry to evaluate testis and sperm cells of yearling beef bulls implanted with zeranol prior to weaning.

## Experimental Procedure

Cattle. Forty-five 3/4 Angus x 1/4 Simmental bull calves averaging 30 days of age were weighed and randomly divided into three treatment groups: control-no implant (0I), one zeranol implant (1I) and two zeranol implants (2I). Calves in the treated groups were implanted with 36 mg of zeranol under the skin on the lower backside of the ear according to manufacturer's recommendation. Ninety days later calves in the 2I group were given a second zeranol implant. After weaning at an average age of 205 days, calves were transported approximately 300 miles to a feedlot facility. Bulls were fed a grower ration consisting of alfalfa hay offered ad libitum and 5.5 lb of corn daily for 90 days followed by a finishing ration for 155 days until slaughter. The finishing ration, fed ad libitum, consisted of 84.0% corn, 10.5% corn silage, 4.8% soybean meal and .7% mineral supplement. Bulls were slaughtered at an average age of 450 days. Five days before slaughter, scrotal circumferences were measured and, from 10 randomly selected bulls of each treatment group, an electroejaculated semen sample was collected and placed on ice. At slaughter, testes and vas deferens from all bulls were collected and placed on ice and testis weights were recorded. From bulls which had been electroejaculated, a testicular biopsy was also taken for measurement by flow cytometry. Within 6 hours of collection, vas sperm were recovered from the vas deferens of electroejaculated bulls.

Cell Preparation and Staining. Flow cytometry measurements were made on ejaculated sperm samples the day of collection. If necessary, samples were diluted with physiological saline solution to lower the cell concentration prior to measurement.

Vas sperm were collected by stripping the contents of the vas deferens into saline solution. Vas sperm and testicular cells were obtained on the same day and there were too many samples to be run by flow cytometry that day. Therefore, vas sperm were mixed with glycerol and frozen at -100° C until flow cytometry measurements were made approximately 2 months later.

Each testicular biopsy, weighing approximately 1 g, was placed in saline solution at the time of collection. Six to eight hours after collection, the biopsy was minced with scissors to form a cellular suspension, filtered free of tissue clumps and measured by flow cytometry to determine DNA and RNA content of each cell.

Testicular cells and ejaculated and vas sperm were stained with acridine orange (AO) dye and measured by flow cytometry. The procedure involves treating the cells with a solution containing detergent (allows dye better access to cell components) and mild acid, then staining with AO. Testicular cells contain both DNA (double-stranded) and RNA. When AO binds with DNA, it fluoresces green, and, when it binds to RNA, it fluoresces red. Measurement of green and red fluorescence allows quantification of the relative DNA and RNA content and, on this basis, proportions of tetraploid, diploid and haploid cells in a sample can be determined. The haploid cells are the developing germ cells.

In sperm cells, the staining procedure is called the sperm chromatin structure assay (SCSA). RNA is not present in sperm and, because of condensation of the chromatin (DNA and associated proteins) associated with maturation, the

acid treatment potentially induces partial denaturation of the DNA so that both double- and single-stranded DNA are present. AO dye will fluoresce green when bound to double-stranded DNA and red with single-stranded DNA. Thus, ratios of green to red fluorescence measure the susceptibility of DNA to denaturation and provide information on the chromatin structure of the sperm. The amount of denaturation was quantified by alpha-t ($\alpha_t$), defined as the ratio of red to total (red + green) fluorescence. The mean ($X\alpha_t$) and coefficient of variation ($CV\alpha_t$) of the $\alpha_t$ distribution were used to describe the extent of denaturation. Previous studies have shown that higher $\alpha_t$ values are associated with poor semen quality and decreased fertility.

To measure morphological abnormalities, sperm from each sample were stained with Eosin dye and smeared onto glass slides. At least 200 cells per bull were scored by light microscopy to estimate percentage of abnormal sperm heads.

## Results and Discussion

Growth of Bulls. Performance data, testicular weights and scrotal circumferences are shown in table 1. There were no significant differences in initial, weaning or slaughter weights among the three treatment groups. Gain to weaning (420, 409 and 434 lb for 0I, 1I and 2I, respectively) and feedlot gain (504, 476 and 485 lb for 0I, 1I and 2I, respectively) also did not vary with treatment.

Bulls in the nonimplanted group had heavier testicular weights and larger scrotal circumferences at slaughter than bulls given one or two implants. The implanted groups (1I and 2I) did not differ in testicular weight or scrotal circumference.

Testicular Samples. Testicular haploid, diploid and tetraploid cells can be distinguished by FCM on the basis of cellular DNA and RNA content after staining with AO. A higher proportion of haploid cells is associated with more efficient spermatogenesis. Within the haploid cell population, maturing germ cells can be separated into proportions of round, elongating and elongated spermatids because during maturation chromatin structure becomes more compact and this is associated with decreased green fluorescence due to restriction of AO binding. Decreasing red fluorescence is also noted, resulting from decreasing RNA content as the spermatids mature.

Proportions of cell types in the testicular biopsies, determined by computer analysis after measuring samples by flow cytometry, are presented in table 2. Bulls in either the 1I or 2I groups had an increased proportion of diploid cells and decreased proportion of haploid cells relative to 0I bulls ($P<.05$). Proportions of spermatid types within the haploid cell population did not vary significantly with treatment.

Sperm Samples. Mature sperm, whether obtained from the vas deferens or an ejaculated semen sample, are characterized by a nucleus with highly condensed chromatin and devoid of RNA. Normal sperm stained with AO should emit largely green fluorescence (reflecting ds DNA content) and minimal red fluorescence. In sperm with abnormal chromatin, the DNA will be more susceptible to denaturation, resulting in increased red fluorescence and higher $\alpha_t$ values.

The $\bar{X}\alpha_t$ and $CV\alpha_t$ of ejaculated and vas sperm samples are listed in table 3. For both sperm samples the $X\alpha_t$ was highest from 2I bulls. The $CV\alpha_t$ for

ejaculated sperm samples did not vary significantly with treatment, although the 0I group had the lowest and the 2I group the highest average values. For vas sperm, differences were seen between treatments, with 0I bulls having lower average $CV\alpha_t$ values (18.42) than the 1I (23.49) and 2I (27.14) groups.

Percentages of morphologically abnormal sperm measured by light microscopy are also presented table 3. For ejaculated samples, bulls in the 2I group tended to have the highest average proportion of abnormal sperm. Treatment effects were more pronounced for vas sperm, since 0I bulls had a lower average proportion of abnormals than implanted bulls (27.6, 40.7 and 41.7% for 0I, 1I and 2I, respectively.)

Although effects of zeranol on testicular development and spermatogenesis of bulls were demonstrated between treatments, considerable variation among bulls within treatments was also evident. Several bulls of the 0I group had high levels of abnormal sperm and $\alpha_t$ values. Bulls of the 2I group were consistently lower in proportion of haploid cells and and $CV\alpha_t$ of the vas sperm than the average of the 0I group. More variability existed among the 1I bulls; some had testicular and sperm flow cytometric values in the "normal" range (similar to 0I) and some were in the "poor" range (similar to 2I).

In conclusion, this research investigated the effects of preweaning zeranol implants on growth and spermatogenesis of bulls. Weaning and slaughter weights were not improved by implantation, but sperm cells in implanted bulls were adversely affected by zeranol. The detrimental effect of reproductive function is of major concern if the bulls are being considered for breeding purposes, since it would likely result in reduced male fertility.

TABLE 1. MEAN LIVE WEIGHTS, TESTICULAR WEIGHTS AND SCROTAL CIRCUMFERENCES[a]

| Measurement | Treatment group | | | SEM[b] |
|---|---|---|---|---|
| | Control | 1 | 2 | |
| Initial wt[c], lb | 130 | 135 | 135 | 9.5 |
| Weaning wt, lb | 550 | 544 | 569 | 24.5 |
| Slaughter wt, lb | 1168 | 1127 | 1142 | 28.4 |
| Testicular wt, g | 637[d] | 496[e] | 463[e] | 28.8 |
| Scrotal circumference, cm | 37.8[d] | 35.1[e] | 34.3[e] | .63 |

[a] n = 15 per group.
[b] SEM = standard error of the mean.
[c] Initial wt = wt at time of first implant (about 30 days of age).
[d,e] Means in the same row with different superscripts vary ($P<.01$).

TABLE 2. MEAN VALUES OF TESTICULAR VARIABLES[a]

| Variable | Treatment group | | | SEM[b] |
|---|---|---|---|---|
| | Control | 1 | 2 | |
| % diploid cells | 10.7[c] | 15.5[d] | 20.9[d] | 2.61 |
| % tetraploid cells | 11.1 | 14.1 | 13.2 | 1.21 |
| % haploid cells | 78.2[c] | 70.4[d] | 65.9[d] | 2.86 |

[a] n = 10 per group.
[b] SEM = standard error of the means.
[c,d] Means in the same row with different superscripts vary (P<.05).

TABLE 3. MEAN VALUES OF SPERM VARIABLES

| Sperm type | Variable | Treatment group | | | SEM[a] |
|---|---|---|---|---|---|
| | | Control | 1 | 2 | |
| Ejaculated[b] | $\bar{X}\alpha_t$ | .31[d] | .31[d] | .36[e] | .02 |
| | $CV\alpha_t$ | 16.18 | 17.50 | 19.40 | 2.06 |
| | % abnormal | 24.6 | 28.6 | 41.4 | 5.42 |
| Vas[c] | $\bar{X}\alpha_t$ | .23[d] | .23[d] | .26[e] | .01 |
| | $CV\alpha_t$ | 18.42[d] | 23.49[e] | 27.14[e] | 2.21 |
| | % abnormal | 27.6[d] | 40.7[e] | 41.7[e] | 5.19 |

[a] SEM = standard error of the mean.
[b] n = 9 for control group; n = 7 for one and two implant groups.
[c] n = 10 per group.
[d,e] Means in the same row with differing superscripts vary (P<.05).