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THE RELATIONSHIP BETWEEN PREPUBERAL HORMONE LEVELS AND SERTOLI CELL NUMBER OF SPERMATID PRODUCTION IN THE BEEF BULL

A Thesis

Presented for the

Master of Science

Degree

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Patricia D. Whaley

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ABSTRACT

Producers of beef cattle must become more efficient and the bull is often the component in beef cattle production that either limits or enhances efficiency. Testicular size is positively correlated with sperm producing ability in the bull. Factors affecting testicular size are, consequently, potential indicators of sperm producing capacity. To determine whether prepuberal hormonal levels and Sertoli cell numbers were related to postpuberal testicular size, groups of crossbred bulls were injected with 10 ug of GnRH ethyamide when either 60 (n=8), 90 (n=14), 120 (n=18), or 180 (n=14) days of age. Jugular blood was collected at 0, 1, 2, and 4 hours after GnRH injection and plasma was assayed for gonadotrophins, testosterone and estradiol. Animals were then unilaterally castrated. The testis was weighed and measured. Sections (5 um) were evaluated for the number of Sertoli cells per testis. A second challenge was administered when the bulls were approximately 14 months of age; blood was collected and assayed as before. The second testis was removed, weighed and measured. Sections (5 um) were evaluated for percentage of tubular areas occupied by spermatids.

Prepuberal FSH, testosterone and estradiol did not vary significantly with age ($p>0.1$) nor were they correlated with Sertoli cell number ($p>0.1$). Basal LH levels differed significantly with age ($p>0.01$). Basal LH levels were correlated ($r = -.57$) with Sertoli cell number. Basal prepuberal levels of LH were negatively correlated

($r = -.31$) and basal testosterone levels were positively correlated ($r = .51$) with the number of cross sections containing spermatids. Basal prepuberal LH levels were not significantly different between high and low testicular weight ($p>0.1$). Sertoli cell number was higher in animals with greater testicular weight ($p<0.1$). Sertoli cell number was not correlated with spermatid production. These results were probably due to the fact that approximately one-half of the animals in the study were still in the prepuberal state at 14 months, as determined by histological examination. The response of hormonal levels to GnRH stimulation was not a useful predictor of spermatid production of testicular weight, as stimulated levels were correlated with neither. It is concluded that prepuberal LH and testosterone were correlated with degree of testicular development but not with testicular weight, another criterion of testicular development. Studies of bulls of more advanced age are required to determine if prepuberal LH and testosterone levels are potentially useful as indicators of full testicular function.

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CHAPTER I

INTRODUCTION

Economic pressure has forced producers to become more efficient in their management techniques. Bulls have always posed special management problems. The current U.S.. beef market prefers steers to bulls as meat animals. Artificial insemination technology has reduced the number of bulls needed for breeding purposes. The cost of maintaining a bull calf until puberty is wasted if the bull is not of substantial breeding soundness.

Testicular size is positively correlated with sperm-producing ability in the bull (Gipson et al., 1985). This size is determined by the Sertoli cell population within the testis (Erickson and Blend, 1976). However, prepuberal testicular measurements are a poor indicator of postpuberal sperm-producing capacity; ergo, a bull must be at least one year of age before a reliable estimate of his capacity as a sire can be assessed. If an earlier determination of reproductive merit could be made, a producer could coordinate his selection procedures more efficiently for greater economic benefit.

As the Sertoli cell is mitotically active only briefly during prepuberal development (Attal and Courot, 1963, and Monet-Kuntz, 1984), its number and hence, the potential for testicular growth is fixed at an early age. Therefore, factors that are either produced by the Sertoli cell or that affect Sertoli cell mitosis

are potential indicators of the sperm-producing capabilities of the testis.

CHAPTER II

LITERATURE REVIEW

Function and Control of Gonadotrophins and Steroids in the Adult Male

The role of the reproductive hormones in the adult begins prepuberally. Study of levels during this period is important in the understanding of their ultimate function. At puberty, maturation of the hypothalamus increases the production and release of a decapeptide, gonadotrophin-releasing hormone (GnRH) (Amann and Schanbacher, 1983). GnRH acts on cells in the anterior pituitary gland called gonadotropes to stimulate the synthesis and release of the gonadotrophins Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) (Fraser, 1979). LH and FSH are glycoproteins consisting of an α and β subunit. The α subunits of both hormones are identical in structure, only the β subunits differ (Hadley, 1984).

Release of GnRH by the hypothalamus is pulsatile, this leads to a pulsatile release of the gonadotrophins which, in turn, leads to pulsatile release of the steroids (Desjardin, 1981). Administration of GnRH caused a thirtyfold increase in LH secretion and a sevenfold increase in FSH secretion in Hereford bulls (Schanbacher and Echternkamp, 1978). Although GnRH stimulates synthesis and secretion of both LH and FSH, the mechanism of action is not similar. Administration of drugs preventing alpha-adrenergic neurotransmission

causes an inhibition of LH secretion by the hypothalamus. FSH release is unaffected. This indicates that regulation or FSH secretion by the hypothalamus is not mediated by cholinergic neurotransmission as was previously thought, but by an as yet unknown mechanism (Chappel et al., 1984). A large number of structural analogs to GnRH have been synthesized. Antagonistic analogs appear to bind to receptor sites on the anterior pituitary, but do not stimulate gonadotrophin release. Agonistic (stimulating) analogs often induce a release greater than that of the natural hormone (Hafez, 1980).

LH stimulation induces the Leydig cells to undergo hypertrophy, resulting in an increase in the number of mitochondria and the amount of smooth endoplasmic reticulum which are associated with the enzymes required for testosterone production (de Kretser, 1982). Estrogen is produced in the Leydig cells through the aromatization of testosterone to estradiol; however, total production is 0.1% of androgen production (Waites et al., 1985). Estradiol is also produced by the Sertoli cell, in response to FSH through conversion of testosterone (Dorrington et al., 1978).

Both testosterone and estrogen affect LH secretion. Santen (1975) showed that, in man, testosterone increases the amplitude but decreases the frequency of LH release. Estradiol decreases amplitude but not frequency. This suggests that each steroid has a different site of action--testosterone at the hypothalamus, estradiol at the pituitary. In the bull, however, estradiol decreases both amplitude and frequency--evidence of its effect on the hypothalamus and pituitary (Schanbacher, 1982). Another hormone,

prolactin (PRL), produced by lactotropes in the anterior pituitary, may play a role in the control of the number of LH receptors on the Leydig cell. Inhibition of PRL secretion by ergot alkaloids results in a decrease in LH receptor number, while treatment with PRL prevents loss of LH receptors in animals which have been hypophysectomized (Zipf et al., 1978).

FSH stimulation causes production of Androgen Binding Protein (ABP) by the Sertoli cell. ABP transports testosterone to the epididymus, where it promotes viability and maturation of spermatozoa. Also produced by the Sertoli cell is the protein, inhibin. Inhibin, as the name suggests, is an inhibitory factor which controls the secretion of FSH from the pituitary (Steinberger and Steinberger, 1976). Lumpkin et al. (1981) showed that inhibin altered FSH release but not LH release and that exposure of the pituitary to inhibin prior to a GnRH challenge suppressed the release of FSH.

FSH and LH are required for the initiation of spermatogenesis. LH has an indirect effect through its stimulation of androgen production by the Leydig cells, as FSH and testosterone can also initiate spermatogenesis (Setchell, 1982). FSH induces production of ABP by the Sertoli cell, which is secreted into the lumen of the seminiferous tubules. Testosterone is taken up by the Sertoli cell, probably through some form of facilitated transport. Within the Sertoli cell it is bound to the ABP. The ABP functions to sequester testosterone near the spermatocytes whose maturation may be androgen dependent and to transport testosterone to the epididymus to promote maturation of the spermatozoa (Hadley, 1984). Once established,

large doses of testosterone can maintain spermatogenesis in the rat. This is not true in other species, such as the sheep and the macaque, in which FSH is also required for maintenance (de Kretser, 1984).

Prepuberal Testicular Development in the Mammal

The mammalian testis is composed of two regions, the interstitial tissue and the seminiferous tubules. Maturation of these two regions is under both external and internal hormonal control (Amann and Schanbacher, 1983).

The Interstitial tissue

Interstitial tissue consists primarily of interstitial or Leydig cells, site of LH receptors and testosterone synthesis in the testis (Setchell, 1982). At birth, the testis contains a large number of interstitial cells (Gier and Marion, 1970). Fetal Leydig cells decline in number and regress soon after birth. Functional Leydig cells are rare in calves 1 to 3 months old, in man and rhesus monkeys until shortly after birth, and in the rat at 4 days (Hooker, 1970). This corresponds with a reduction in the number of LH receptors observed in the lamb (Barenton et al., 1983), pig (Peyrat et al., 1981), and the rat (Lee and Burger, 1983). Leydig cells produce testosterone under LH stimulation. Transient high levels of LH and testosterone occur at birth followed by a decline to a low level which remains until just prior to puberty (Waites et al., 1985). In cattle, this period of high LH and testosterone occurs from 1

to 4 months of age (McCarthy et al., 1979). The short term increase in testosterone at birth may be produced by a reduction in an estradiol-mediated block to GnRH discharge resulting in an increase in LH release (Amann et al., 1986). LH decrease may be due to negative feed-back by the 5 α-reduced androgens, 5 α-andronstanediol and 5 α-dihyprotestosterone, produced during this period (Kennedy et al., 1985).

The Seminiferous Tubules

Seminiferous tubules are made up of both somatic cells (myoid and Sertoli cells) and germ cells. The somatic cells regulate the environment within the tubule to promote spermatogenesis (Setchell, 1982).

Myoid cells. Myoid cells form the wall of the seminifous tubule and are probably responsible for the peristaltic-like movement of the tubules (Setchell, 1982).

Sertoli cells. Sertoli cells extend from the outer tubular wall to the lumen of the tubule. They contain the receptors for FSH and testosterone (Tindall et al., 1985). As previously stated, the testis, at birth, is dominated by interstitial tissue, but the seminiferous tubule dominates thereafter as a result of the proliferation of Sertoli cells and germ cells.

Prepuberal Sertoli cells proliferation is instrumental in determining adult size. de Reveris et al. (1980) concluded from their study of hemicastrated prepuberal animals that the proliferative

activities of the Sertoli and germ cells before puberty rather than the initial numbers at birth were the limiting factor of their final number in the adult. Testicular hypertrophy which occurs with hemi-castration is primarily due to an increased number of Sertoli cells (Cunningham et al., 1978). This phenomenon was also observed in immature rats (Orth et al., 1984). Weight per testis in hemicastrated lambs was higher than in intact lambs (Hochereau-de Reviers et al., 1984). A larger degree of testicular hypertrophy occurred in uni-laterally castrated bulls in the early stages of puberal development than in those castrated later (Wildeus, 1985). Sertoli cell number remains constant, in bulls, from 6 months of age (Attal and Courot, 1963). Monet-Kuntz (1984) has shown that, in rams, from day 40 onward Sertoli cell number remained constant. Those animals castrated (unilaterally) at a time early in Sertoli cell proliferation show greater testicular hypertrophy.

Sertoli cell numbers and germ cell numbers are related in mammals (Kluin et al., 1984). Depletion of Sertoli cells during neo-natal life causes reduced sperm production in humans (Orth, 1985; Johnson et al., 1984). Berndtson et al. (1985) demonstrated, in the bull, that testes containing larger numbers of Sertoli cells had greater daily sperm production than those with fewer cells. Testes of maturing mice, in all ages studied, showed a numerical relationship between Sertoli cells and germ cells (Kluin et al., 1984).

Proliferation of Sertoli cells is under gonadotrophin control in mammals. Vogel et al. (1983) found that male rats treated with a single injection of anti-serum to GnRH at 5 days of age had

testicular weights of about 60% of controls at all ages from 10-90 days, suggesting that GnRH reduction at a critical period impairs early testicular growth and causes a permanent reduction in growth. In the rat, FSH is instrumental in Sertoli cells proliferation. Cunningham et al. (1978) showed that changes in serum FSH were associated with testicular hypertrophy in hemicastrated rats. FSH concentration was twice normal levels in hemicastrated rats while LH concentration was unchanged (Orth et al., 1984). This is also true in cattle (Schanbacher et al., 1987). However, in sheep and swine, proliferation appears to be controlled by LH. Prepuberal, hypophysectomized lambs can maintain total number of Sertoli cells by injection of LH alone, but not FSH and in normal, unilaterally castrated, or cryptorchid lambs, Sertoli cell number is correlated with plasma LH levels between 40 and 55 days (de Reviers et al., 1980). Kilgour et al. (1983) demonstrated that lambs treated with anti-LH antisera had lower testicular weights and a lower number of Sertoli cells/testis. Seminiferous tubule diameter at 14 weeks of age was positively correlated with mean LH concentration and basal LH concentration for weeks 8-14 (Echternkamp and Lunstra, 1984). Concentrations of LH, from 42 to 84 days of age, in boars, were positively correlated with measures of testicular development ($r = .24$ to $.49$) while concentrations of FSH were not related to development (Schinckel et al., 1984).

Differentiation of Sertoli cells to mature cells is completed from 20 to 28 weeks postnatal (Curtis and Amann, 1981; and Sinowatz and Amselgruber, 1979). Mature Sertoli cells, in close proximity,

form junctional complexes which divide the tubules into basal and adluminal compartments. This blood-testis barrier separates the spermatogonia (basal compartment) from the spermatocytes, spermatids and spermatozoa (adluminal compartments). The barrier prevents proteins and antibodies from contacting the haploid germ cells and also prevents proteins produced by these haploid germ cells from causing an immune reaction, as well as, regulating the entry of compounds which may be toxic (Setchell, 1982).

Germ cells. The testis contains five types of germ cells: spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa (Setchell, 1982). Prepuberal germ cell proliferation parallels that of the Sertoli cells; however, germ cell numbers continue to increase after Sertoli cells proliferation has ceased (Sinowatz and Amselgruber, 1979). These cells continue to be produced and mature throughout the life of the animal.

CHAPTER III

MATERIALS AND METHODS

Experimental Design

This study consisted of two parts: (1) an evaluation of prepuberal gonadotrophin and steroid secretion and possible interrelationships between hormone levels and Sertoli cell numbers, and (2) an evaluation of the relationship between prepuberal hormone profiles and spermatid production. Both studies were performed on Crossbred bull calves (54) at the Tobacco and Plateau Experiment Stations.

In the first study, animals were evaluated at either 60 (n=8), 90 (n=14), 120 (n=18), or 180 (n=14) days of age. A 12 milliliter blood sample was obtained from all animals, followed by injection of 10 ug of a Gonadotrophin-Releasing hormone (GnRH) analog, des-Gly, [D-Ala]-GnRH ethylamide, in all except the 60 day animals. Further blood samples were collected at 1, 2, and 4 hours postinjection. Blood was centrifuged at 3000 rpm for 20 minutes. Plasma was removed and stored at -20 C until assayed for gonadotrophins, estradiol, and testosterone. Samples from each animal were pooled for estradiol analysis. Due to a shortage of plasma, testosterone values for the 60 day animals were unattainable. Prepuberal secretion patterns were then established.

Following blood collection, bulls were unilaterally castrated. The testis was weighed, measured and transverse sections (4mm) were

excised from the center and each pole and fixed with Bouins fluid. The fixed, paraffin embedded tissue was sectioned at 5 um and stained with hemotoxylin and orange G. Sections were evaluated for Sertoli cell population. Interrelations between the established hormone patterns and Sertoli cell numbers were established.

In the second study, conducted at 14 months of age, a second injection of the synthetic GnRH was given. Plasma was collected and assayed as previously indicated. The remaining testis was removed and evaluated for spermatid production. Postpuberal hormonal secretion patterns were evaluated. The relationship between prepuberal hormone secretion, Sertoli cell content of the first testis and spermatid production was determined.

Radioiodination Procedures

LH. LH was iodinated by the Chloramine T method. The procedure was a modification of the one used by Brown et al. (1983). Dr. L. H. Reichert of the Albany Medical School, New York, provided both the purified, iodination and reference grades of LH. The detailed protocol and validation of the tracer is described in Appendix 1.

FSH. A modification of the iodogen method employed by Dr. D. J. Bolt (1983 unpublished) was used for iodination FSH. Dr. Bolt, USDA, Beltsville, MD, also provided the purified and reference grades of FSH. Detailed procedures are described in Appendix 1. Validation of the tracer is included in Appendix 1.

Radioimmunoassay Techniques

LH. The technique used for measuring LH was a basic RIA developed by Niswender et al. (1969) as modified by D. J. Bolt (unpublished). LH antiserum (#15 anti-ovine LH) was provided by Dr. Gordon Niswender, Colorado State University. Crossreactivity of the antibody with FSH, thyroid-stimulating hormone, and growth hormone was not significant (Niswender et al., 1969). A reference curve (31-4000 pg) was constructed using triplicate samples of the standard (LER 1956). LH concentration was determined through comparison to the curve. An antibody dilution of 1:40,000 and a tracer activity of 18,000 cpm LH-^{125} I was used to obtain an average total binding capacity of 30.0%. Nonspecific binding was <2.5%. Intra- and inter-assay variation was determined by measure of a standard sample of pooled plasma from steers in each assay. Intraassay variation of these pooled samples was <7.0% and interassay variation was <11.0%. The addition of tracer (18,000 cpm) to stock LH antibody (1:400) provided a maximum binding test, a further measure of tracer quality. Average maximum binding was 60%. Variation among sample replicates was <3.0%. Details of the RIA protocol are included in Appendix 2.

FSH. Measurement of FSH was accomplished using the RIA developed by Bolt and Rollins (1983). FSH reference standard (USDA-bFSH-B1) and FSH antiserum (USDA-5-0122) were supplied by Dr. D. J. Bolt. Crossreactivity of the antibody (FSH-subunit) with bovine luteinizing hormone, bovine growth hormone, and bovine prolactin was 0.09%;

reactivity with bovine thyroid-stimulating hormone was 0.5% (Bolt and Rollins, 1983). A reference curve (1-128 ng) was constructed using triplicate samples of the standard (bFSH-B1). FSH concentration was determined through comparison to the curve. An antibody dilution of 1:10,000 and a tracer activity of 20,000 cpm of FSH-^{125}I was used to obtain an average total binding capacity of 32.0%. Nonspecific binding was <2.0%. Intra- and interassay variation was determined using the method previously described for LH. Intra-assay variation was <9.0% and interassay variation was 12.0%. The average maximum binding capacity was 60%. Variation among sample replicates was <3.0%. Details of the RIA protocol are included in Appendix 2.

Estradiol. Estradiol (E_2) was measured using the RIA protocol developed by Thompson et al. (1978). Reference standard was obtained from Sigma Chemical Co. First antibody (#244 anti-E -6-BSA serum) was provided by Dr. Gordon Niswender. Crossreactivity of the antiserum was measured against: estriol (0.12%), estrone (25.6%), estrone-sulfate (0.88%), progesterone (0.0%), pregnenelone (23.9%), testosterone (0.06%), and androstenedione (3.1%) (personal assay). A reference curve (2.5-250 pg) was constructed using duplicate samples of the standard. Recovery of estradiol during extraction of the plasma was measured by adding 2,000 cpm of [2,4,6,7, ^{3}H]-E_2 (Amersham Co.) to the extraction mixture. Estradiol was measured by comparison to the curve. An antibody dilution of 1:50,000 and a tracer activity of 20,000 cpm E_2-^{3}H was used to obtain an average total binding

capacity of 60%. Details of the protocol are described in Appendix 2.

Testosterone. Testosterone (T) was assayed using a modification of a progesterone RIA protocol developed by Gordon and Sherwood (1978). Reference standard was obtained from Sigma Chemical Co. Testosterone first antibody (#250 anti-T-11-BSA serum) was provided by Dr. Gordon Niswender. Cross reactivity of the antibody with dihydrotestosterone is 69% (Price et al., 1986). A reference curve (10-1000 79) was constructed using duplicate samples of the standard. Testosterone concentration was ascertained by comparison with the curve. An antibody dilution of 1:20,000 and a tracer activity of 20,000 cpm [1,2,6,7 ^{3}H]-T (Amersham Co.) was used to obtain an average total binding capacity of 50%. Nonspecific binding was 2.5%. Validation of all assays was accomplished by recovering known amounts of standards added to plasma of known hormone content. Detailed explanation of the protocol is included in Appendix 2.

All buffers and reagents used in the radioiodinations and radioimmunoassays are described in Appendix 3.

Histological Evaluation

The 5 um sections obtained from testes removed prepuberally were evaluated for Sertoli cell populations using the procedure described by Erickson and Blend (1976): (1) Total testicular volume (V) was calculated using the formula $V=R^2 h$, where R is testicular width and h is testicular length. (2) The volume occupied by seminiferous tubules was estimated by making an evaluation of the tubular:

interstitial tissue ratio for each treatment, in this case, age. The ratio was determined by the mean number of incidents of either tubular or interstitial tissue found in 600 "hits" or points within a cross section of a testis from 10 animals in a treatment (Chalkley, 1943). (3) The volume occupied by 1000 tubular cross sections was estimated by using the formula $V= R^2 h$, where R is tubule diameter (10 cross sections of 10 animals within treatment) and h is section thickness (5 um). (4) The number of cells/tubular cross section was determined by counting only those cells with nucleoli; this prevents overestimating cell number/cross section. (5) Volume occupied by seminiferous tubules was divided by the volume occupied by 1000 cross sections and multiplied by the number of cells per 1000 cross sections to determine the number of Sertoli cells per testis.

Sections obtained from the second testis were evaluated for spermatid production. Three hundred cross sections per testis were examined for the incidence of spermatids and grouped according to either a high (>20%) or low (<15%) number of cross sections containing spermatids. Animals were also grouped according to testicular weight.

Statistical Analysis

In the first study, the effect of age on basal and GnRH stimulated levels of LH, FSH, estradiol, testosterone was tested for significance using a one-way analysis of variance and Student-Newman-Keuls (SNK). Correlations (Pearson's product-moment correlation coefficient) were calculated between Sertoli cell number and hormone levels.

In the second study, the effect of spermatid incidence and testicular weight group on pre- and postpuberal hormone levels were tested for significance using a one-way analysis of variance and orthogonal contrasts. Correlation coefficients between hormone levels and histology were calculated at the 0.05 level of significance.'

The Statistical Analysis System provided the programs for computing means, standard errors, correlations (SAS, 1984a), analysis of variance, SNK, and orthogonal contrasts (SAS, 1984b). The coefficient of variation was used as an index of variation.

CHAPTER IV

RESULTS

Study 1: Prepuberal Hormone Secretion and Interrelationship with Sertoli Cell Number

Table I shows the means and standard errors of both basal and GnRH stimulated levels of LH, FSH, 17β-estradiol and testosterone in the prepuberal bull at varying ages in response to a GnRH challenge. Ages studied were 60, 90, 120, and 180 days of age. LH levels increased significantly through day 120 ($p<0.01$), then decreased significantly through day 180 ($p<0.01$) (Figure 1). Testosterone increased significantly between days 90 and 180 ($p<0.05$). Both FSH and testosterone showed significant differences in stimulated levels at the different ages ($p<0.01$). LH and FSH levels increased in response to GnRH at all ages, but the difference between ages for LH was not significant ($p>0.1$). Testosterone concentration only increased on days 120 and 180 and differences between ages was significant ($p<0.05$). Estradiol level did not differ with age ($p>0.1$) but a trend of high to low existed. Estradiol did not increase as a result of GnRH stimulation (data not shown). The coefficient of variation for basal LH levels was 35%. Coefficients of variation for stimulated FSH levels was 37% and for stimulated testosterone levels was 57%.

As testicular data from the unilateral castration of the 60 day calves was lost, comparison of Sertoli cell number was limited

Table I. Prepuberal Basal and GnRH Stimulated Levels of LH, FSH, E_2 and T at Varying Ages*

	LH(ng/ml)		FSH(ng/ml)		E_2(pg/ml)	T(ng/ml)	
Age(d)	Basal	Stim**	Basal	Stim	Basal	Basal	Stim
60(n=8)	0.3 ± 0.03[c]	--	11.6 ± 1.6	--	4.8 ± 0.5	--	--
90(n=14)	1.1 ± 0.1[b]	27.8 ± 2.1	18.2 ± 3.2	58.9 ± 9.5[a]	5.3 ± 0.9	1.2 ± 0.1[a]	0.99 ± 0.1[a]
120(n=18)	1.8 ± 0.2[a]	22.3 ± 2.9	20.8 ± 2.7	41.8 ± 4.6[b]	3.3 ± 0.3	2.3 ± 0.4[b]	4.3 ± 0.6[b]
180(n=14)	1.0 ± 0.3[b]	29.4 ± 8.8	12.6 ± 0.6	22.7 ±3.3[c]	4.2 ± 0.5	3.4 ± 0.8[b]	9.2 ± 0.7[c]

* Mean ± standard error

** Stimulated

a,b,c Means in the same column with different letters are significantly different ($p<0.05$)

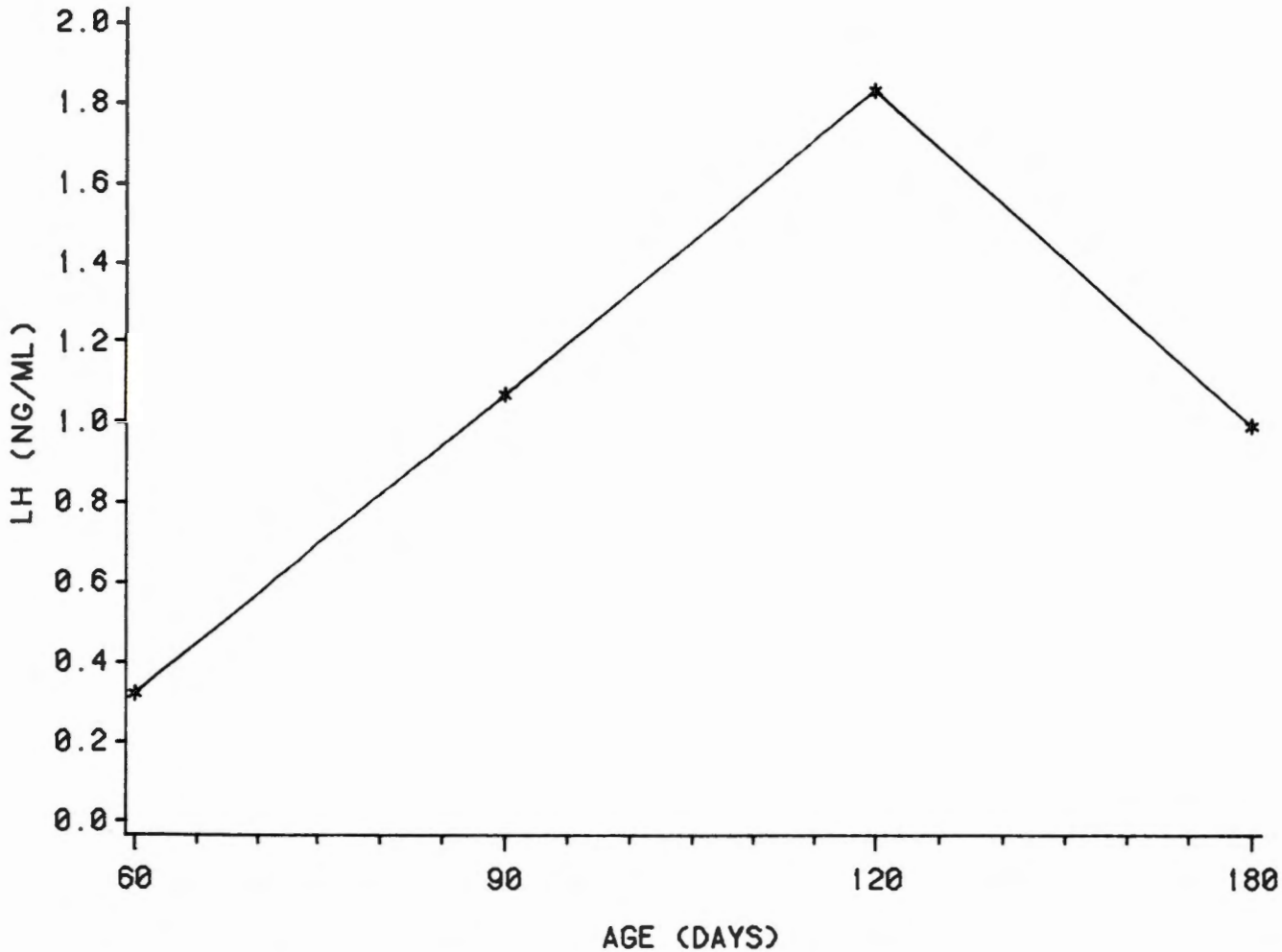

Figure 1. Basal LH Levels for Calves at Varying Ages

to the 90, 120 and 180 day animals. Sertoli cell number did not differ ($p>0.1$) from days 90 through 120, but a twofold increase in cell number was observed between day 120 and day 180 ($p<0.01$) (Table II). Both mean basal and stimulated prepuberal LH levels differed significantly with Sertoli cell number ($p<0.1$). The peak basal LH concentration (1.8 + 0.2 ng/ml) coincided with the lowest number of Sertoli cells (1.435 + 0.14 x 10 cells/testis). The coefficient of variation for both levels was 39%. Neither basal nor stimulated levels of FSH, testosterone, or estradiol showed a significant correlation with Sertoli cell numbers ($p>0.1$). Sertoli cell number was negatively correlated ($r = -.57$) with basal LH levels. Sertoli cell number was correlated with testicular weight at 14 months of age ($r = .26$), correlation ($r = .07$) with stimulated LH levels.

Study 2: Relationship of Prepuberal Hormone Levels and Sertoli Cell Number to Spermatid Production

When the bulls were 14 months of age, the remaining testis was removed and prepared for histological analysis. Bulls were categorized as to testicular content of spermatids. Figure 2 shows the mean basal LH levels for calves with either a high (>20%) or low (<15%) percentage of tubular cross sections with spermatids. Prepuberal (60, 90, 120, or 180 days at unilateral castration) basal LH levels were significantly ($p<0.1$) higher in the group with a low number of cross sections containing spermatids. Basal prepuberal

Table II. Number of Sertoli Cells at Varying Ages*

Age (d)	Sertoli Cell Number ($x10^{16}$)
90	1.5 ± 0.1^{a}
120	1.4 ± 0.1^{a}
180	3.1 ± 0.2^{b}

* Mean ± standard error

a,b Means in the same column with different letters are significantly different ($p<0.01$)

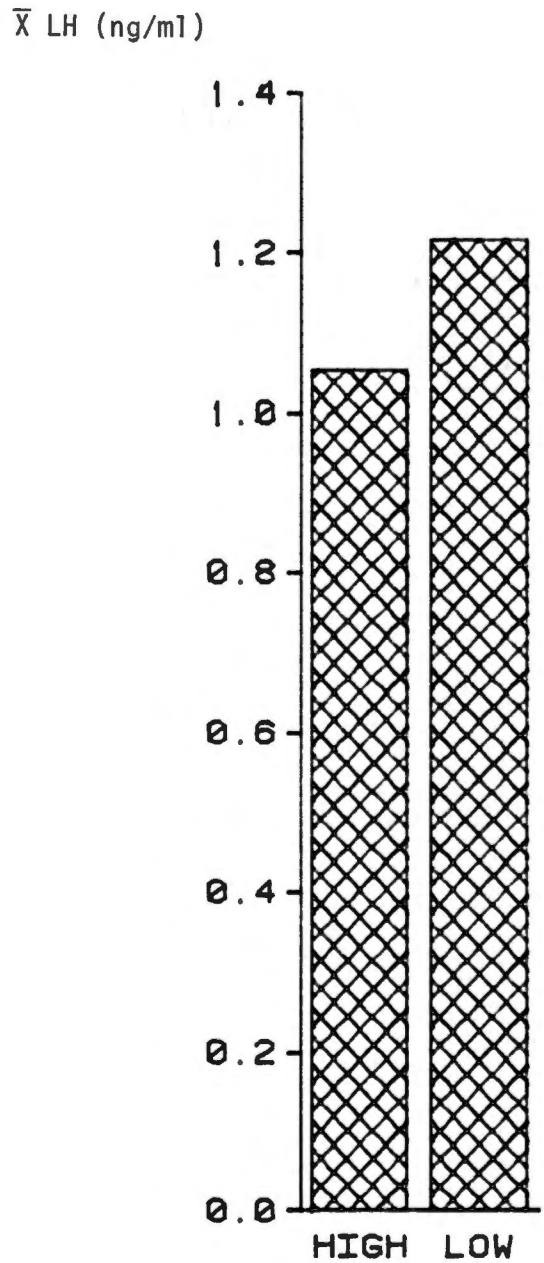

Figure 2. Basal LH Levels for Bulls Grouped by Presence of Spermatids

testosterone levels also differed between the two groups ($p<0.05$) (Figure 3). There were no differences in FSH or estradiol between groups ($p>0.1$) (Table III).

At the second GnRH challenge (14 months), no significant differences existed between groups for either basal or stimulated levels of LH, FSH, estradiol or testosterone ($p>0.1$) (Table IV). Basal LH levels (60, 90, 120, or 180 days) were negatively correlated ($r = -.31$) with the number of spermatids (Figure 2). Basal testosterone levels show a positive correlation ($r = .51$) with spermatid number (Figure 3).

Among animals grouped by a high (>260 gm) or low <235 gm) postpuberal testicular weight, none of the prepuberal hormone levels differed ($p>0.1$) (Table V).

Sertoli cell number did not differ ($p>0.1$) among bulls grouped by absence or presence of spermatids. Number of Sertoli cells did, however, differ among animals grouped by final testicular weight (Figure 4). Bulls with a higher mean second testis weight, had a higher mean number of Sertoli cells per testis ($2.42 + .39$ vs. $1.77 + .28 \times 10^{16}$ cells/testis) ($p<0.1$).

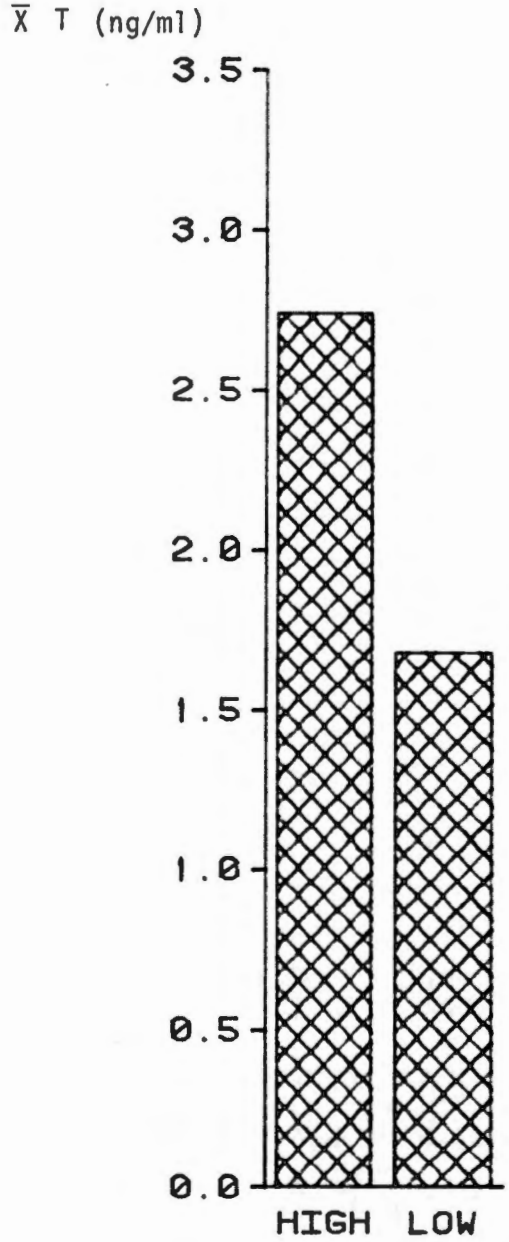

Figure 3. Basal Testosterone Levels for Bulls Grouped by Presence of Spermatids

Table III. Basal Prepuberal Hormone Levels for Bulls Grouped by Presence of Spermatids*

Group	LH(ng/ml)	FSH(ng/ml)	E_2(pg/ml)	T(ng/ml)
High (n=10)	1.0 ± 0.2[a]	14.4 ± 1.0	4.1 ± 0.3	2.3 ± 0.5[a]
Low (n=10)	1.2 ± 0.2[b]	19.2 ± 3.5	4.5 ± 0.4	1.7 ± 0.3[b]

* Mean ± standard error

a,b Means in the same column with different letters are significantly different ($p<0.05$)

Table IV. Postpuberal Basal and GnRH Stimulated Levels of LH, FSH, E_2 and T for Bulls Grouped by Presence of Spermatids*

Group	LH (ng/ml)		FSH (ng/ml)		E_2(pg/ml)	T(ng/ml)	
	Basal	Stim**	Basal	Stim	Basal	Basal	Stim
High (n=10)	1.4 ± 0.3	36.4 ± 2.7	26.7 ± 2.4	51.5 ± 3.8	4.1 ± 0.3	12.4 ± 1.1	16.9 ± 0.9
Low (n=10)	1.2 ± 0.1	40.6 ± 4.4	25.4 ± 3.6	53.3 ± 6.1	4.5 ± 0.4	12.0 ± 1.4	18.3 ± 1.4

* Mean ± standard error

** Stimulated

Table V. Basal Prepuberal Hormone Levels for Bulls Grouped by Testicular Weight*

Testicular Wt. Group	LH(ng/ml)	FSH(ng/ml)	E_2(ng/ml)	T(ng/ml)
High (n=16)	1.0 ± 0.2	15.7 ± 2.0	4.8 ± 0.5	2.6 ± 0.6
Low (n=16)	1.3 ± 0.2	18.3 ± 2.9	3.9 ± 0.4	1.8 ± 0.4

* Mean ± standard error

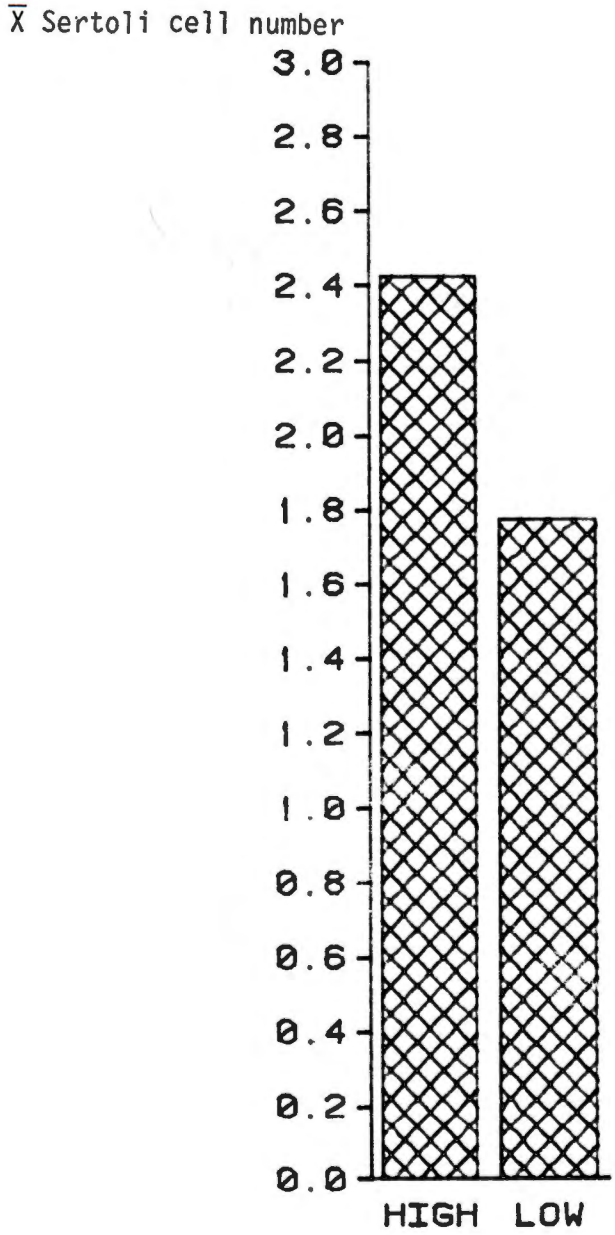

Figure 4. Sertoli Cell Numbers for Bulls Grouped by Testicular Weight

CHAPTER V

DISCUSSION

Study 1: Prepuberal Hormone Secretion and Interrelationship with Sertoli Cell Number

LH and FSH secretion increased in response to the prepuberal administration of GnRH, a phenomenon also observed by Tannen and Convey (1977). This indicates that the anterior pituitary is capable of responding to a GnRH challenge at an early age. Basal levels of LH and FSH are a product of both tonic and infrequent episodic release of the gonadotrophins. Episodic release of LH is rare at 6 weeks, occurs about every 4 hours at 10 weeks, and every 1.8-2.3 hours in 14 and 18 week old bulls (Amann et al., 1986). FSH level remains relatively constant throughout the prepuberal period (Pelletier et al., 1981). Infrequent episodic release of gonadotrophins prior to 10 weeks of age may be due to a low level of hypothalamic activity or to a small number of GnRH receptors on the anterior pituitary (Lacroix and Pelletier, 1979). In this case, LH and FSH are released from the pituitary in response to GnRH; therefore, low level hypothalamic activity must be responsible for the infrequent release of gonadotrophins.

The transient rise in LH level observed between 90 and 120 days in this study concurs with studies conducted by McCarthy et al. (1979) and D'Occhio et al. (1986). The mechanism for this rise, postulated by Amann et al. (1986) and Schanbacher (1984), involves

a reduction in the estradiol-mediated negative feedback on GnRH secretion by the hypothalamus. In their studies, serum estradiol levels decreased markedly between 6 and 10 weeks of age, with a concomitant rise in serum LH. Although comparable ages were studied, in this study such a decrease in estradiol levels was not observed until calves exceeded 90 days of age. Therefore, another mechanism is also involved in this short-term LH release.

Plasma testosterone levels were concomitant with the rise but not the decline in LH secretion (Table I, page 19). Testosterone levels seen in this study (see Table I) agreed with those observed by Bedair and Thibier (1979). They observed that androstenedione levels did, however, parallel LH secretion. Analysis for this and the 5 α-reduced androgens may be necessary in ascertaining the cause of the transient increase in LH release.

Sertoli cell proliferation is important in determining adult testis size. The proliferation of Sertoli cells in the prepuberal animal was the limiting factor in adult testis cell number (Erickson and Blend, 1976; and de Reviers et al., 1980). Testicular size has been positively correlated with reproductive capacity (Gipson et al., 1985; and Lunstra et al., 1978). Sinowatz and Amselgruber (1986) and Curtis and Amann (1981) have observed that the number of Sertoli cells increase between 8 and 28 weeks, with one large increase occurring between 20 and 28 weeks (202 + 81 to 7.927 + 660 x 10^9 cells/testis). Sertoli cell number remained constant after this time. In this study, increase in cell number was observed

between 120 days (12 weeks) and 180 days (25 weeks) (Table II, page 22), which is comparable to the results of Curtis and Amann (1981). However, cell numbers were greater in this study, perhaps due to measurement error. Only plasma LH concentrations were correlated with Sertoli cell number (r = .40). FSH, estradiol, and testosterone were all unrelated to Sertoli cell number. The lack of a relationship between FSH and Sertoli cells is interesting, however, since FSH is responsible for the proliferation of Sertoli cells that occurs in hemicastrates (Schanbacher et al., 1987).

Study 2: Relationship of Prepuberal Hormone Levels and Cell Number to Spermatid Production

Prepuberal LH and testosterone levels were correlated with the extent of testicular development at 14 months. High levels of plasma LH were associated with low numbers of spermatids. This differs from a study conducted by Schinckel et al. (1984), in boars, in which postpuberal testicular traits were positively correlated with prepuberal LH concentration. Prepuberal LH levels were also positively correlated with postpuberal testicular traits in rams (r = .61) (Echternkamp and Lunstra, 1984).

Prepuberal LH levels were positively correlated with postpuberal testosterone level (r = .44). Bulls with a low postpuberal testosterone level had a higher percentage of cross sections containing spermatids (r = -.51). This concurs with a study conducted by Walker et al. (1984) in which bulls immunized against testosterone had greater parenchymal weight and 30% greater daily sperm production

at 12 months and 21% greater daily sperm production at 18 months.

Neither prepuberal FSH nor estradiol were related to spermatid production. Production of estradiol by the Leydig cell or other cells (Waites et al., 1985) may be affecting the amount of estradiol produced by the Sertoli cell, resulting in no apparent relationship between levels of estradiol and number of Sertoli cells. Inhibin is produced by Sertoli cells associated with mature spermatids and causes a reduction in plasma FSH but does not affect LH secretion (Steinberger and Steinberger, 1976). Increased levels of mature spermatids should, therefore, be associated with decreased levels of FSH. This was not the case in this study, as the difference in FSH levels between high and low producers of spermatids was not significant ($p>0.1$) (Table III, page 26).

These results were probably due to the fact that approximately one-half of the animals in the study were still in the prepuberal state at 14 months, as determined by histological examination. The response of hormonal levels to GnRH stimulation was not a useful predictor of spermatid production of testicular weight, as stimulated levels were correlated with neither.

It is concluded that prepuberal LH and testosterone were correlated with degree of testicular development but not with testicular weight, another criterion of testicular development. Studies of bulls of more advanced age are required to determine if prepuberal LH and testosterone levels are potentially useful and indicators of full testicular function.

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APPENDIXES

APPENDIX 1

RADIOIODINATION PROCEDURES

LH: Chloramine-T Technique (Brown et al., 1983)

Protein: LER 1027-2

Materials needed:

0.5 M Phosphate buffer (pH 7.5) PB)

0.05 M Phosphate buffer (pH 7.5) (PB)

Chloramine-T (1 ug/ul in 0.05 M PB)

Sodium Metabisulfite (2 ug/ul in 0.05 M PB)

Bovine Serum Albumin (BSA)

Proceed in a step-wise addition:

1. 2.5 ug LER 1072-2 in 25 ul of 0.5 M PB (pH 7.5)
2. Add 500 uCi ^{125}I (Amersham Co.)
3. Add 15 ul of Chloramine-T
4. Mix gently, for 1 minute
5. Add 60 ul of Sodium Metabisulfite
6. Add 100 ul of eluent buffer (0.05 M PB + 0.1% BSA)
7. Load on a G 25-M Sephadex column for separation
8. Elute with 0.05 M PB + 0.1% BSA

FSH: Iodogen Technique (Bolt and Rollins, 1983)

Protein: USDA bFSH-BP3

Materials needed:

0.5 M PB (pH 7.4)

0.25 M PB (pH 7.4)

0.05 M PB (pH 7.4)

0.5 M Sodium Diphosphate solution

Sodium Metabisulfite (25 ug/ml in 0.25 M PB)

Potassium Iodide (1 mg/ml in 0.5 M Sodium Di)

BSA

Plate the reaction vessel with a solution of iodogen in chloroform at a concentration of 2 ug/50 ul. (Wheaton 1.5 ml flat bottom serum vial, acid washed).

1. 5 mg in 5 ml; iodogen/chloroform
2. 40 ul of #1 on 940 ul of chloroform

Proceed in a step-wise addition:

1. 2.5 ug bFSH-BP3 in 35 ul of 0.5 M PB
2. Add 400 uCi ^{125}I (Amersham Co.)
3. Vortex for 10 minutes (thorough, but gentle)
4. Add 200 ul Sodium Metabisulfite
5. Add 200 ul Potassium Iodide
6. Load on G 25-M Sephadex column for separation
7. Elute with 0.5 M PB + 0.1% BSA

Column Preparation

Gel filtration was used to separate LH and FSH radiolabelled compounds. Sephadex G 25-M gel (Pharmacia Co.) suspended in 0.05 M sodium phosphate buffer was placed into a 1 x 20 cm glass column. Approximately 10 mls of the elution buffer (0.05 M sodium phosphate buffer containing 0.1% BSA) was used to rinse the column just prior to use. Buffer pH for LH was 7.5 and for FSH was 7.4.

Isolation Technique

Upon loading iodinated material onto the column, isolation of the labelled protein is accomplished through fractionating the eluted material in 1 ml fractions, monitoring radioactivity using a portable geiger counter. Radioactivity is eluted from the column in two peaks; the first is the protein and the second is free iodide. The labelled protein is eluted after approximately 12 fractions, while elution of free iodide is complete at about 25 fractions.

TRACER VALIDATION

Trichloroacetic Acid Test

Materials needed:

Diluted radiolabelled protein

Four 1.5 ml plastic microcentrifuge tubes

18% Trichloroacetic acid in 0.09 M PB

1% BSA in 0.5 M PB (pH 7.5)

1. Add 25 ul of the diluted tracer to the microcentrifuge tubes
2. Set aside two tubes as controls
3. To the two remaining tubes, add 100 ul of cold (4°C) 1% BSA, then add 200 ul TCA and vortex rapidly for 10 seconds
4. Centrifuge treated tubes at 3000 rpm for 5 minutes
5. Aspirate supernatant from the treated tubes and dispose of properly

6. Count control and treated tubes on a gamma counter.

Calculation of Labelled Hormone

$$\% \text{ labelled hormone} = \frac{\text{average cpm of treated tubes}}{\text{average cpm of control tubes}}$$

APPENDIX 2

RADIOIMMUNOASSAY TECHNIQUES

LH: Niswender as modified by D. J. Bolt (1981)

Tube	Buffer	Sample	1st Ab	Tracer
TC	--	--	--	100 ul
NSB	700 ul	--	--	100 ul
BO	500 ul	--	200 ul	100 ul
Standards	300 ul	200 ul	200 ul	100 ul
QC	100 ul	400 ul	200 ul	100 ul
Max BO	500 ul	--	200 ul	100 ul
Blank	500 ul	--	200 ul	100 ul
Sample	*100 ul	400 ul	200 ul	100 ul

*Sample volume must equal 500 ul; therefore, if 400 ul of sample is used--add 100 ul buffer, if 300 ul of sample is used, add 200 ul of buffer, etc.

TC = total counts of the total number of counts per minute added to each tube

NSB = nonspecific binding, amount of interference by impurities in the assay tubes, buffer, etc.

BO = total binding (%) capacity of the working dilution of tracer and antibody to be used as the basis for determining hormone concentrations

Standards = known amounts of hormone used to construct the standard reference curve

QC = a standard unknown plasma sample used in assay to control intra- and interassay variation

Max BO = maximum binding (%) capacity of the tracer and antibody to control the quality of the tracer

Sample = unknown amount of hormone that is to be measured

Mix standards = standard ampule contains 32 ng/800 ul

Number tubes: 31, 62, 125, 500, 1000, 2000 and 4000 and place 800 ul of assay buffer in each. Place the 800 ul from the ampule into the tube labelled 4000 (pg). Mix well and perform a serial dilution.

Proceed in a step-wide addition:

1. Add sample or standard to assay tube
2. Add 200 ul of anti-LH antibody (1:40,000)
3. Add 100 ul of LH-^{125}I
4. Vortex
5. Incubate for 48 hrs. at room temperature
6. Add too ul EDTA (Sigma Chemical Co.)
7. Add 400 ul NRS (Miles Scientific, Inc.)
8. Add 200 ul 2nd Ab (Miles Scientific, Inc.)
9. Vortex
10. Add 1 ml PEG (Sigma Chemical Co.)
11. Incubate for 3-16 hrs. at 4°C
12. Centrifuge at 3000 rpm for 20 minutes
13. Decant supernatant and discard
14. Count "pill" on gamma counter

First Ab recipe: $\frac{x}{300} = \frac{\text{total volume (ul)}}{\text{Ab concentration needed}}$ = volume of stock Ab

Total volume = [# of tubes + 25 (for error)] x 200 ul

Ab concentration = 1:10,000

Stock Ab = 1:300

Then take: Total volume - volume of stock needed = amount of buffer needed

Tracer recipe: need 18,000 cpm/tube, therefore,

[# of tubes + 30] x 18,000 = total cpm needed

Total cpm - stock cpm/ul = volume of stock tracer needed

Total volume = [# of tubes + 30] x 100 ul

Total volume - stock volume = amount of assay buffer needed

Second Ab recipe (Goat anti-rabbit IGg): need 1:5 dilution

Total volume = [# of tubes + 25] x 200 ul

Total volume - 5 = volume of stock Ab needed

Total volume - Ab volume = amount of assay buffer needed

FSH: Bolt and Rollins, 1983

Follows LH protocol with exception of the standard curve parameters, Ab dilution, and tracer cpm

Standard: standard aliquot = 1024 ng/800 ul

number tubes: 1, 2, 4, 8, 16, 32, 64, and 128 (ng)

Working Ab dilution = 1:40,000

Tracer: 20,000 cpm/tube

Estradiol: Thompson et al., 1978

Proceed in a step-wise addition:

1. Add 2 ml of sample to extraction tube
2. Add 2000 cpm of ^3H-E_2 for recovery
3. Add 10 ml of Bensene:Methanol (90:10)
4. Vortex vigorously 1-2 minutes
5. Freeze aqueous layer with liquid Nitrogen
6. Decant organic layer onto an LH-20 Sephadex column
7. Collect fraction
8. Evaporate organic layer with Nitrogen gas
9. Add 500 ul of assay buffer
10. Remove 100 ul to count recovery
11. Transfer 200 ul to assay tube (in duplicate)
12. Add 100 ul diluted anti-estradiol antiserum (1:50,000)
13. Add 100 ul tracer (^3H-E_2:20,000 cpm)
14. Vortex gently
15. Incubate for 24 hr. at 4°C
16. Add 500 ul of Dextran coated charcoal to all but TC tube
17. Vortex tubes and incubate for 15 minutes
18. Centrifuge for 10 minutes at 3000 rpm
19. Decant supernatant into scintillation vials (Fisher Co.)
20. Add 10 ml scintillation fluid (Scintiverse II, Fisher)
21. Count vials for 1 minute in a liquid scintillation counter

Testosterone: Gordon and Sherwood, 1978

Proceed in a step-wise addition:

1. Add 200 ul of sample to extraction tube (in duplicate)
2. Add 3 ml of Diethyl Ether

3. Vortex vigorously for 1-2 minutes
4. Freeze the aqueous layer with liquid Nitrogen
5. Decant the organic layer into evaporation tube
6. Evaporate layer with Nitrogen gas
7. Add 200 ul assay buffer
8. Vortex
9. Add 200 ul of anti-testosterone antiserum (1:20,000)
10. Add 200 ul tracer (^{3}H-T:20,000 cpm/tube)
11. Vortex gently
12. Incubate for 24 hr. at 4°C
13. Add 200 ul of Dextran coated charcoal to all but TC tube
14. Vortex and incubate for 15 minutes
15. Centrifuge for 10 minutes at 3000 rpm
16. Decant supernatant into scintillation vials
17. Add 10 ml scintillation fluid
18. Count for 1 minute

APPENDIX 3

BUFFERS AND REAGENTS

Sodium Phosphate Buffers

1. Dibasic F.W. = 268.07 (hydrous or anhydrous)
   to make up 500 ml of .5M Sodium Phosphate (dibasic): add 67 gms to volmetric flask (500 ml), fill about half way with double distilled H_2O, stir until dissolved, full to volume and stir for 10-15 minutes. Filter the solution into storage container through a 0.45 um filter (will need vacuum) and store at room temperature. Buffer is good for 4-6 weeks.

2. Monobasic F.W. = 137.99 (hydrous or anhydrous)
   To make up 500 ml of .5M Sodium Phosphate monobasic: add 34.49 gms. to volumetric flask (500 ml), fill half way with double distilled H_2O, stir until dissolved; fill to volume and stir for 10-15 minutes. Filter the solution into a storage container through a 0.45 um filter (will need vacuum) and store at room temperature. Buffer is good for 4-6 weeks.

Reagents for Gonadotrophin RIA

1. Phosphate Buffer (PB): see phosphate buffer pH chart for mixture (pH) use .5M Sodium Phosphates and dilute with double distilled H_2O to desired molarity of buffer.
   NOTE: May use 1M, 3M, or 10M, NaOH to adjust pH (base) or 5'-sulfosalicylic acid (acid).

2. Basic RIA buffer: mix solution using 0.01M PBS with a pH of 7.5 use a volumetric flask; readjust pH after mixing; add to 0.01M PBS - pH 7.5, add material to flask before adding liquid only fill about half way with PBS and dissolve materials before completing the mixture.

	liter	½ liter
0.1% BSA	1 gm BSA	0.5 gm
0.135 M NaCl	7.89 gm	3.95 gm
0.005 M $NaHCO_3$	.420 gm	0.21 gm
0.01% Thimerosal	100 mg	50 mg

3. EDTA: mix a 0.1 M solution using double distilled water adjust the pH to 7.5 ± .05. Use a volumetric flask.

	liter	½ liter
(Di)NaEDTA	37.22 gm	18.6 gm
0.01% Thimerosal	100 mg	50 mg

4. 2% Normal Rabbit Serum v/v: mix using a .01 M PBS with a pH of 7.5 readjust pH after mixing solution.

	liter	½ liter
Normal Rabbit Serum	20 ml	10 ml
0.01 Thimerosal	100 mg	50 mg

5. Polyethylene glycol (PEG) w/v: mix a 5% solution in 0.01M PBS with a pH of 7.5. Readjust pH after mixing.

	liter	½ liter
PEG	50 gm	25 gm
0.01% Thimerosal	100 mg	50 mg

6. First Ab Buffer: mix using 0.01M PBS with a pH of 7.5; readjust the pH after mixing. Use a volumetric flask

	liter	½ liter
0.135 M NaCl	7.89 gm	3.95 gm
0.005 M $NaHCO_3$	0.420 gm	0.21 gm
0.05 M (Di) NaEDTA	18.6 gm	9.3 gm
1:300 Normal Rabbit Serum	3.3 mls	1.65 mls
0.01 Thimerosal	100 mg	50 mg

Dextran coated charcoal

Mix: 0.625 gm Norit A charcoal (Sigma Chemical Co.)
0.0625 gm Dextran T-70 (Pharmacia Co.)
100 ml Assay buffer

Assay Buffer (Estradiol and Testosterone) pH = 7.0 ± .1

For 2 liters mix:
gm. dosium phosphate (monobasic)
32.7 gm sodium phosphate (dibasic)
2 gm sodium azide
18 gm sodium chloride
2 gm gelatin

VITA

Patricia D. Whaley, daughter of Charles and Kathleen Whaley, was born in Johnson City, Tennessee, on June 16, 1961. She was graduated from Doyle High School, Knoxville, Tennessee, in June 1979. The following September, she entered The University of Tennessee at Knoxville and in June 1983 she received a Bachelor of Science degree in Agriculture, with a concentration in Animal Science.

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