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RELATIONSHIP OF *IN VITRO* GONADOTROPIN BINDING TO BOVINE TESTES AND THE ONSET OF SPERMATOGENESIS¹

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SUMMARY

Concentrations of luteinizing hormone (LH) and follicle stimulating hormone (FSH) were determined in serum of crossbred beef bulls during pubertal development. Serum LH ranged between .5 and 2.0 ng/ml with highest concentrations observed as 12 months of age. Serum FSH varied considerably among individuals but mean concentrations were within the normal range (50 to 100 ng/ml). Neither LH nor FSH increased at the time when testosterone increased or spermatogenesis entered meiosis.

Specific binding of ¹²⁵I-labeled human chorionic gonadotropin (hCG) and ¹²⁵I-labeled FSH to the bovine testis was demonstrated by autoradiographic localization techniques. Binding of ¹²⁵I-hCG and ¹²⁵I-FSH was preferentially located over interstitial tissue and seminiferous epithelial cells, respectively. An abundance of binding sites in newborn and 4-month-old calf testes shows that receptors for LH and FSH are present in the immature bovine testis.

Initiation of spermatogenesis was assessed by estimating the number of testicular sperm by histological and cytometric techniques follow-

ing castration of bull calves at monthly intervals from birth to 12 months. Although spermatogenesis was qualitatively complete at 7 months, spermatogenesis and total daily sperm production were highest in 12-month-old animals. A high correlation ($r = .98$, $P < .01$) was found between sperm per gram testis and total daily sperm production. A parallel increase in serum testosterone of young bulls and testes weight ($r = .95$, $P < .01$) and total daily sperm production ($r = .87$, $P < .05$) suggest that initiation of spermatogenesis could be dependent on increased testosterone.

(Key Words: LH, FSH, Testosterone, Spermatogenesis, Bulls.)

INTRODUCTION

Initiation and maintenance of spermatogenesis in domestic animals, particularly in bulls, are poorly understood. Hafs *et al.* (1968) and Odell *et al.* (1970) concluded that gonadotropic hormones are responsible for initiating the chain of events that result in normal spermatogenesis and sexual maturity in bulls. Three questions concerning this hypothesis are presented: (1) do serum concentrations of gonadotropins change relative to onset of spermatogenesis; (2) is initiation of spermatogenesis dictated by availability of testicular receptors for LH and FSH; and (3) does the testis secrete testosterone in response to gonadotropins during the different stages of pubertal development? Two lines of evidence suggest that pituitary function does not change when spermatogenesis commences in bulls. First, it has been shown that the pituitary of prepubertal bull calves releases LH in response to gonadotropin releasing hormone (GnRH) much like that of mature bulls (Mongkonpunya *et al.*, 1973), and secondly, Karg *et al.* (1976) reported no significant changes in plasma LH concentrations when Brown Swiss bulls were sampled three times weekly from birth to 72 weeks of

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age. Bulls less than 6 months of age do not secrete testosterone in response to naturally occurring episodic LH release (Lacroix *et al.*, 1977) or GnRH-induced LH release (Mongkonpunya *et al.*, 1973). While these studies do not exclude an important role of the hypothalamus during pubertal development, they do indicate that testes function (including testicular sensitivity to gonadotropin) is established at this time. A better understanding of the relationship between hypothalamus, pituitary and testes in bulls is needed before additional studies on sexual development in this species are conducted.

The objectives of this study were to determine (1) the endocrine relationships between the pituitary and testes of bulls during pubertal development and onset of spermatogenesis and (2) the presence of gonadotropin receptors in the bovine testis during this period.

MATERIALS AND METHODS

Thirty-nine crossbred bull calves (3/4 Limousin) born in early spring and maintained under routine management conditions were used in the present study. At monthly intervals from birth to 12 months, blood was collected from three calves by venipuncture after which they were castrated. Serum was harvested and stored at -20 C until all samples could be assayed for concentrations of LH, FSH and testosterone. Serum LH and FSH were assayed by double antibody radioimmunoassay procedures described previously (Schanbacher and Echterkamp, 1978). Minimum sensitivity was .5 ng/ml for LH and 30 ng/ml for FSH. Serum testosterone was assayed in ether extracts without chromatography (Schanbacher, 1976).

After castration, testes were dissected free of extraneous tissue, weighed, and the seminiferous epithelium assessed by histological techniques (Berndtson and Desjardins, 1974).

To determine whether gonadotropins bind to bull testes at different ages, autoradiographic localization studies were performed on testes from 0-, 4- and 12-month-old bulls following *in vitro* binding of ^{125}I -hCG and ^{125}I -FSH (Desjardins *et al.*, 1975). In brief, both gonadotropins were radioiodinated with 1 mCi of ^{125}I and 1 μg of chloramine T as described previously (Schanbacher and Ford, 1977). Specific activity of the radioiodinated preparations was estimated at 50 uCi/ μg for hCG and 50 uCi/ μg for FSH. Pieces of frozen testes were

cut at 6 μm with a cryomicrotome (Lipshaw Manuf. Co., Detroit, MI), and serial sections were transferred to microscope slides. Sections (two per slide) were dried on a warm stage and incubated at room temperature with either ^{125}I -hormone only (~ 2 ng or 100,000 cpm) or ^{125}I -hormone plus 250-fold excess mass (500 ng) of unlabeled hormones in 50 μl of .5 M phosphate buffer (pH 7.5) for 2 hours. Unbound hormone was washed away by three 5-min rinses with ice cold phosphate buffer. The sections were then air dried and processed for autoradiography using Kodak NTB-2 emulsion. After 2 weeks exposure, the slides were developed, stained with hematoxylin and eosin and grain density observed by photography over various testicular compartments. Binding specificity for FSH was demonstrated by the ability of excess unlabeled FSH, but not hCG, to compete for binding to seminiferous tubules; whereas, binding specificity for hCG was demonstrated by the ability of excess unlabeled hCG, but not FSH, to compete for binding to interstitial tissue of the bovine testis.

Testicular sperm were counted in testicular homogenates and parameters of sperm production were calculated by the procedures of Amann (1970). A time divisor of 3.27 days was used to determine total daily sperm production from total testicular sperm.

RESULTS

Serum LH, FSH and Testosterone. LH was detectable in serum of bull calves at birth (.89 ng/ml) and remained low but variable throughout 12 months. A gradual increase after 6 months resulted in maximum concentrations (2.03 ng/ml) at 12 months of age (figure 1).

Serum FSH varied among animals; however, mean concentrations remained relatively constant (between 50 and 100 ng/ml) during this study (figure 1). No change in FSH concentration was detected which could be related to onset of spermatogenesis.

Serum concentrations of testosterone were low in bull calves at birth and remained low (<.6 ng/ml) through 5 months. Testosterone concentrations increased to .74 ng/ml at 6 months and gradually increased thereafter to peak concentrations (10.14 ng/ml) at 11 months of age (figure 1).

Histology of and Gonadotropin Binding to Bovine Testes. Histological sections from the developing calf showed that neonatal testes

Figure 1. Serum testosterone, LH and FSH in bull calves between birth and 12 months of age. Values were determined for three different animals at monthly intervals ($\bar{X} \pm \text{SEM}$, $n=3$).

contained numerous Leydig cells and small inactive seminiferous tubules (figure 2A). Tubules remained quiescent in most calves; only spermatogonia and Sertoli cells were present during the first 90 days of life. Thereafter, mitotic activity was more prevalent and tubule diameters were increased. Primary spermatocytes were seen at 4 months of age and Sertoli cell cytoplasm was extensive (figure 2B). Elongated, step 9 spermatids (stage IX) were observed at 5 and 6 months and mature, step 14 spermatids (stage V) were observed at 6 and 7 months of age. Quantitatively active spermatogenesis and mature tubule diameters were not apparent until 11 months of age. Figure 2C shows a histological section from a representative 12-month-old bull testis.

Autoradiographs prepared after ^{125}I -FSH was applied topically to the surface of frozen sections of bull testes showed the accumulation of silver grains over the seminiferous tubules. Furthermore, accumulation of these silver grains was greatly reduced when adjacent sections were simultaneously exposed to a

Figure 2. Photomicrograph (X 95) of Zenker formal-fixed bovine testes embedded in paraffin, sectioned at $5\text{ }\mu\text{m}$, stained with PAS and counter-stained with hematoxylin. Testes sections are shown for a newborn calf (A), a 4-month-old calf (B) and a 12-month-old bull (C).

250-fold excess mass of unlabeled FSH. Unlabeled hCG was without effect. Labeling intensity of seminiferous tubules from calves at birth (figure 3B) and at 4 months of age (figure 4B) was as prevalent as that observed for seminiferous tubules of 12-month-old bulls (figure 5B).

Autoradiographs developed after topical application of ^{125}I -hCG showed that silver grains were concentrated over the interstitial tissue of the testis from 12-month-old bulls (figure 5D). Density of these silver grains was greatly reduced when testicular sections were simultaneously exposed to a 250-fold excess mass of unlabeled hCG (figure 3C, 4C and 5C). On the other hand, unlabeled FSH did not affect the labeling intensity of ^{125}I -hCG to interstitial tissue. Although identification of interstitial tissue in frozen sections of testes from newborn calves was difficult, close examination showed that the density of silver grains was greatest over interstitial tissue and least

Figure 3. Photomicrograph (X 95) of frozen sections of newborn calf testis processed for topical autoradiographic localization of ^{125}I -FSH (A and B) and ^{125}I -hCG (C and D). Sections A and C were co-incubated with 250-fold excess mass of unlabeled hormone; whereas, B and D were incubated with labeled hormone only. Black arrows point to seminiferous tubules and white arrows to interstitial cells.

Figure 4. Photomicrograph (X 95) of frozen sections of a 4-month-old calf testis processed for topical autoradiographic localization of ^{125}I -FSH (A and B) and ^{125}I -hCG (C and D). Sections A and C were co-incubated with 250-fold excess mass of unlabeled hormone; whereas, B and D were incubated with labeled hormone only. Black arrows point to seminiferous tubules and white arrows to interstitial cells.

over the supporting elements of seminiferous tubules and blood vessels (figure 3D). Testes from 4-month-old calves bound ^{125}I -hCG to the interstitial compartment (figure 4D) as did those from newborn and 12-month-old animals.

Spermatogenesis. As indicated by the presence of sperm in testicular homogenates, Limousin crossbred calves do not produce sperm until they are at least 6 months of age (table 1). All calves 7 months and older produced sperm and spermatogenic efficiency was highest in 12-month-old bulls (100×10^6 sperm per gram testis). Total daily sperm production was not detected before 6 months but increased to 8.58×10^9 sperm per day at 12 months of age. A highly significant correlation existed between sperm per gram testis and total daily sperm production ($r = .98$, $P < .01$).

Although serum concentrations of LH and FSH may be the primary effector of spermatogenesis, serum concentrations of testosterone

correlate well with paired testes weights ($r = .95$, $P < .01$), sperm per gram testis ($r = .85$, $P < .05$) and total daily sperm production ($r = .87$, $P < .05$). These correlations were calculated from the mean values for animals 6 through 12 months of age ($n=7$).

Discussion

The data presented in this study characterize the relationships between the gonadotropins, LH and FSH, and testicular function during pubertal development; namely, testosterone secretion and spermatogenesis. From these results, it is suggested that increased serum gonadotropins are not necessary for increased testosterone secretion or the initiation of spermatogenesis; however, this possibility cannot be ruled out because the animals in this study were not sampled at frequent intervals throughout the prepubertal period. Karg *et al.* (1976) collected blood three times weekly

Figure 5. Photomicrograph (X 95) of frozen sections of a 12-month-old calf testis processed for topical autoradiographic localization of ^{125}I -FSH (A and B) and ^{125}I -hCG (C and D). Sections A and C were co-incubated with 250-fold excess mass of unlabeled hormone; whereas B and D were incubated with labeled hormone only. Black arrows point to seminiferous tubules and white arrows to interstitial cells.

during a comparable period and was likewise unable to detect appreciable changes in serum LH. Reports by other investigators have shown serum LH to increase temporarily between the first and fifth month of life (Lacroix *et al.*, 1977) or to increase gradually from birth until adulthood (Gombe *et al.*, 1973; Mori *et al.*, 1974).

Serum concentrations of FSH were similar to those determined for mature bulls at all ages studied but varied markedly between individuals. Similar variation in serum FSH has been reported for individual bulls (Schanbacher and Echternkamp, 1978) and for calves of different ages (Karg *et al.*, 1976).

Although the roles of LH and FSH in initiation and maintenance of spermatogenesis are not precisely known, considerable information is available regarding their site of action within the testis and the biochemical events that occur in the testis after gonadotropic stimulation. Receptors for both LH and FSH have been localized within the testes of laboratory rats. Autoradiographic techniques have been used to show receptor binding of ^{125}I -hCG to interstitial cells (DalLago *et al.*, 1975; Desjardins *et al.*, 1975; Frowein and Engel, 1975) and ^{125}I -FSH to cells within the seminiferous tubules (Means, 1973; Desjardins *et al.*, 1975). Using *in vitro* binding techniques,

TABLE 1. ANIMAL WEIGHTS AND TESTICULAR FUNCTION IN CROSSBRED BULL CALVES FROM BIRTH TO 12 MONTHS OF AGE^a

Age (months)	Animal weight (kg)	Paired testes weight (g)	Sperm per gram testis (10^6) ^b	Total daily sperm production (10^9) ^b
0	...	5.6 ± .2	N.D.	N.D.
1	...	10.1 ± 2.7	N.D.	N.D.
2	...	14.6 ± .4	N.D.	N.D.
3	...	20.3 ± 1.5	N.D.	N.D.
4	...	31.7 ± 2.8	N.D.	N.D.
5	...	48.7 ± 1.3	N.D.	N.D.
6	176 ± 9	50.7 ± 6.0	3.1 ± .2	.04 ± .003
7	181 ± 6	91.6 ± 11.4	12.5 ± 2.1	.32 ± .09
8	245 ± 4	159.2 ± 49.3	22.4 ± 6.6	1.13 ± .56
9	274 ± 9	165.8 ± 7.2	35.3 ± .4	1.37 ± .17
10	337 ± 12	324.8 ± 51.0	65.9 ± 11.3	5.62 ± 2.39
11	385 ± 17	342.6 ± 29.2	71.2 ± 2.3	6.75 ± .64
12	408 ± 14	309.4 ± 29.9	100.3 ± 1.6	8.58 ± .91

^aValues are means ± SEM for three animals.

^bN.D. = non detectable.

Desjardins *et al.* (1975) showed the amount of ^{125}I -hCG binding per 100 μg of DNA to be low in 5-day-old rats but to increase dramatically between 25 and 55 days of age. Binding capacity also increases when expressed on a per mg protein basis (Frowein and Engel, 1975). On the other hand, *in vitro* binding of ^{125}I -FSH was relatively high in young rats and was at mature levels by 15 days of age. Means (1973) reported twice as much radioactive FSH bound per 100 mg of testicular tissue in 16-day-old rats as in 60-day-old rats. Similar analyses were not conducted in bull calves in the present study; nonetheless, it is clear that gonadotropin receptors exist in the prepubertal bull testes and that these receptors are able to bind radioiodinated preparations of hCG and FSH. Properties of LH/hCG and FSH receptors in membrane preparations from rat and bull testes have been published elsewhere (Bhalla and Reichert, 1974; Means and Huckins, 1974; Cheng, 1975; Mendelson *et al.*, 1975).

The density of silver grains over specific compartments of the bovine testis and the ability of unlabeled hormones to compete for binding sites indicate that an abundance of specialized gonadotropin receptors exists in the immature calf testis. Considering the levels of immunologically active LH and FSH in serum of these calves and the abundance of gonadotropin receptors, it would appear that gonadotropin binding at the testicular level is not the rate-limiting step for the initiation of testicular function in young bulls. The prepubertal bull does not secrete adult levels of testosterone and GnRH-induced LH release results in a relatively small increase in serum testosterone (Mongkonpunya *et al.*, 1973). It would seem fair to conclude that pubertal development in bulls is dictated at the testicular level, and that the mere presence of gonadotropin receptors in the testis is not sufficient to elicit the responses necessary for testosterone secretion and spermatogenesis. Comprehensive reviews on the interaction of LH (Catt and Dufau, 1973) and FSH (Means, 1973) with testicular receptors have been compiled in an effort to understand the dependency of the testis on gonadotropins.

The increase in paired testes weight of crossbred Limousin bull calves was similar to that reported in developing Holstein bulls by MacMillan and Hafs (1968). Testes weight at birth in Holstein bulls was approximately 5 g and increased to approximately 407 g by 12 months of age. The relative high correlation

between testes weight and animal weight in the present study suggests an important relationship between the sexual development and physical development of young bulls.

Sperm were not present in testicular tissue of crossbred calves until 7 months of age. MacMillan and Hafs (1968) found testicular sperm in one Holstein bull at 5 months, but only after 8 months of age did all bulls produce sperm. Total daily sperm production by the 12-month-old bulls in this study was similar to that reported by Amann (1970) for beef and dairy bulls. The parallel increase in total daily sperm production and serum testosterone and the positive correlation between them suggest that testosterone could be the primary hormone that affects the rate of spermatogenesis. This finding is not surprising when the well documented dependency of spermatogenesis on testosterone (Steinberger, 1971; Desjardins *et al.*, 1973) is considered. Numerous investigators (Rawlings *et al.*, 1972; Cotta *et al.*, 1975; Karg *et al.*, 1976; Secchiari *et al.*, 1976; Lacroix *et al.*, 1977) have reported the pubertal increase in serum testosterone of bulls; however, few authors (Swanson *et al.*, 1971; Lunstra *et al.*, 1978) have associated this endocrine change with sperm production.

From these results, it can be concluded that bovine spermatogenesis is associated with increased serum concentrations of testosterone. Furthermore, testosterone secretion and initiation of spermatogenesis do not appear to be dependent on increased serum gonadotropins but rather on a change in testicular sensitivity to the gonadotropins. The specific binding of LH/hCG and FSH to receptors within the interstitial tissue and seminiferous tubules respectively confirms the existence of gonadotropin testicular interactions in bulls; however, the separate effects of these hormones on the initiation and maintenance of spermatogenesis are yet to be determined.

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