Effects of treatment with LH releasing hormone before the early increase in LH secretion on endocrine and reproductive development in bull calves

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Between 6 and 20 weeks of age an early increase in LH secretion has been reported in Hereford bull calves. Delaying this early increase in LH secretion delays testicular development. This study was designed to determine whether a premature increase in LH secretion during the early postnatal period enhances testicular development. Ten age- and body weight-matched Hereford bull calves were divided into two groups. One group ($n = 5$) received 200 ng LH releasing hormone (LHRH) i.v. every 2 h for 14 days, between 4 and 6 weeks of age. On the basis of blood samples taken every 15 min for 10 h, mean serum LH and testosterone concentrations and LH pulse frequency were increased by LHRH treatment ($P < 0.05$). Serum concentrations of FSH were not significantly influenced by treatment ($P > 0.05$). In treated animals at 24 weeks of age, mean serum testosterone concentrations and LH pulse amplitude were increased ($P < 0.05$). The concentrations of spermatozoa in electroejaculates collected at 52 weeks of age were greater in LHRH-treated compared with control calves. Testicular growth was enhanced by LHRH treatment and histological evaluation of the testis at 54 weeks of age showed increased spermatogenesis and also larger numbers of Sertoli cells per tubule cross-section as a result of LHRH treatment. We conclude that treatment with LHRH before the early increase in LH secretion altered testicular development and suggest that the early increase in LH secretion in bull calves may be critical for initiating and regulating the progression of reproductive maturation.

Introduction

In cattle (Amann, 1983; Evans *et al.*, 1995) sexual maturation can be divided into three phases: the infantile, prepubertal and pubertal. The infantile period is characterized by low episodic discharge of LH (Rawlings *et al.*, 1978; Amann *et al.*, 1986; Rodriguez and Wise, 1989); during this period the frequency of LHRH release is low (Rodriguez and Wise, 1989). From 6 to 20 weeks of age, an early transient increase in LH secretion has been reported in what is called the prepubertal phase (Karg *et al.*, 1976; Amann *et al.*, 1986; Evans *et al.*, 1995). The pituitary is sensitive to exogenous LHRH stimulation during the infantile period in Holstein bull calves (Rodriguez and Wise, 1991). However, LHRH pulse frequency increases two-fold during transition from the infantile to the prepubertal period; pituitary sensitivity to LHRH also increases at this time (Rodriguez and Wise, 1989). During the infantile and prepubertal periods, serum FSH concentrations are also high (Evans *et al.*, 1995). At the time of the early increase in gonadotrophin secretion, serum concentrations of testosterone

are slightly increased (Rawlings and Cook, 1986; Rawlings and Evans, 1995). The pubertal period is the period of active reproductive development from 20 weeks of age until puberty (defined as 50×10^6 sperm ml^{-1} ejaculate with a minimum of 10% motility; Wolf *et al.*, 1965). Puberty in beef bull calves occurs at about 1 year of age, at a scrotal circumference of about 28 cm (Wolf *et al.*, 1965; Lunstra *et al.*, 1978).

The functional significance of the early increase in LH secretion is not fully known. The early increase occurs as spermatogenesis is initiated, just before tubule lumination and as the Sertoli cells and Leydig cells are differentiated (Curtis and Amann, 1981). The results of previous experiments led to the suggestion that the early increase in LH secretion is important for testicular and early sexual development in cattle (Evans *et al.*, 1995). Suppression of the early increase in LH secretion leads to a delay in testicular development (Chandolia *et al.*, 1997). The magnitude of the early increase in LH secretion was shown to be negatively correlated with age at puberty in Hereford bull calves (Evans *et al.*, 1995). It has also been suggested that an early increase of LH secretion is important for normal fertility in rats (Pinilla *et al.*, 1994) and that its inhibition decreases testicular growth and

Received 17 March 1997.

spermatogenesis in this species (van den Dungen *et al.*, 1989, 1990). This study was designed to evaluate the significance of the early increase in LH secretion for sexual maturation in bull calves. We hypothesized that increasing LHRH pulse frequency before the normal increase in LHRH/LH secretion would enhance or advance both endocrine and testicular development. Therefore, this study examined the effects of pulsed LHRH administration during the infantile period (4–6 weeks of age) on testicular and reproductive endocrine development in Hereford bull calves.

Materials and Methods

Animals and treatment

Ten, spring born, age and body weight matched (± 5 days) Hereford bull calves were weaned from their dams at 3 weeks of age and kept indoors until 14 weeks of age. The calves were fed up to 6 l day⁻¹ of a milk replacer (40% crude protein, 20% fat; Prairie Micro-Tech, Alpine Agro, Regina, SK) and a solid starter ration (complete calf ration, CO-OP[®] Saskatoon, SK) and hay were fed *ad libitum*. As soon as the calves were consuming 0.5 kg of starter ration per calf per day the supply of milk replacer was gradually reduced over a period of 40 days. From 14 weeks of age the calves were moved to pasture and the starter ration was gradually replaced by a standard beef grower ration *ad libitum* (Rawlings and Evans, 1995). From 25 weeks of age the calves were kept in a dirt paddock on the beef grower ration.

Two days before commencement of treatments, calves were cannulated in one jugular vein as described by Evans *et al.* (1993). From 4 to 6 weeks of age, 200 ng LHRH (Sigma Chemical Co, St. Louis, MO), dissolved in sterile saline, was administered by a jugular catheter every 2 h to each of five calves; control calves ($n = 5$) received saline. The dose was based on a previous study by Rodriguez and Wise (1991) and the treatment was designed to give LH pulses of an amplitude similar to those of control calves but at a frequency as great or greater than the expected peak LH pulse frequency seen at the height of the early increase in LH secretion in normal Hereford bull calves (Evans *et al.*, 1995).

Blood sampling, and body and testicular growth

At 4 (first day of treatment), 6 (last day of treatment), 12, 18 and 24 weeks of age, blood samples were taken every 15 min for 10 h. Blood samples were collected every day during the treatment period at 08:00 h. Body weight was measured and blood samples were taken every 2 weeks from birth to 52 weeks of age. Scrotal circumference was measured every 2 weeks from 20 weeks of age. At 52 weeks of age semen samples were collected from animals by electroejaculation and concentrations of spermatozoa were estimated by haemocytometer.

Histology and flow cytometry of testicular tissue

At 54 weeks of age the animals were castrated and the testes were weighed. Pieces of the left testes were fixed in Bouin's

solution for histological evaluation. Right testes were immediately frozen at -20°C for flow cytometry. Tissue for histology was embedded in paraffin wax and 5 μm sections were stained with haematoxylin and eosin. In a cross-section of the testis from each animal, the diameters of 15 round seminiferous tubules at stage VI of spermatogenesis were measured and a mean was calculated. Germinal and Sertoli cells (elongated and round spermatids, pachytene spermatocytes, A and B spermatogonia) were counted in stage VI of spermatogenesis as described by Curtis and Amann (1981). This stage corresponds to stage VII of spermatogenesis in rats and is considered the most sensitive to hormonal changes (Russell and Clermont, 1977). The counts were corrected for section thickness and cell-nuclear diameter (Abercrombie, 1946). Testicular germ cells were also sorted on the basis of DNA content by flow cytometry as reported by Aravindan *et al.* (1990) and Medhamurthy *et al.* (1993). In brief, approximately 150 mg testicular tissue was minced in 3 ml Dulbecco's phosphate-buffered saline (D-PBS), vortexed and washed in D-PBS (pH 7.4) and treated with 0.5% (w/v) pepsin (Serafein Biochemica, Heidelberg) at pH 2.0 for 1 h at 37°C . After centrifugation at 800 g for 10 min, the cells were resuspended for 20 min at 4°C in a staining solution containing 25 μg ethidium bromide ml⁻¹ (Sigma Chemical Co.), 40 μg ribonuclease A ml⁻¹ (Sigma) and 0.3% (w/v) nonidet P-40 (Sigma) in D-PBS. After adding 0.5 ml FACSflow solution (Becton Dickinson, Mississauga, Ontario), about 10 000 cells were analysed using a FACSflow cytometer (Becton Dickinson). The cells were sorted on the basis of DNA content and are expressed as 'C' values (Medhamurthy *et al.*, 1993): HC, elongated spermatids; 1C, round spermatids; 2C, cells with a diploid DNA content (for example, G1-spermatogonia, G1-Leydig cells, G1-Sertoli cells); and 4C, cells during mitotic or primary meiotic division, after DNA synthesis but before cell division (for example, leptotene, zygotene, pachytene and diplotene primary spermatocytes; spermatogonial and non-germinal cells during the G2 phase of the cell cycle; Toppari *et al.*, 1989; Chandolia *et al.*, 1991). The fact that HC cells bound relatively less ethidium bromide than did 1C cells, in spite of the fact that they possessed an equal amount of DNA, permitted differential quantitation of HC and 1C cells (Medhamurthy *et al.*, 1993).

Radioimmunoassays

All serum samples were analysed for concentrations of LH and FSH using double-antibody radioimmunoassays (Rawlings and Evans, 1995). The first antibody used for the FSH assay was NIDDK-anti-oFSH-1, and FSH concentrations are expressed in terms of USDA-bFSH-11. The sensitivity of the assay, assessed as the lowest concentration of FSH capable of significantly displacing labelled FSH from the antibody (Student's t test), was 0.125 ng ml⁻¹. Intra- and interassay coefficients of variation (CV) were 8.4 and 12.7%, or 7.0 and 13.2%, respectively, for sera with mean FSH concentrations of 1.51 or 2.99 ng ml⁻¹. The CVs were compiled by replicating reference sera in each assay. Concentrations of LH are expressed in terms of NIDDK-bLH4. The sensitivity of the assay was 0.06 ng ml⁻¹. Intra- and interassay CVs were 10.6

Fig. 1. Serum LH concentrations in representative calves treated with LH-releasing hormone (a, b) and controls (c, d) at 4 (a, c) and 6 (b, d) weeks of age in Hereford bull calves. Blood samples were taken every 15 min for 10 h. Arrows indicate time of injections. *Pulses determined by pulsar analysis.

and 8.9%, or 7.9 and 14.2%, respectively, for reference sera with mean LH concentrations of 0.24 or 0.74 ng ml⁻¹.

Serum concentrations of testosterone were measured by radioimmunoassay (Rawlings and Evans, 1995) in samples taken every other week and every hour from the periods of intensive bleeding. The sensitivity of the assay was 0.04 ng ml⁻¹. The intra- and interassay CVs were 9.8 and 11.2, or 12.8 and 14.2%, respectively, for reference sera with mean concentrations of testosterone of 4.03 or 7.63 nmol l⁻¹.

Statistical analysis

The PC-pulsar program (J. Gitzen and V. Ramirez, University of Illinois, IL) was used to analyse the episodic patterns of serum concentrations of LH and FSH in blood samples collected every 15 min for 10 h. From this analysis, basal and mean serum concentrations, pulse amplitude and frequency were recorded. Pulses were identified using standard deviation criteria of height (G values) and duration (Merriam and Wachter, 1982). Basal concentrations were determined by subtraction of the pulses from the 10 h gonadotrophin profile.

Data for scrotal circumference, body weight, basal and mean serum concentrations of LH and FSH, pulse amplitude of LH and FSH, and frequency and mean serum concentrations of testosterone were analysed for the effect of treatment, age and interactions using univariate and multivariate repeated measures analysis of variance ($P < 0.05$; GLM procedures in the Statistical Analysis System: SAS/STAT[®] version 6; Cary, NC, USA). Duncan's test was used to compare individual means ($P < 0.05$). The concentrations of spermatozoa in semen, testis mass at castration, tubule diameter and corrected numbers of testicular cells from histological sections and number of cells sorted by flow cytometry in control and treated calves were compared by one-way analysis of variance ($P < 0.05$). Data are presented as means $\pm$ sem.

Results

Response of gonadotrophins and testosterone to treatment with LH-releasing hormone

In blood samples taken daily during the period of LHRH treatment, mean serum concentrations of LH were higher in the

Fig. 2. Mean (a) and basal (c) serum concentrations of LH, and LH pulse amplitude (b) and frequency (d) (means $\pm$ SEM) in blood samples collected every 15 min for 10 h at 4, 6, 12, 18 and 24 weeks of age in Hereford bull calves treated with LH-releasing hormone (■) or vehicle (□) every 2 h from 4 to 6 weeks of age. Values with different superscripts show differences between ages within groups ($P < 0.05$); *significant differences ($P < 0.05$) between treatment and control at a particular age; †approached significance ($P < 0.06$).

calves treated with LHRH (overall mean: 0.25 ± 0.03 ng ml⁻¹) compared with control calves (overall mean: 0.17 ± 0.02 ng ml⁻¹; $P < 0.05$). On the basis of samples taken every 15 min for 10 h, on the first and last days of treatment with LHRH (4 and 6 weeks of age), LH pulse frequency was increased but the amplitude was lower compared with that of control calves (Figs 1 and 2; $P < 0.05$). At 6 weeks of age the apparent increase in mean serum LH concentrations in treated compared with control calves approached significance ($P < 0.06$; Fig. 2). At 24 weeks of age, LH pulse amplitude was significantly higher in calves treated with LHRH compared with controls ($P < 0.05$). Basal serum concentrations of LH were highest at 6 weeks of age in treated calves compared with other ages ($P < 0.05$) but did not differ significantly from those of control calves ($P > 0.05$; Fig. 2). LH pulse amplitude declined from 4 to 24 weeks of age in control calves ($P < 0.05$). LH pulse frequency in control calves increased to a peak at 12–18 weeks of age and then declined ($P < 0.05$; Fig. 2). In blood samples

taken every other week, from 2 to 52 weeks of age, there were no differences in mean serum LH concentrations between treatment groups ($P > 0.05$). Concentrations of LH were variable, with high values in young calves and an increase as calves approached maturity ($P < 0.05$; Fig. 3).

On the basis of blood samples taken every 15 min for 10 h, there was no consistent response of parameters of FSH secretion to exogenous administration of LHRH at 4 or 6 weeks of age (Figs 4 and 5), although FSH pulse amplitude was decreased at 6 weeks of age in calves given LHRH ($P < 0.05$). Some pulses of FSH were apparent after LHRH injections but pulsatile patterns of FSH were difficult to interpret (Fig. 4). In calves treated with LHRH, mean and basal serum concentrations of FSH increased at 6 weeks, decreased to a nadir at 12 weeks of age and then gradually increased until 24 weeks of age ($P < 0.05$; Fig. 5). Although a similar significant trend was not seen for control calves ($P < 0.05$), mean and basal serum concentrations of FSH did not differ between

Fig. 3. Mean serum concentrations of (a) LH, (b) FSH and (c) testosterone in blood samples taken every other week from 2 to 52 weeks of age, in Hereford bull calves treated with LH-releasing hormone (●) or vehicle (▽) every 2 h from 4 to 6 weeks of age.

control and treated calves ($P < 0.05$) except at 18 weeks of age when values were lower in treated calves ($P > 0.05$). FSH pulse frequency, in both groups of calves, was highest ($P < 0.05$) at 4 weeks of age, was decreased by 6 weeks of age and was lowest at 24 weeks of age ($P < 0.05$). At 18 weeks of age, FSH pulse amplitude was higher in control calves compared with calves treated with LHRH (Fig. 5; $P < 0.05$). In blood samples taken every 2 weeks from birth to 52 weeks of age, serum concentrations of FSH did not differ between groups ($P > 0.05$). Serum concentrations of FSH did change with time ($P < 0.05$) but no consistent trend was noted with maturity of the bull calves (Fig. 3).

On the basis of blood samples taken every hour for 10 h, mean serum testosterone concentrations were higher in calves treated with LHRH compared with those of controls at 4, 6 and 24 weeks of age but peak concentrations of testosterone were greater in calves treated with LHRH at 6 and 24 weeks of

age compared with at 4 weeks of age (Fig. 6; $P < 0.05$). In control calves, mean serum testosterone concentrations rose progressively from 4 to 24 weeks of age ($P < 0.05$; Fig. 6). In blood samples taken every 2 weeks from birth to 52 weeks of age, serum concentrations of testosterone did not differ between groups ($P > 0.05$). Serum concentrations of testosterone increased from 32 weeks of age to maturity ($P < 0.05$; Fig. 3).

Body and testicular growth and sperm concentration

Body weight did not differ between groups of calves but scrotal circumference appeared higher at all ages in calves treated with LHRH, with significant differences ($P < 0.05$) at 52 weeks (29.70 ± 0.58 versus 28.00 ± 0.30 cm; treated versus control) and 54 weeks (30.58 ± 0.72 versus 28.60 ± 0.40 cm)

Fig. 4. Serum FSH concentrations in representative calves treated with LH-releasing hormone (a, b) and controls (c, d) at 4 (a, c) and 6 (b, d) weeks of age in Hereford bull calves. Blood samples were taken every 15 min for 10 h. Arrows indicate time of injections. *Pulses determined by pulsar analysis.

of age. The masses of right (244 ± 24.2 versus 227 ± 14.2 g; treated versus control) and left (242 ± 23.3 versus 228.2 ± 8.8 g) testes at castration (54 weeks of age) appeared to be higher in treated calves compared with controls, but the apparent difference was not significant ($P > 0.05$). The concentration of spermatozoa in electroejaculates at 52 weeks of age was significantly higher in calves treated with LHRH ($132.5 \pm 24.70 \times 10^6 \text{ ml}^{-1}$) compared with controls ($63.0 \pm 20.09 \times 10^6 \text{ ml}^{-1}$; $P < 0.05$).

Histology and flow cytometric sorting of cells

In calves treated with LHRH, diameters of seminiferous tubules at stage VI of spermatogenesis were greater ($245.8 \pm 7.68 \mu\text{m}$) compared with those of controls ($224.0 \pm 4.29 \mu\text{m}$; $P < 0.05$). In stage VI of spermatogenesis, numbers of elongated and round spermatids, pachytene spermatocytes, A and B spermatogonia and Sertoli cells were significantly higher in treated calves compared with controls ($P < 0.05$; Table 1). Flow cytometric sorting of cells revealed a greater percentage of elongated spermatids (HC cells) in treated calves compared with controls ($P < 0.05$; Table 2).

Discussion

In previous studies, the early increase in LH secretion in Hereford bull calves started after 6 weeks of age and was complete by 20 weeks of age (Evans *et al.*, 1995; Rawlings and Evans, 1995). This increase in LH secretion reflected an increase in frequency of LHRH pulses (Rodriguez and Wise, 1989) and also an increase in pituitary responsiveness to LHRH (Rodriguez and Wise, 1991). In the present study, based on changes in LH pulse frequency observed in blood samples taken every 15 min for 10 h, the early increase in LH secretion was from 6 to 24 weeks of age in control animals. However, mean serum concentrations of LH did not change significantly to mirror the change in LH pulse frequency, probably because LH pulse amplitude decreased as LH pulse frequency increased, reflecting the low pituitary LH stores seen in young calves (Amann *et al.*, 1986). Owing to these changes and the fact that LH pulses occur every 4–5 h in young bull calves, mean concentrations of LH in blood samples taken every other week were quite variable; however, an overall reflection of the developmental pattern of LH secretion (the early increase and the prepubertal increase) was apparent in blood samples taken every other week from birth to puberty (Fig. 3; Evans *et al.*,

Fig. 5. Mean (a) and basal (c) serum concentrations of FSH, and FSH pulse amplitude (b) and frequency (d) (means $\pm$ SEM) in blood samples collected every 15 min for 10 h at 4, 6, 12, 18 and 24 weeks of age in Hereford bull calves treated with LH-releasing hormone (■) or vehicle (□) every 2 h from 4 to 6 weeks of age. Values with different superscripts show differences between ages within groups ($P < 0.05$); *significant differences ($P < 0.05$) between treatment and control at a particular age.

1995). In young postnatal bull calves, serum concentrations of FSH tend to be high and then decrease but this trend is never as obvious as for LH (Evans *et al.*, 1995; Rawlings and Evans, 1995). In the present study, blood samples taken every 15 min for 10 h indicated that FSH pulse frequencies increased from 4 to 24 weeks of age. However, no particular trend in mean serum concentrations of FSH was observed in blood samples taken every other week.

In the present study, the goal of increasing LH pulse frequency over the period before the early increase in LH secretion (4–6 weeks of age) to at least the peak values seen during the normal early increase in LH secretion was achieved. However, this did not appear to perturb the occurrence of the early increase in LH secretion. The amplitude of induced LH pulses was lower than for endogenous pulses, again, probably reflecting the low pituitary LH stores in young postnatal bull calves (Amann *et al.*, 1986). The response of FSH secretion to administration of LHRH was unclear. Although FSH secretion was determined to be pulsatile by pulsar analysis in the young

calves studied and many injections of LHRH appeared to induce small pulses in FSH secretion, pulse delivery of LHRH was not reflected by a measurable increase in FSH pulse frequency. Secretion of FSH is clearly pulsatile in ram lambs (Rawlings *et al.*, 1991) and in previous studies pulsatile FSH secretion in young bull calves has been observed (Evans *et al.*, 1993; Rawlings and Evans, 1995). In other studies in bulls it was concluded that FSH secretion was not pulsatile (Amann and Walker, 1983) or that serum FSH concentrations were reported as means (Stumpf *et al.*, 1993). In ewes, where the regulation of secretion of FSH has been better studied, the pulsatile secretion of FSH was unclear in jugular blood samples but in hypophyseal portal blood a clear pulsatility was seen (Midgley *et al.*, 1992); the masking of FSH pulses in jugular blood samples may be due to the longer half life of FSH compared with LH (Akbar *et al.*, 1974). Other studies in ewes and steers have led to the suggestion that LHRH regulates pulsatile LH secretion but has a more chronic, trophic role in regulating FSH secretion (Clarke *et al.*, 1984; Goodman, 1994).

Fig. 6. Mean ($\pm$ SEM) serum testosterone concentrations in blood samples taken every hour for 10 h at 4, 6, 12, 18 and 24 weeks of age in Hereford bull calves treated with LH-releasing hormone (■) or vehicle (□) every 2 h from 4 to 6 weeks of age. Values with different superscripts show differences between ages within groups ($P < 0.05$); *significant differences ($P < 0.05$) between treatment and control at a particular age.

The unclear acute responsiveness of FSH secretion to LHRH may also reflect a lack of pituitary sensitivity to LHRH owing to the very high serum concentrations of inhibin observed in young calves (Miyamoto *et al.*, 1989; MacDonald *et al.*, 1991).

Decreased FSH secretion at 18 weeks of age in calves treated with LHRH may reflect increased production of inhibin by Sertoli cells (which multiply at this age; Amman, 1983) since the number of Sertoli cells is greater than in control calves. At 24 weeks of age, in calves treated with LHRH, LH pulse amplitude was higher than in controls. The reason for the higher LH pulse amplitude at this age is not clear. Testosterone production was also increased in treated calves at this age, based on blood samples taken each hour for 10 h. It is possible that increased LHRH stimulation between 4 and 6 weeks of age enhanced the increase in pituitary LH stores and sensitivity to LHRH normally seen at this time (Rodriguez and Wise, 1991) and this may have resulted in increased LH pulse amplitude as the LHRH pulse frequency decreased at 24 weeks of age. The responses of LH and FSH to LHRH observed in blood samples taken every 15 min for 10 h were not reflected in samples taken every other week. Again, sampling every other week gives an indication of trends in mean serum concentrations of LH and FSH over the first year of life but does not accurately reflect

subtle changes in pulsatility, especially if pulse frequency and amplitude are inversely related as they are in young postnatal calves at the time when LH pulse frequency first increases.

LHRH-induced testosterone secretion was observed in the present study at 4 and 6 weeks of age in blood samples taken every hour for 10 h, apparently as a result of increased secretion of LH. Serum concentrations of testosterone were also increased at 24 weeks of age, presumably in response to increased secretion of LH. These increases in testosterone secretion in young bull calves were quite small compared with the amounts of testosterone secreted in older bulls and were not reflected in blood samples taken every other week (Rawlings *et al.*, 1972; Amann, 1983). In young bull calves, the testis produces other androgens, such as androstenedione and 5 α -reduced androgens, as well as testosterone, but in the adult, testosterone is the major product (Rawlings *et al.*, 1972; Rawlings and Cook, 1986). As the bull calves in the present study reached maturity, a clear increase in serum concentrations of testosterone was seen in blood samples taken every 2 weeks.

Testicular size at 52 and 54 weeks of age was increased by treatment with LHRH from 4 to 6 weeks of age; this was reflected in a greater seminiferous tubule diameter and increased spermatogenesis. It did not appear that control calves were simply slower to complete the first waves of spermatogenesis compared with calves treated with LHRH, in which all cell types, including Sertoli cells, were present in greater numbers than in controls. It is interesting to note that treatment with LHRH over 2 weeks in early life produces an effect on spermatogenesis at 54 weeks of age. Since significant initial effects of treatment with LHRH on serum concentrations of FSH were not observed, the stimulation of spermatogenesis in bull calves probably reflects increased secretion of LH and androgen between 4 and 6 weeks of age. However, it is also possible that stimulation of spermatogenesis occurred as a result of an increase in secretion of LH and androgen at 24 weeks of age, which is about the time that Sertoli cells are differentiating and proliferating (Curtis and Amann, 1981). A positive correlation has been found between numbers of A spermatogonia and numbers of Sertoli cells (de Reviers *et al.*, 1980; Hochereau-de Reviers *et al.*, 1987). As bulls approached maturity, increases in mean serum concentrations of LH and testosterone were noted, but there was no significant difference between bull calves treated with LHRH and controls. Although caution must be exercised when interpreting mean serum hormone concentrations based on blood sampling every 2 weeks, as a reflection of changes in secretion of hormones

Table 1. Mean ($\pm$ SEM) numbers of germ cells (corrected) and Sertoli cells per tubular cross-section in stage VI of spermatogenesis at 54 weeks of age in Hereford bull calves treated with LHRH or vehicle (control group) every 2 h from 4 to 6 weeks of age

Group	Elongated spermatids	Round spermatids	Pachytene spermatocytes	B-spermatogonia	A-spermatogonia	Sertoli cells
LHRH	30.29 $\pm$ 1.14*	36.29 $\pm$ 1.14*	9.23 $\pm$ 0.33*	4.53 $\pm$ 0.15*	3.89 $\pm$ 0.14*	3.98 $\pm$ 0.14*
Control	17.57 $\pm$ 0.53	26.01 $\pm$ 0.77	7.55 $\pm$ 0.26	3.70 $\pm$ 0.12	2.93 $\pm$ 0.09	2.86 $\pm$ 0.10

*Significant difference between treatment groups ($P < 0.05$).

Table 2. Mean ($\pm$ SEM) percentage of germ cells and other testicular cells (HC, elongated spermatids; 1C, round spermatids; 2C, diploid cells and 4C, cells undergoing division with 4N DNA) sorted by a flow cytometer on the basis of DNA content at 54 weeks of age in Hereford bull calves treated with LHRH or vehicle (control group) every 2 h from 4 to 6 weeks of age

Group	HC cells	1C cells	2C cells	4C cells
LHRH	6.84 $\pm$ 0.71*	25.48 $\pm$ 2.15	42.46 $\pm$ 2.64	28.29 $\pm$ 1.63
Control	3.29 $\pm$ 0.27	19.05 $\pm$ 2.74	45.76 $\pm$ 3.93	33.70 $\pm$ 5.72

Testicular cells were quantitated by quantitative ethidium bromide binding to germ cell DNA followed by flow cytometry.

*Significant difference between treatment groups ($P < 0.05$).

produced episodically, these results do suggest that treatment did not markedly alter the peripubertal profile of serum concentrations of LH and testosterone. Therefore, the effects of treatment with LHRH on secretion of LH and testosterone in young calves must have produced testicular changes at the time of treatment that resulted in enhanced testicular development at maturity.

Amann (1983) suggested that attempts to induce puberty with LHRH were not successful because treatments were applied too late in development and that very early treatment, particularly before the early increase in LH secretion, may be more successful. The present data support this contention and a longer period of LHRH treatment than was used in the present study, from 4 weeks of age, may have an even more profound effect on reproductive development in bull calves and could perhaps induce early puberty. The magnitude of the early increase in LH secretion, between 6 and 20 weeks of age in bull calves, is negatively correlated with age at puberty (Evans *et al.*, 1995). On the basis of the present study and previous observations (Amann, 1983), the early increase in secretion of LH and androgen appears to be important for initiating testicular and reproductive development and perhaps even determining sperm output in the pubertal bull.

The authors thank S. Cook and L. Kumor for technical help, B. Blakley for flowcytometry, B. Kerr and his staff for the care and management of the animals, and NIDDK and USDA for the provision of reagents for the gonadotrophin assays. This research was funded by the Natural Sciences and Engineering Research Council of Canada.

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