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H. Jiménez-Severiano
CENID Fisiologia Animal

M. J. D'Occhio
University of Queensland

D. D. Lunstra
USDA-ARS

M. L. Mussard
Ohio State University

J. W. Koch
Texas A & M University - College Station

See next page for additional authors

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Research

Effect of chronic treatment with the gonadotrophin-releasing hormone agonist azagly-nafarelin on basal concentrations of LH in prepubertal bulls

H. Jiménez-Severiano[1,2*], M. J. D'Occhio[3†], D. D. Lunstra[4], M. L. Mussard[1,2], J. W. Koch[1‡], L. R. Ehnis[1§], W. J. Enright[5] and J. E. Kinder[1,2,¶]

[1]*Department of Animal Science, University of Nebraska, Lincoln, NE 68583-0908, USA;* [2]*Department of Animal Sciences, The Ohio State University, 2029 Fyffe Road, Columbus, OH 43210-1095, USA;* [3]*Animal Sciences and Production Group, Central Queensland University, Rockhampton, Queensland, Australia 4701;* [4]*The US Department of Agriculture, Agriculture Research Service, Roman L. Hruska US Meat Animal Research Center, Clay Center, NE 68933-0166, USA; and* [5]*Intervet International, 5830 AA Boxmeer, The Netherlands*

Administration of GnRH agonist for an extended period inhibits pulsatile LH release but enhances testicular function of bulls. The mechanism whereby long-term administration of GnRH agonist enhances testosterone concentration in the blood of bulls has not been determined. The aim of this study was to determine whether chronic treatment with the GnRH agonist, azagly-nafarelin, increases blood concentrations of LH and FSH in prepubertal bulls. Two different doses of the GnRH agonist were administered via Alzet mini-osmotic pumps for 28 days. Blood samples were collected at 20 min intervals for 24 h at days 2, 13 and 25 of treatment. Agonist-treated groups had reduced testosterone pulse frequency ($P < 0.05$) and increased mean and basal concentrations of testosterone ($P < 0.05$) compared with untreated control bulls. Basal LH concentrations were higher in agonist-treated bulls during all three periods ($P < 0.05$) and overall (1 ng ml^{-1} higher, compared with control bulls; $P < 0.001$). Frequency of LH pulses in the agonist-treated groups was reduced to less than one pulse in 24 h. Agonist-treated bulls tended to have ($P < 0.10$) or had ($P < 0.05$) a slight but significant increase in blood FSH concentration. In conclusion, the higher blood testosterone concentration in bulls after prolonged treatment with GnRH agonist may result, at least in part, from changes in the testes induced by enhanced basal concentration of LH.

Introduction

Chronic treatment with GnRH or GnRH agonist induces desensitization of the anterior pituitary gland (Labrie *et al*., 1980; Bint Akhtar *et al*., 1983; Schürmeyer *et al*., 1984; Lincoln *et al*., 1986). In many species, including rats (Labrie *et al*., 1980), rhesus monkeys (Bint Akhtar *et al*., 1983; Sundaram *et al*., 1984), dogs (Vickery *et al*., 1985), humans (Schürmeyer *et al*., 1984) and sheep (Fraser and Lincoln, 1980; Lincoln *et al*., 1986), pituitary desensitization is characterized by decreased gonadotrophin secretion and reduced responsiveness to further GnRH stimulation. Pituitary desensitization to GnRH and the resulting decrease of LH concentration in blood induces impairment of gonadal function, which can eventually lead to azoospermia and infertility (Labrie *et al*., 1980; Bint Akhtar *et al*., 1983; Vickery *et al*., 1985). Cattle appear less sensitive to the negative effects of chronic administration of GnRH (Melson *et al*., 1986; Ronayne *et al*., 1993; D'Occhio and Aspden, 1996). For example, chronic treatment with GnRH or GnRH agonist in bulls is associated with enhanced testicular function, as evidenced by increased concentrations of testosterone in the blood (Melson *et al*., 1986; Ronayne *et al*., 1993; D'Occhio and Aspden, 1996), greater testicular size (D'Occhio and Aspden, 1996; Aspden *et al*., 1998), increased numbers of testicular LH receptors (Melson *et al*., 1986) and enhanced steroidogenic and spermatogenic capacities (Aspden *et al*., 1998). Paradoxically, increased steroidogenic capacity occurs in bulls treated with agonist even though bulls have

*Present address: CENID Fisiologia Animal, INIFAP-SAGARPA. Apdo. Postal 2–29, Queretaro, Qro. CP 76020, Mexico
†Present address: University of Queensland – Gatton Campus, Gatton, Queensland, Australia 4343
‡Present address: Department of Animal Science, Texas A&M University, College Station, TX 77843–2471, USA
§Present address: University Outreach and Extension, University of Missouri, 299 East Second St., Lebanon, MO 65536, USA
¶Correspondence
Email: kinder.15@osu.edu

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a classical downregulation of the pituitary gland. Downregulation of the pituitary gland is associated with a lack of responsiveness to exogenous GnRH stimulation (Melson *et al*., 1986; D'Occhio and Aspden, 1996), decreased numbers of GnRH receptors (Melson *et al*., 1986), lower pituitary content of LH and FSH (Melson *et al*., 1986; Aspden *et al*., 1996) and lower amounts of LH-β and FSH-β subunit mRNA (Aspden *et al*., 1996, 1997). Similarly, female cattle treated with GnRH agonist show enhanced luteal function (Davis *et al*., 2000) and have increased blood concentrations of progesterone (Gong *et al*., 1995; Davis *et al*., 2000) and oestradiol (Bergfeld *et al*., 1996a).

The mechanism(s) whereby agonists induce functional and morphological changes in the testes of bulls is not known. It is possible that the apparent enhancement of testicular function in bulls treated with GnRH agonist may result from changes in LH status, including alterations in basal secretion, characteristics of pulsatile secretion or mean plasma concentrations. A lack of pulsatile secretion of LH in cattle during agonist treatment has been associated with normal (Melson *et al*., 1986; Ronayne *et al*., 1993; Bergfeld *et al*., 1996a,b) or slightly higher (Gong *et al*., 1995; D'Occhio and Aspden, 1996) than normal mean plasma concentrations of LH. The sampling frequencies in these studies unfortunately did not allow a precise description of individual components of the LH secretory profile. Accordingly, the primary aim of the present study was to provide a thorough description of the characteristics of LH secretion in bulls treated with GnRH agonist. This information is fundamental to understanding the biological basis for increased steroidogenic function in bulls treated with GnRH agonist that have a downregulated pituitary gland. It is hypothesized that chronic treatment with GnRH agonist in bulls results in increased basal concentrations of LH in the blood and that this provides the basis for increased testicular LH receptors and greater steroidogenic function. Changes in FSH secretion were also monitored in order to obtain a complete understanding of the characteristics of gonadotrophin secretion in male cattle during treatment with GnRH agonist.

Materials and Methods

Experimental animals

All protocols and procedures used in this experiment were approved by the Institutional Animal Care and Use Committee at the University of Nebraska, Lincoln. Eighteen bull calves (1/4 Hereford, 1/4 Angus, 1/4 Pinzgauer, 1/4 Red Poll) were used. At the beginning of the experiment, bull calves averaged 152 ± 0.6 days of age, and 182 ± 3.1 kg body weight (BW). The GnRH agonist used was azagly-nafarelin ([D-Nal(2)^6, aza-Gly^{10}]GnRH; Intervet International BV, Boxmeer).

Treatments

Bulls were randomly allotted to three groups and each group was assigned to one of three treatments ($n = 6$ bulls per group) as follows: a placebo group treated with the vehicle (control), or treated with a relatively small (1 μg kg^{-1} BW day^{-1}; A1) or a relatively large (3 μg kg^{-1} BW day^{-1}; A3) dose of azagly-nafarelin. This GnRH agonist is considered to be 200 times more potent than the native GnRH in rats. The large dose of azagly-nafarelin was similar in potency to a similar GnRH agonist used in previous studies with young bulls (Ronayne *et al*., 1993). The vehicle used was 5% (w/v) mannitol diluted in distilled water. Treatments were administered subcutaneously via an Alzet mini-osmotic pump (model 2ML4; Alza Corp., Palo Alto, CA) for 28 days. Pumps were inserted beneath the skin overlying the ribs, under local anaesthetic. Before insertion, pumps were pre-incubated overnight in 0.15 mol sodium chloride l^{-1} at 37°C, to ensure an immediate release of the hormone after insertion of the pumps.

Blood sampling

Blood samples were taken every 2 h from 2 to 20 h after inserting the pumps. At the end of this period, serial blood samples (5 ml) were collected every 20 min for 24 h (day 2). Additional serial blood samplings were made on days 13 and 25 of the treatment period. Animals were fitted with indwelling jugular catheters® (Tygon flexible plastic tubing, ID: 1.27 mm, OD: 2.29 mm; Norton Performance Plastics, Akron, OH) one day before the start of each collection period. Blood samples were allowed to clot at room temperature and were then stored at 4°C. Blood was centrifuged within 36 h of collection at 1500 ***g*** for 20 min at 4°C. Serum was then decanted into polypropylene vials and stored at –20°C until assayed for LH, FSH and testosterone concentrations.

Hormone radioimmunoassays

Concentrations of LH were determined in all samples by a double-antibody radioimmunoassay (Wolfe *et al*., 1989). The limit of detection was 140 pg ml^{-1}. The intra- and inter-assay coefficients of variation (CV) were 4.8 and 6.6%, respectively. Concentrations of FSH were determined in selected samples (every 2 h) from the serial blood sampling periods and in all the other samples collected during the experiment. Determinations were made by a double-antibody radioimmunoassay (Wolfe *et al*., 1989). The limit of detection was 154 pg ml^{-1}. The intra- and inter-assay CVs were 2.4 and 3%, respectively.

Concentrations of testosterone were determined in every other serial sample that was collected, and in all the other samples collected during the experiment. Duplicate 25 μl aliquots of sample were double-extracted with ether, and extract residues were

re-suspended in 600 μl Tris-buffered saline with 0.1% (w/v) gelatin (TBS-gel), to be assayed by a double-antibody radioimmunoassay, using a micro-scale method for liquid scintillation counting (Grotjan and Steinberg, 1978). The assay used a sheep anti-T antibody (GDN no. 250; 200 μl of a 1:120 000 dilution), provided by G. Niswender (Colorado State University, Fort Collins, CO), [1,2,6,7-^{3}H (N)] testosterone (24 000 d.p.m per tube; New England Nuclear, Boston, MA); and donkey anti-sheep gamma globulin (DSG 1002; 200 μl of a 1:55 dilution; ImmunoVision, Springdale, AR) as second antibody. The standard curve was prepared with testosterone in solution with ethanol (3.6–461.4 pg (25 μl)$^{-1}$), allowed to dry and re-suspended in TBS gel. The limit of detection was 50 pg ml^{-1}. The intra- and inter-assay CVs were 4.4 and 7.9%, respectively.

The validation of the radioimmunoassay for testosterone was as follows. Fifteen different bull serum samples were assayed at 10, 20 and 40 μl. These serial dilutions generated binding inhibition curves that paralleled the testosterone standard curve. Furthermore, the average ratios ± SD and correlations of the testosterone concentrations obtained between the different pairs of volumes were 0.94 ± 0.13 (r = 0.996), 0.99 ± 0.11 (r = 0.99) and 0.93 ± 0.17 (r = 0.995) respectively, for 10 versus 20 μl, 20 versus 40 μl and 10 versus 40 μl. Three bovine serum samples were used to evaluate recovery of mass. Different amounts of added testosterone were utilized (50, 100, 200, 400 and 800 pmol per tube), and the average ± SD recovery from these samples was 97 ± 3.9%.

Hormone secretory pattern and statistical analyses

Secretory patterns of LH and testosterone were evaluated for mean and basal concentration and for pulse frequency and amplitude, using computerized algorithms (Merriam and Wachter, 1982; Pulsar software modified for IBM-PC by J. F. Gitzen and V. D. Ramirez). This method removes long-term trends, such as diurnal rhythms, from the series of observations and calculates a base line by generating a smoothed series that omits peaks or trends with time constants less than 6 to 12 h. It identifies peaks by criteria of both height and duration of deviation from the base line, measured relative to the expected random variability of the series. The smoothing time was set to 12 h. The assay standard deviation used in the algorithm was modelled as a quadratic function of the dose for each hormone. G values used were: $G(1)$ = 3.8, $G(2)$ = 2.6, $G(3)$ = 1.9, $G(4)$ = 1.5 and $G(5)$ = 1.2.

Data for LH and testosterone secretory patterns, and mean concentrations of FSH on days 2, 13 and 25 of the treatment were analysed as a completely randomized design, using the MIXED procedure of SAS (Littell *et al*., 1996) with the statement repeated, the options sub = animal (treatment), and autoregressive (1) covariance structure within animal. Treatment, day, and treatment-by-day interaction were included in the model. The PDIFF option of SAS was used to compare least square means among treatments. Except for FSH concentration, all variables were Log_{10} (Y+1) transformed so as to be consistent with the assumptions of the ANOVA.

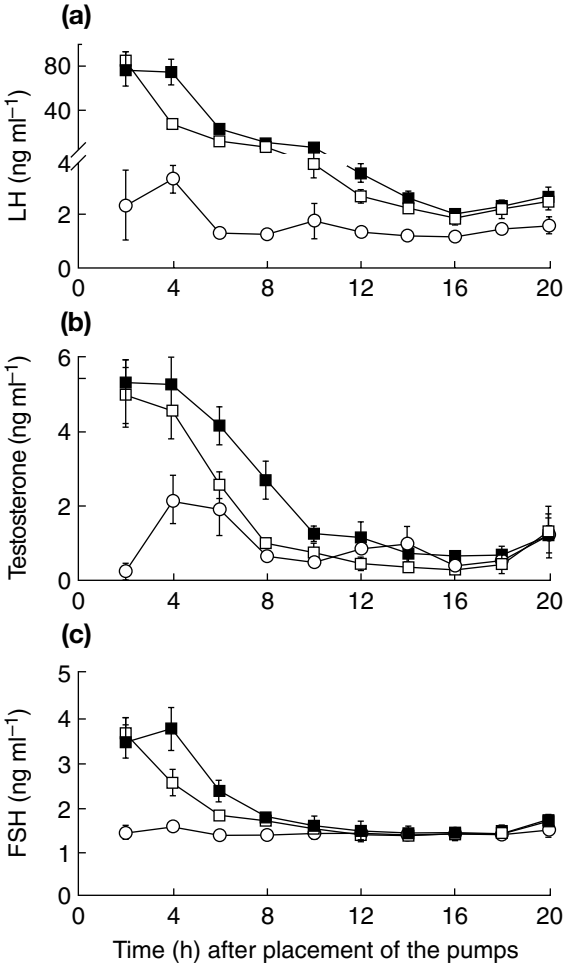

Fig. 1. Mean (± SE) serum concentrations of (a) LH, (b) testosterone and (c) FSH in prepubertal bulls from 2 to 20 h after insertion of the pumps. Bulls were treated with vehicle (control; ○), or with a small (1 μg kg^{-1} body weight (BW) day^{-1}; A1: □), or large (3 μg kg^{-1} BW day^{-1}; A3: ■) dose of the GnRH agonist azagly-nafarelin for 28 days.

Results

Response to GnRH agonist during the initial 20 h of treatment

Hormonal concentrations in bulls from 2 to 20 h after insertion of the mini-osmotic pumps are shown (Fig. 1). Two hours after insertion of the pumps, concentrations of LH (Fig. 1a) reached 84.49 ± 7.99 and 77.19 ± 15.23 ng ml^{-1}, respectively, for groups A1 and A3, compared with 2.33 ± 1.33 for the control group. Thereafter, LH concentrations decreased in both groups to values

Table 1. Average (± SEM) characteristics for LH secretory patterns in prepubertal bulls on days 2, 13 and 25 of treatment

Day of treatment	Treatment*	Mean (ng ml^{-1})	Basal (ng ml^{-1})	Pulse frequency (pulses 24 h^{-1})	Pulse amplitude (ng ml^{-1})
2	Control	1.67 ± 0.21^{e}	0.99 ± 0.12^{b}	3.00 ± 1.00^{a}	10.11 ± 3.15
	A1	2.36 ± 0.28^{d}	2.35 ± 0.27^{a}	0.00^{b}	–
	A3	2.76 ± 0.32^{d}	2.74 ± 0.32^{a}	0.00^{b}	–
13	Control	1.60 ± 0.19	0.75 ± 0.07^{b}	4.33 ± 0.84^{a}	7.35 ± 1.36
	A1	1.75 ± 0.08	1.70 ± 0.07^{a}	0.17 ± 0.17^{b}	3.16‡
	A3	1.47 ± 0.09	1.44 ± 0.08^{a}	0.17 ± 0.17^{b}	0.84‡
25	Control	1.14 ± 0.24	0.62 ± 0.11^{e}	1.83 ± 0.65^{d}†	8.63 ± 0.85
	A1	1.29 ± 0.06	1.28 ± 0.06^{d}	0.00^{e}	–
	A3	1.17 ± 0.16	1.10 ± 0.12^{d}	0.67 ± 0.42^{de}†	1.99 ± 1.17
Overall	Control	1.47 ± 0.13	0.79 ± 0.07^{b}	3.06 ± 0.52^{a}	8.61 ± 1.16
	A1	1.80 ± 0.14	1.78 ± 0.14^{a}	0.06 ± 0.06^{b}	3.16‡
	A3	1.80 ± 0.20	1.76 ± 0.20^{a}	0.28 ± 0.16^{b}	1.61‡

*Bulls were treated with vehicle (control), or with a small (1 μg kg^{-1} body weight (BW) day^{-1}; A1) or large (3 μg kg^{-1} BW day^{-1}; A3) dose of the GnRH agonist azagly-nafarelin for 28 days.
†Any two means with a dagger for the same variable and day tend to be different from each other ($P < 0.10$), but the effect is not significant.
‡The standard error could not be calculated.
Means with different superscripts within variable and day are different: $^{a,b}P < 0.001$; $^{d,e}P < 0.05$.

comparable to control animals by 18 h (1.32 ± 0.25 ng ml^{-1}). Testosterone concentrations (Fig. 1b) followed a similar pattern to LH in both agonist-treated groups, with values of 4.99 ± 0.76 ng ml^{-1} (A1) and 5.2 ± 1.1 ng ml^{-1} (A3) 2 h after insertion of the pumps, decreasing to values similar to the control group between 6 h (2.55 ± 0.37 ng ml^{-1} for group A1) and 12 h (1.09 ± 0.42 ng ml^{-1} for group A3) after insertion of the pumps. Concentrations of FSH (Fig. 1c) reached 3.69 ± 0.34 ng ml^{-1} by 2 h in group A1, and 3.77 ± 0.48 ng ml^{-1} by 4 h in group A3. Concentrations of FSH then decreased to values similar to the control group (1.58 ± 0.11 ng ml^{-1}).

Characteristics of the LH secretory patterns on days 2, 13 and 25 of treatment

Characteristics of the LH patterns during the three 24 h sampling periods are shown (Table 1). The interaction of treatment by day was significant for mean LH concentration ($P < 0.05$). Both agonist-treated groups had higher ($P < 0.05$) mean concentrations of LH, compared with the control group on day 2 only. The overall effect of treatment on mean LH concentration was not significant.

For basal concentration of LH, the effects of treatment ($P < 0.001$) and treatment-by-day interaction ($P < 0.05$) were significant. During the three periods of sampling, both agonist-treated groups had higher ($P < 0.05$) basal concentrations of LH compared with the control group. The overall basal concentrations of LH in groups A1 and A3 were 1 ng ml^{-1} above the values detected in the control group ($P < 0.001$).

The interaction of treatment by day was significant ($P < 0.05$) for frequency of LH pulses. No LH pulses were detected in almost all bulls treated with the GnRH agonist during the three sampling periods. In group A1, only one bull had one pulse during the sampling on day 13. In group A3, one bull had two pulses on day 25, and another bull had one pulse on day 13 and two pulses on day 25. During the three periods of sampling and overall, pulse frequency was less ($P < 0.001$) in both agonist-treated groups compared with the control group.

Owing to the small number of pulses of LH in the agonist-treated groups, on some days the standard error for pulse amplitude could not be calculated and it was not possible to make statistical comparisons among treatments; therefore, only numerical data for this variable are included (Table 1). Individual LH and testosterone secretory patterns from one bull of each group on day 13 of treatment are shown (Fig. 2). Basal concentrations of LH and testosterone were higher in both groups treated with the GnRH agonist (Fig. 2b,c) compared with the control group. Patterns of LH and testosterone secretory episodes also differed between control and agonist-treated bulls. In control bulls (Fig. 2a), LH pulses were characterized by a sharp increase, followed by a gradual decrease until returning to basal values; in addition, a pulse of testosterone usually followed each pulse of LH. In contrast, the few pulses of LH detected in bulls treated with the GnRH agonist (Fig. 2c) were of small amplitude, had a longer period to reach the peak and return to basal concentrations, and were not clearly associated with subsequent increases of testosterone.

Characteristics of testosterone secretory patterns on days 2, 13 and 25 of treatment

Treatment with both doses of GnRH agonist either tended to increase ($P < 0.10$) or increased significantly ($P < 0.05$) mean concentration of testosterone at each day of sampling and overall (Table 2) compared with the control group, except on day 13, when mean concentrations of testosterone were similar in group A3 and the control group ($P > 0.10$). Bulls in group A1 tended to have higher mean concentrations of testosterone compared with those in group A3 on day 13 ($P = 0.09$), although the effect was not significant, and had higher mean testosterone concentrations on day 25 ($P < 0.05$). Basal testosterone concentration was higher ($P < 0.05$) in both agonist-treated groups compared with the control group at each day of sampling. Furthermore, the overall basal concentration of testosterone was higher ($P < 0.05$) in groups A1 (12-fold) and A3 (8-fold) compared with the control group. Bulls in group A1 tended to have higher basal concentrations of testosterone compared with those in group A3 on day 13 ($P = 0.07$) and had higher basal concentrations on day 25 ($P < 0.05$).

Treatment with both doses of GnRH agonist reduced ($P < 0.05$) the number of testosterone pulses in the 24 h sampling periods, except for group A3 at day 25. Agonist-treated groups were different from each other in number of testosterone pulses only on day 25 ($P < 0.05$). Most testosterone pulses in the agonist-treated bulls differed in shape compared with the bulls in the control group, in a way similar to that described for LH pulses (Fig. 2). A detectable pulse of LH did not always precede pulses of testosterone in these groups. Considering the apparent absence of LH pulses preceding testosterone, it was unclear whether the increases in testosterone were induced by release of LH, or were sustained increases of testosterone responding to the constantly higher basal concentrations of LH. Owing to the few pulses of testosterone in the agonist-treated groups, on some days the standard error for pulse amplitude could not be calculated and it was not possible to make statistical comparisons among treatments; therefore, only numerical data for this variable are included (Table 2).

Concentrations of FSH on days 2, 13 and 25 of treatment

Data for mean concentration of FSH are shown (Table 3). The effects of treatment and treatment-by-day interaction were significant ($P < 0.05$). Bulls treated with the small dose of GnRH agonist tended to have increased ($P = 0.08$) FSH concentrations compared with the control group on day 13, and had higher FSH concentrations on days 2, 25 and overall ($P < 0.05$). Overall concentration of FSH tended ($P = 0.08$) to be higher in group A3, and was higher on days 2 and 25 ($P < 0.05$) compared with the control group.

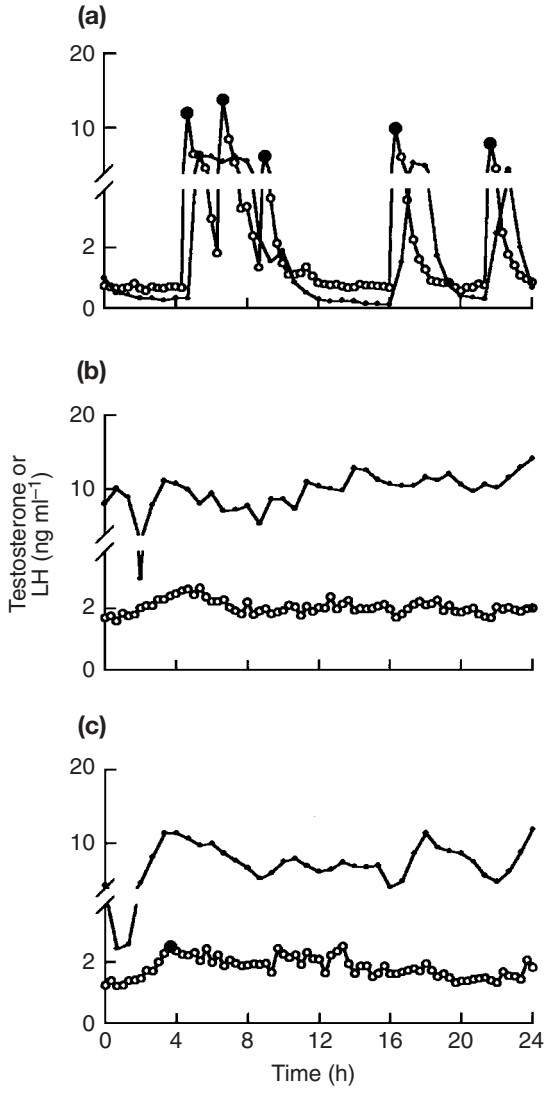

Fig. 2. Individual secretory patterns of LH and testosterone in prepubertal bulls on day 13 of treatment. Bulls were treated with (a) vehicle (control), or with (b) a small (1 μg kg^{-1} body weight (BW) day^{-1}) or (c) a large (3 μg kg^{-1} BW day^{-1}) dose of the GnRH agonist azagly-nafarelin for 28 days. Samples were taken every 20 min (LH) or every 40 min (testosterone) for 24 h, starting at 08:00 h. Open circles denote LH concentration, small black circles denote testosterone concentration and large black circles denote detected LH pulses.

Discussion

The most significant finding in the present study was a clear and consistent increase in basal concentration of LH in bulls treated with the GnRH agonist, azagly-nafarelin, regardless of the dose used. Although Ronayne *et al*. (1993) reported a small increase in basal concentration of LH in GnRH agonist-treated bulls, this increase was observed only during the first day of treatment and not on subsequent days. Furthermore, Melson *et al*. (1986) suggested an increase in basal LH concentration in bulls treated with nafarelin acetate, but this action

Table 2. Average (± SEM) characteristics for testosterone secretory patterns in prepubertal bulls on days 2, 13 and 25 of treatment

Day of treatment	Treatment*	Mean (ng ml^{-1})	Basal (ng ml^{-1})	Pulse frequency (pulses 24 h^{-1})	Pulse amplitude (ng ml^{-1})
2	Control	$0.79 \pm 0.16^{e\dagger}$	0.40 ± 0.07^{e}	2.67 ± 0.88^{d}	2.76 ± 0.64
	A1	$2.44 \pm 0.82^{de\dagger}$	2.45 ± 0.82^{d}	0.17 ± 0.17^{e}	$1.18^{\ddagger}$
	A3	2.76 ± 0.83^{d}	2.76 ± 0.84^{d}	0.17 ± 0.17^{e}	$0.77^{\ddagger}$
13	Control	1.64 ± 0.39^{e}	0.47 ± 0.10^{e}	3.67 ± 0.71^{d}	4.89 ± 0.95
	A1	$6.35 \pm 1.52^{d\dagger}$	$5.98 \pm 1.45^{d\dagger}$	1.00 ± 0.37^{e}	4.08 ± 1.30
	A3	$3.88 \pm 1.31^{de\dagger}$	$3.57 \pm 1.18^{d\dagger}$	1.00 ± 0.45^{e}	2.56 ± 1.28
25	Control	$1.35 \pm 0.43^{e\dagger}$	0.45 ± 0.07^{f}	2.00 ± 0.68^{d}	6.08 ± 1.67
	A1	7.54 ± 1.04^{d}	7.54 ± 1.03^{d}	0.17 ± 0.17^{e}	$1.69^{\ddagger}$
	A3	$3.76 \pm 1.32^{e\dagger}$	3.68 ± 1.34^{e}	1.17 ± 0.31^{d}	1.73 ± 0.47
Overall	Control	$1.26 \pm 0.21^{e\dagger}$	0.44 ± 0.04^{e}	2.78 ± 0.45^{d}	4.57 ± 0.69
	A1	5.45 ± 0.82^{d}	5.32 ± 0.80^{d}	0.44 ± 0.17^{e}	2.90 ± 0.10
	A3	$3.47 \pm 0.65^{de\dagger}$	3.34 ± 0.62^{d}	0.78 ± 0.21^{e}	0.93 ± 0.56

*Bulls were treated with vehicle (control), or with a small (1 μg kg^{-1} body weight (BW) per day; A1) or large (3 μg kg^{-1} BW per day; A3) dose of the GnRH agonist azagly-nafarelin for 28 days.
†Any two means with a dagger for the same variable and day tend to be different from each other ($P < 0.10$), but the effect is not significant.
‡The standard error could not be calculated.
Means with different superscripts within variable and day are different: $^{d,e,f}P < 0.05$.

Table 3. Mean concentrations (± SEM) of FSH (ng ml^{-1}) in prepubertal bulls on days 2, 13 and 25 of treatment

	Day of treatment			
Treatment*	Day 2	Day 13	Day 25	Overall
Control	1.30 ± 0.05^{e}	1.22 ± 0.11^{h}	1.11 ± 0.11^{e}	$1.21 \pm 0.05^{e\dagger}$
A1	1.64 ± 0.08^{d}	1.48 ± 0.14^{g}	1.51 ± 0.12^{d}	1.54 ± 0.07^{d}
A3	1.67 ± 0.08^{d}	1.21 ± 0.08^{h}	1.39 ± 0.09^{d}	$1.42 \pm 0.06^{de\dagger}$

*Bulls were treated with vehicle (control), or with a small (1 μg kg^{-1} body weight (BW) day^{-1}; A1) or large (3 μg kg^{-1} BW day^{-1}; A3) dose of the GnRH agonist azagly-nafarelin for 28 days.
†Any two means with a dagger in the same day tend to be different from each other ($P < 0.10$), but the effect is not significant.
Means with different superscripts within day are different or tend to be different: $^{d,e}P < 0.05$; $^{g,h}P < 0.10$.

of the GnRH agonist was not clearly shown. Therefore, previous studies have shown inconsistent effects of the GnRH agonists on LH concentration. The schedule of sampling used in the present study (every 20 min for 24 h) allows for a more accurate characterization of the LH secretory patterns, compared with single samples per day or serial samplings made in shorter periods of time, as used in previous studies. In the present study, the increased basal concentration of LH in the agonist-treated bulls was more marked at day 2 of treatment (137–176% above the basal concentration in control bulls). At days 13 and 25, basal LH in agonist-treated groups was still 77–100% above the values detected in control animals, indicating that increased basal concentrations of LH in bulls chronically treated with the GnRH agonist can be maintained over extended periods of time.

The reduced number or the lack of LH pulses in bulls treated with GnRH agonists has been previously reported (Melson *et al*., 1986; Ronayne *et al*., 1993; D'Occhio and Aspden, 1996). Given that endogenous pulses of GnRH are not suppressed by chronic treatment with the GnRH agonist (Caraty *et al*., 1990), the suppression of LH pulses indicates that the pituitary gland of cattle becomes desensitized to endogenous GnRH pulses, similar to the response in other species. Pituitary desensitization is supported by findings in a related study in which bulls treated with a GnRH agonist failed to respond to exogenous administration of natural sequence GnRH, or did not present the classical LH increase after castration (D'Occhio and Aspden, 1996).

Another possibility is that the treatment with the GnRH agonist changes the pulsatile pattern of LH, from a lesser frequency and greater amplitude similar to that found in the control bulls, to a greater frequency and lesser amplitude pattern of LH secretion. If this had occurred, pulses might not have been detected by the method used, and basal LH would have been over-estimated. However, it is unlikely that this is the situation. The algorithm used to analyse the LH episodes makes no assumptions about ideal peak shapes. Instead, it requires that peaks

have some combination of height and width. A peak is detected if it is of great amplitude, even if it is narrow, or if it has moderate amplitude, but extends for several points in width (Merriam and Wachter, 1982). Furthermore, in the authors' experience, this method has been able to detect LH pulses with amplitudes of 0.25 ng ml^{-1}.

In the present study, and in previous studies (Melson *et al*., 1986; Ronayne *et al*., 1993; Bergfeld *et al*., 1996b; D'Occhio and Aspden, 1996), bulls chronically treated with GnRH agonists have increased concentrations of testosterone in the blood. The reasons for greater concentrations of testosterone when LH pulses are essentially not occurring and when mean LH concentration appears to be normal are unclear. Results from the present experiment and data reported by others (Chase *et al*., 1988; Mendis-Handagama *et al*., 1998) support the hypothesis that the enhanced testicular steroidogenesis and increased blood concentrations of testosterone most likely result from the increased basal concentration of LH in bulls chronically treated with GnRH agonist. Previous studies in rams immunized against GnRH and treated with LH for 12 to 20 days indicate that the infusion of LH producing the highest basal concentration of LH also resulted in the greatest testosterone response to LH administration (Chase *et al*., 1988). In adult rats, continuous infusion of LH for 2 weeks induced hypertrophy and hyperplasia of Leydig cells, associated with a sixfold increase in testosterone secretory capacity, with no change in testis volume (Mendis-Handagama *et al*., 1998). Moreover, chronic treatment of intact rats with LH or hCG results in increased capacity for LH-stimulated testosterone production and induction of steroidogenic enzyme synthesis (Payne *et al*., 1980; O'Shaughnessy and Payne, 1982). Signs of enhanced steroidogenesis are also reported for bulls treated for 10 days with implants of the GnRH agonist deslorelin (Aspden *et al*., 1998). The testes of these bulls had increased amounts of StAR protein and the steroidogenic enzymes $P450_{scc}$ and 3β-hydroxysteroid dehydrogenase, and 17α-hydroxylase mRNA, indicating increased testicular stimulation by LH, because the synthesis of these enzymes is stimulated by LH (Saez, 1994).

In addition to the basal concentration of LH, greater numbers of testicular receptors for LH might be important for the increased synthesis of testosterone in bulls treated with GnRH agonist, as shown by Melson *et al*. (1986). Evidence that LH induces increased numbers and activity of its own receptors has been shown by Kero *et al*. (2000). This study used transgenic female mice expressing a chimaeric protein derived from the α subunit of bovine LH and the carboxy-terminus peptide extension of the hCG β subunit. The animals exhibited chronically increased serum LH concentrations, associated with increased numbers of adrenal LH receptors and steroidogenic capacity.

In animals with increased basal LH concentration, the testicular response observed corresponds to the so-called long-term trophic effect of the hormone on cellular structure and function, which requires mRNA and protein synthesis (Saez, 1994). This long-term response explains the changes in the Leydig cell previously reported, which might be important to maintain increased testosterone synthesis for extended periods in bulls chronically treated with GnRH or GnRH agonists.

The effect of GnRH agonists on FSH concentration in bulls has not been well characterized. Aspden *et al*. (1996) reported decreased serum FSH concentrations, associated with decreased amounts of FSH-β subunit mRNA in the pituitary, in castrated bulls treated with deslorelin. In contrast, Melson *et al*. (1986) reported no effect of nafarelin acetate on serum FSH concentrations, but noted a decreased pituitary content of FSH in mature intact bulls. In the present study, there was a slight increase in FSH concentrations in bulls treated with the GnRH agonist. Whether this increase was biologically important to the extent that it altered testicular function (directly or indirectly on Leydig cells or any other testicular component) is unknown. There is evidence in immature hypophysectomized rats that treatment with highly purified FSH can induce Leydig cell hypertrophy and hyperplasia, associated with increased numbers of LH receptors and steroidogenic capacity (Teerds *et al*., 1989; Vihko *et al*., 1991).

In summary, the present study has shown that chronic infusion with the GnRH agonist, azagly-nafarelin, increases basal LH and induces a modest increase in FSH mean concentration, associated with increased concentration of testosterone in bulls. In addition, the number and amplitude of LH pulses were reduced by the treatment. In conclusion, these data support the working hypothesis that the functional changes in the testes of bulls chronically treated with GnRH agonists, associated with enhanced steroidogenesis and testosterone secretion, most likely result from enhanced basal secretion of LH. A possible effect of the small increase of FSH concentration on testicular function cannot be disregarded. The mechanisms inducing increased tonic LH secretion by the downregulated pituitary remain to be elucidated.

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