Increasing sensitivity of the pituitary to GnRH from early to late anoestrus in the beagle bitch

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The sensitivity of the pituitary to GnRH in early and late anoestrus and the indirect response of the ovary were investigated in six adult beagle bitches. Plasma concentrations of LH and oestradiol were determined after i.v. injection of graded doses of GnRH (0, 0.01, 0.1, 1, 10 and 100 µg kg⁻¹). The responses were measured by the LH and oestradiol concentration profiles over time. The responses of LH and oestradiol were significantly dose dependent ($P = 0.002$ and $P < 0.001$, respectively). The responses of LH and oestradiol were significantly higher ($P = 0.02$ and $P = 0.001$, respectively) in late anoestrus than in early anoestrus. The responses of LH and the responses of oestradiol were positively correlated ($r = 0.97$, $P = 0.001$). It is concluded that during the course of anoestrus in the bitch pituitary sensitivity to GnRH increases while the ovary responds accordingly.

Introduction

The breeding cycle of the female dog is not as precisely timed as in common laboratory or farm animals. After ovulation, the following luteal period is on average 75 days followed by a variable period before the onset of the next follicular phase (Okkens *et al.*, 1985; Concannon, 1986; Shille *et al.*, 1989; Bouchard *et al.*, 1991). The term 'anoestrus' is used to describe this variable period. Anoestrus in the cyclic female dog persists for at least 90 days (Shille *et al.*, 1989; Bouchard *et al.*, 1991). Although anoestrus in bitches is generally described as a period of ovarian quiescence without external signs of oestrogen activity (Concannon, 1986; Feldman and Nelson, 1987), gonadotrophin and oestradiol secretion do not remain at basal values throughout this period (Shille *et al.*, 1989). An increasing secretion of gonadotrophins (Concannon *et al.*, 1986; Shille *et al.*, 1987) and a fluctuating secretion of oestradiol (Gräf, 1978; Olson *et al.*, 1982) during the progression of anoestrus have been reported. It is not yet known how these changes in gonadotrophin and oestradiol secretion are regulated.

The pituitary sensitivity to GnRH might be an important factor in the neuroendocrine regulation of these changes. An increasing response of LH to stimulation with GnRH during the progression of anoestrus has been determined in mares (Silvia *et al.*, 1987) and female brown hares (Caillol *et al.*, 1986; Caillol *et al.*, 1990). The purpose of this study was to determine whether pituitary sensitivity to GnRH increases in the female dog during the progression of anoestrus and

whether the ovary responds accordingly during this time. We, therefore, studied the effect of a wide range of doses of GnRH on the secretion of LH and oestradiol in early and late anoestrus.

Materials and Methods

Animals

Six healthy adult female beagle dogs, 3.8 ± 0.7 (mean $\pm$ SD) years of age and weighing 14.5 ± 2.6 (mean $\pm$ SD) kg, were housed in pairs in separate indoor kennels, with access to separate outdoor runs for 2 h day⁻¹. They were fed a standard commercial diet and water was continuously available. They were given an iron supplement orally (Ferroxi fumaras; 65 mg; Brocacef, Maarsse) once a day to prevent anaemia. Packed cell volume (PCV) was measured using a micro cell-counter (Sysmex F800, Kobe) every 2 weeks.

The dogs were examined three times a week for vulvar swelling and vaginal discharge, and when these were first present the plasma concentration of progesterone was determined. Day 1 was defined as the day on which the progesterone concentration exceeded 16 nmol l⁻¹; that is when ovulation was assumed to occur (Concannon *et al.*, 1977; Okkens *et al.*, 1985). The duration of the interval between day 1 of the preceding cycle to day 1 of the cycle in which experiments were carried out was 284 ± 70 (mean $\pm$ SD, $n = 6$) days. Anoestrus is defined as the period from the first day, following the luteal period, on which the progesterone concentration did not exceed 3.2 nmol l⁻¹ to the day on which vulvar swelling or vaginal discharge was first present.

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Box 1. Administration of six doses of GnRH to six dogs in early and late oestrus in latin square designs

		Early anoestrus								Advanced anoestrus					
		Day 90			Day 132					Day 160			Day 202		
Dog (number)	8505	△	▲	□	▼	■	▽	Dog (number)	8505	△	■	□	▽	▼	▲
	8603	■	▼	△	□	▽	▲		8603	▽	▲	■	▼	□	△
	8605	▽	△	■	▲	□	▼		8605	▲	▼	△	□	■	▽
	8607	▼	■	▲	▽	△	□		8607	■	▽	▼	△	▲	□
	8703	□	▽	▼	■	▲	△		8703	▼	□	▽	▲	△	■
	8704	▲	□	▽	△	▼	■		8704	□	△	▲	■	▽	▼

△: 0; ▲: 0.01; □: 0.1; ■: 1.0; ▽: 10; ▼: 100 µg GnRH kg⁻¹.

Administration of GnRH and collection of blood samples

Synthetic GnRH (Fertagyl; Intervet International BV, Boxmeer) dissolved in 0.9% NaCl was given in a series of six injections of 0, 0.01, 0.1, 1, 10 and 100 µg kg⁻¹ to six dogs at weekly intervals in a 6 × 6 latin square design (Spriet and Simon, 1985). This schedule was similar to the one used in a pituitary–gonadal challenge test in male dogs (Knol *et al.*, 1993). This treatment was given in early (days 90–132) and in late (days 160–202) anoestrus of the same cycle (Box 1).

GnRH (0.1 mg ml⁻¹) was diluted to 20 ml in 0.9% NaCl for injection in the cephalic vein of one of the front legs. Blood samples were collected by venepuncture from a jugular vein at –20, –10, 0, 5, 10 and consecutive 10 min intervals until 160 min after injection. All blood samples were collected in ice-cooled heparinized glass tubes and these were centrifuged for 10 min at 1800 g; the plasma was removed and stored at –25°C.

Radioimmunoassays

Concentrations of progesterone in the peripheral blood were estimated by a previously validated radioimmunoassay (Okkens *et al.*, 1986); the intra- and interassay coefficients of variation were < 11 and < 14% (*n* = 12), respectively, and the sensitivity was 0.3 nmol l⁻¹. LH concentrations were estimated by a heterologous radioimmunoassay (Nett *et al.*, 1975; Knol *et al.*, 1993). The sheep antibody CSU204, donated by G.D. Niswender (Colorado State University, CO), radioiodinated bLH7981 as prepared for our bovine LH assay (Dieleman and Bevers, 1987) and the canine pituitary standard LER 1685-1 (LE Reichert, Albany Medical College, New York) were used in this assay.

Concentrations of oestradiol were estimated by a solid-phase radioimmunoassay using ¹²⁵I (Coat-A-Count TKE; Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer's instructions with slight modifications as described by Dieleman and Bevers (1987). The sensitivity was 7 pmol l⁻¹. The intra-assay coefficient of variation ranged from 23.6 to 10.5% for the standards added to plasma of an ovariectomized bitch, over the range 10–165 pmol l⁻¹, with

recoveries of 80.3–115% (*n* = 6). The interassay coefficient of variation was 11.8% (*n* = 20).

Statistical analyses

The pretreatment concentration was defined for each dose in every dog as the mean of the concentrations at –20, –10 and 0 min. When an endogenous surge occurred at any of these times the pretreatment concentration was defined as the average of the other pretreatment concentrations in the same dog in the same period of anoestrus. The response was defined as the area under the curve that represents the induced peripheral concentration. The pretreatment concentration was used as the baseline value. The area was calculated from the time of injection of GnRH to the time at which the peripheral concentration had returned to the pretreatment concentration or to the time of collection of the last sample.

Student's *t* test for paired samples was used for the comparison of the interoestrous intervals and the overall mean pretreatment concentrations of LH and oestradiol. A multivariate analysis of variances (MANOVA) for repeated measurements was used to analyse the differences between the responses in early and in late anoestrus. Correlation was calculated with the Spearman's rank test. The probability limit was stated at 0.05 (Norusis, 1989; Shott, 1990). Values are given as means ± SD.

Results

No adverse effects of GnRH administration or collection of blood samples on health were observed. The PCV dropped slightly in some dogs but remained between 37 and 55%. No external signs of induced ovarian activity were observed.

GnRH-induced LH secretion in early and late anoestrus

The overall mean pretreatment concentration of LH was 1.8 ± 0.2 µg l⁻¹ (*n* = 6) in early anoestrus and 2.1 ± 0.4 µg l⁻¹ (*n* = 6) in late anoestrus; these concentrations did not differ significantly.

Fig. 1. Mean LH concentrations after administration (arrow) of different doses of GnRH to six female dogs in (a) early and (b) late anoestrus. $\triangle = 0$, $\blacktriangle = 0.01$, $\square = 0.1$, $\blacksquare = 1$, $\nabla = 10$ and $\blacktriangledown = 100$ $\mu\text{g GnRH kg}^{-1}$.

Each administration of the doses 0.1, 1, 10 and 100 $\mu\text{g GnRH kg}^{-1}$ induced a distinct and immediate rise in plasma LH concentration during early and late anoestrus. The dose of 0.01 $\mu\text{g GnRH kg}^{-1}$ caused a slight and variable rise, while no distinct rise was seen after administration of vehicle. Peak concentrations occurred 5, 10 or 20 min after injection and concentrations declined gradually to pretreatment concentrations after about 2 h. Occasionally in both early and late anoestrus, minor surges were superimposed on the LH curve; these surges were observed before ($n = 4$) or after ($n = 19$) injection of GnRH or vehicle. The four LH curves induced at the time of such a surge had no aberrant patterns. The superimposed surges were excluded from calculations of mean LH concentrations (Fig. 1) and mean LH responses (Fig. 2).

The LH responses were significantly ($P = 0.002$) dose dependent. The LH responses in late anoestrus were significantly ($P = 0.02$) greater than were the LH responses in early anoestrus (Fig. 2).

GnRH-induced oestradiol secretion in early and in late anoestrus

The overall mean pretreatment concentration of oestradiol was 20.2 ± 4.4 pmol l^{-1} ($n = 6$) in early anoestrus and 21.6 ± 8.4 pmol l^{-1} ($n = 6$) in late anoestrus; these concentrations did not differ significantly.

Administration of 0.1, 1, 10 and 100 $\mu\text{g GnRH kg}^{-1}$ induced a moderate increase in oestradiol concentration that did not return to pretreatment values during the observation period (Fig. 3). Neither 0.01 $\mu\text{g GnRH kg}^{-1}$ nor injection vehicle induced an increase in oestradiol concentrations.

Two large increases in plasma oestradiol concentration were observed after administration of vehicle, once during early and once during late anoestrus; these were excluded from calculations of mean oestradiol concentrations and responses.

The oestradiol responses were significantly dose dependent ($P < 0.001$) and the oestradiol responses in late anoestrus were

Fig. 2. Mean LH responses $\pm$ SD to different doses of GnRH in six female dogs in ($\square$) early and ($\blacksquare$) late anoestrus.

significantly ($P = 0.001$) greater than were those in early anoestrus (Fig. 4).

The LH and oestradiol responses of early and late anoestrus were significantly correlated ($r = 0.97$, $P = 0.001$).

During the stimulation tests, external signs of onset of oestrus like vulvar swelling and vaginal bleeding were absent and the concentration of plasma progesterone remained less than 3.2 nmol l^{-1} . The average interval between the previous oestrus and that following the stimulation tests was 291 ± 57 days ($n = 6$). This period did not differ significantly from the duration of the preceding average cycle. Individual intervals were 207, 260, 277, 314, 317 and 374 days. There were no indications that the LH and oestradiol responses in late anoestrus in the three dogs with shorter intervals were different from those in the three dogs with longer intervals.

Discussion

In most of the experiments GnRH evoked a clear LH curve, from which the response could be deduced directly. The minor

Fig. 3. Mean oestradiol concentrations after administration (arrow) of different doses of GnRH to six female dogs in (a) early and (b) late anoestrus. $\triangle = 0$, $\blacktriangle = 0.01$, $\square = 0.1$, $\blacksquare = 1$, $\nabla = 10$ and $\blacktriangledown = 100$ $\mu\text{g GnRH kg}^{-1}$.

Fig. 4. Mean oestradiol responses $\pm$ SD to different doses of GnRH in six female dogs in ($\square$) early and ($\blacksquare$) late anoestrus.

LH surges that were occasionally superimposed on the LH curve were considered to be independent endogenous LH surges, because they occurred before the administration of GnRH and also after the administration of vehicle. Their occurrence was within the range of the physiological pulse frequency reported by Concannon *et al.* (1986). When a surge occurred before GnRH administration, the pituitary responsiveness did not appear to be affected, since the subsequently induced LH curves did not show aberrant patterns. Consequently, all superimposed surges were excluded from the calculations. The graded GnRH doses induced LH rises that virtually comprised minimal to maximal responses. Saturation was not demonstrated, yet it must have been approximated, considering the relatively minor enhancement in response to the tenfold increase in the higher doses. Because pituitary responsiveness over the entire range of doses was significantly greater 160–202 days after ovulation than after 90–132 days, it may be concluded that pituitary sensitivity to GnRH increases as anoestrus progresses.

Since the subsequent ovulation in these bitches occurred long after the second experimental period (average of 291

days) it can be speculated that pituitary responsiveness increases further as oestrus approaches. However, in the three bitches that ovulated soon after the last experiment, the responsiveness was not different from that of the bitches having a longer interoestrous interval. It is therefore concluded that pituitary responsiveness to GnRH initially increases as anoestrus progresses, possibly reaching a plateau towards the end of anoestrus.

The induced oestradiol curves were less clearly defined and were more variable. This might be due to the influence of FSH responses, which were probably also evoked, since GnRH is thought to be the releasing hormone for both LH and FSH (Fink, 1988). In general, LH enhances synthesis of aromatase substrates in thecal cells, whereas FSH stimulates aromatase activity in granulosa cells. Although LH is regarded as the primary regulatory factor in controlling oestrogen secretion in all but the most immature of follicles, FSH is of importance in the early stages of follicular development (Gore-Langton and Armstrong, 1988). This two-cell theory for the biosynthesis of oestradiol has not been demonstrated in the bitch, but there are no reasons to assume a different type of regulation in this species.

Nevertheless, the oestradiol and LH responses were positively correlated. The oestradiol response was significantly higher during late than during early anoestrus. This is probably a reflection of the enhanced LH response, but an increase in ovarian capacity to produce oestrogen cannot be excluded.

It is concluded that during the course of anoestrus in the female dog pituitary sensitivity to GnRH increases while the ovary responds accordingly.

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