Basal and GnRH-induced secretion of FSH and LH in anestrous versus ovariectomized bitches

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Abstract

The basal and gonadotropin releasing hormone (GnRH)-induced plasma concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were studied in four anestrous and four ovariectomized (OVX) bitches. Blood samples were obtained via jugular venipuncture 40 min before and 0, 10, 20, 30, 60, 90, and 120 min after the i.v. administration of synthetic GnRH in a dose of 10 µg/kg body weight.

The basal plasma FSH and LH concentrations were significantly higher in the OVX bitches than in the anestrous bitches. In the anestrous bitches, the plasma FSH concentration was significantly higher than the pretreatment level at 10, 20, and 30 min, whereas the plasma LH concentration was significantly elevated at 10 and 20 min. The maximal GnRH-induced plasma FSH concentration in the anestrous bitches did not surpass the lowest plasma FSH concentration in the OVX bitches, whereas the GnRH-induced plasma LH concentrations in the anestrous bitches overlapped with the basal plasma LH concentrations in the OVX bitches. In the OVX bitches, GnRH administration did not induce a significant change in the plasma FSH concentration, whereas the plasma LH concentration increased significantly at 10 and 20 min.

In conclusion, the results of the present study indicate that in anestrous bitches GnRH challenge results in increased plasma levels of both FSH and LH, whereas in the OVX bitches, in which the basal plasma FSH and LH concentrations are higher, only a rise in the plasma LH concentration is present after GnRH stimulation. The results also suggest that a test to measure plasma concentration of FSH in single samples appears to have potential in verification of neuter status in bitches.
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1. Introduction

The reproductive cycle of the bitch is under endocrine control exerted by interactions among the hypothalamus, anterior pituitary, and ovary —the so-called hypothalamic–pituitary–ovarian axis. The neuroendocrinological mechanisms responsible for the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary are very complex. It is generally accepted that these gonadotropins are secreted in a pulsatile fashion [1] in response to the similarly pulsatile release of gonadotropin releasing hormone (GnRH). The gonadotropins stimulate ovarian hormone secretion, which in turn regulates

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the gonadotropins via a negative feedback mechanism (long-loop negative feedback). The extent of GnRH release and subsequently gonadotropin release seems to be dependent upon sensitivity of the hypothalamus to estradiol. During progression of anestrus, there is gradual reestablishment of positive feedback of estradiol on the hypothalamus [2], and GnRH secretion [3] and the responsiveness of the pituitary to GnRH increase [4]. In addition, a large amount of LH is released prior to ovulation, due to this positive feedback elicited by the rapidly rising plasma estradiol level [5]. Apart from the feedback of the gonadal hormones, two additional feedback loops have been identified. These are the gonadotropin-dependent inhibition of GnRH production (short-loop negative feedback) and the GnRH-dependent inhibition of GnRH production, known as ultrashort-loop negative feedback [6].

The measurement of plasma FSH and LH concentrations before and after administration of a GnRH analogue during different reproductive conditions, such as during anestrus and after ovariectomy, permits assessment of the capacity of the pituitary to secrete gonadotropins. Knowledge about the characteristics of gonadotropin secretion after intravenous administration of a GnRH analogue in the anestrous bitch is mainly limited to LH [7], the response of which is dosage-dependent [4,8]. Furthermore, an increasing dosage-dependent response of LH to stimulation with GnRH was found in the bitch during the progression of anestrus [4], but another study revealed no apparent correlation between stage of anestrus and LH response [9]. However, the used GnRH dosage in the latter study was minimal. Only one study was concerned with the concomitant secretion of the gonadotropins in the bitch after GnRH administration and showed that in cyclic bitches FSH and LH levels increased within 15 min after GnRH stimulation [10].

Ovariectomy (OVX) influences the long-loop feedback. The loss of inhibitory influence of ovarian steroids and peptides on the hypothalamic–pituitary axis causes a rapid increase in the plasma concentration of both LH [7,11–16] and FSH [12,13,16], while there are indications that the secretion pattern of both remains pulsatile [13]. Little is known about the characteristics of gonadotropin secretory response to exogenous GnRH in the OVX bitch. Both a rise [7,11] and an unpredictable response [14] in the plasma LH concentration have been reported. So far, in OVX bitches there is no information either on GnRH-induced FSH secretion or on the concomitant secretion of FSH and LH after GnRH administration.

The present study was designed to compare the basal and GnRH-induced plasma concentrations of FSH and LH in OVX bitches with those in anestrous bitches.

2. Materials and methods

2.1. Animals, treatment, and collection of blood samples

Four intact Beagle bitches (weighing 11.3–14.1 kg and aged 2–5 years), in which the previous estrous cycle had been monitored and characterized, and four OVX Beagle bitches (weighing 12.8–16.0 kg and aged 9–10 years) were included in this study. All dogs were whelped and raised in the Department of Clinical Sciences of Companion Animals and were accustomed to the laboratory environment and procedures, such as the collection of blood samples. They were housed in pairs in indoor–outdoor runs, fed a standard commercial dog food once daily, and provided with water ad libitum. The four intact Beagles were examined three times per week for the presence of swelling of the vulva and a serosanguinous vaginal discharge, which were considered to signify the onset of proestrus. Plasma concentrations of progesterone were determined three times per week from the start of proestrus until the day on which the plasma progesterone concentration exceeded 16 nmol/L, which is when ovulation was assumed to occur [17–19]. During this study, all bitches were in anestrus (104–118 days after ovulation), as confirmed by plasma progesterone concentrations below 3 nmol/L [20]. The four remaining dogs had been ovariectomized at the Department of Clinical Sciences of Companion Animals at Utrecht University at least 1 year before the start of the experiment and had exhibited no signs of recurrent estrus.

At 09.00 h (time is 0 min) 10 μg/kg synthetic GnRH (Fertagyl[®] batch 20105B; Intervet International BV, Boxmeer, The Netherlands) was administered via the cephalic vein in all Beagle bitches. Blood samples were taken 40 min before and 0, 10, 20, 30, 60, 90, and 120 min after the administration of GnRH.

Blood samples were collected from the jugular vein, immediately placed in chilled EDTA-coated tubes, and centrifuged at 4 °C for 10 min at 1500 × g. Plasma was stored at −25 °C until analysis. All samples from each dog were measured in the same FSH or LH assay.

2.2. Hormone immunoassays and validation of FSH assay

Plasma FSH concentration was measured by the canine immunoradiometric assay (IRMA) of Biocode

SA (Liège, Belgium) coded AHCOO4. This IRMA uses a first and a second monoclonal mouse anti-FSH antibody. These antibodies were raised against rat-FSH and have cross-species reactivity (rat, dog, and horse) (personal communication, Biocode SA). Briefly, the procedure used highly purified canine FSH diluted in horse serum provided by the manufacturer to produce a standard curve ranging from 1.5 to 240 μg/L. An excess of a first monoclonal mouse anti-rat FSH antibody bound to polystyrene tubes captured the canine FSH present in 100 μL standards or samples. Thereafter, a second [^{125}I]-labeled monoclonal mouse anti-rat FSH antibody (50 μL), directed to another determinant on the FSH molecule, was added to the tubes, which were incubated at room temperature for 90 min. According to the manufacturer, the cFSH used as standard was derived from extracts of pituitary glands of dogs collected over several years in Belgium. Pituitary extracts were purified by ion exchange and gel filtration. The monoclonal antibodies were obtained by injecting highly purified rat FSH in mice after which splenocytes of the mice were fused with Sp2O myeloma cells. Finally, diluted positive clones were tested for binding to iodinated cFSH to obtain highly stabilized monoclonal cells. The reported purity of the antibodies (IgG class) is >95%. More detailed information on the reagents is not available.

To validate the FSH IRMA for parallelism, accuracy, and precision, plasma from an OVX bitch 10 min after the administration of 10 μg/kg GnRH was diluted with plasma from an anestrous bitch before GnRH stimulation (dilutions 1:2 to 1:32). Each level of dilution was measured six times. As shown in Table 1, the precision and accuracy are within commonly accepted ranges. In addition, the intra-assay coefficient of variation (CV) was measured in 12 replicates of each of four samples in a single assay. These four quality controls samples were included in duplicate in all assays (n = 12) to determine the interassay CV. The intra-assay and the interassay CV for values above 1.60 μg/L were 3.5 and 15.1%, respectively. Cross-reactivity of canine LH (LER 1685-1; Dr. L.E. Reichert, Albany Medical College, NY, USA) with the first mouse anti-rat FSH monoclonal antibodies bound to the tube was determined by adding the preparation to PBS buffer containing 0.29% (w/v) human immunoglobulin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and 0.36% (w/v) bovine serum albumin (BSA; Povite, Organon, Oss, The Netherlands) at pH of 7.4. Maximal binding in the FSH IRMA was 36.8 ± 2.2%. At 50% of the maximal binding the cross-reactivity of canine LH (LER 1685-1) in this assay was 1.8%. The limit of quantitation of canine FSH was arbitrarily defined as the value of the lowest standard, 1.50 μg/L, and FSH values below this were arbitrarily assigned the value of one-third of this limit, i.e., 0.5 μg/L.

Plasma LH concentration was measured by a heterologous RIA as described previously [4,21]. Briefly, a rabbit antiserum raised against ovine LH (CSU-204, kindly supplied by G.D. Niswender, Colorado State University, CO, USA), radioiodinated bLH-7981 as prepared for our bovine LH assay [22], and canine pituitary standard LER 1685-1 (a gift from Dr. L.E. Reichert) were used in this assay. The intra-assay and interassay CV for values above 0.5 μg/L were 2.3 and 10.5%, respectively, and the limit of quantitation was 0.3 μg/L.

Plasma progesterone concentration was measured by a previously validated ^{125}I RIA [23]. The intra-assay and interassay CV were 6 and 10.8%, respectively, and the limit of quantitation was 0.13 nmol/L.

Table 1
Accuracy and precision in the estimation of follicle stimulating hormone (FSH) by immunoradiometric assay

Dilution	Added dose (μg/L)[a]	Estimated dose (μg/L)[b]	S.D. (μg/L)[c]	C.V. (%)[d]	Recovery (%)[e]
Undiluted	47.76	47.76	0.99	2.07	100
1:2	23.88	22.25	0.95	4.26	93.2
1:4	11.94	10.59	0.41	3.88	88.7
1:8	5.97	5.24	0.31	5.90	87.8
1:16	2.98	2.65	0.13	4.95	88.9
1:32	1.49	1.32	0.13	9.88	88.6

Each dilution was measured six times. All samples were measured in the same assay.
[a] Canine FSH was added by diluting plasma collected from an ovariectomized bitch 10 min after intravenous administration of a GnRH analogue (10 μg/kg) with plasma from an anestrous bitch.
[b] Values were corrected for the FSH responses in the IRMA of 1.57 μg/L of the plasma of the anestrous bitch.
[c] S.D.: Standard deviation.
[d] C.V.: Coefficient of variation, this value indicates the precision of the assay.
[e] Accuracy (recovery) is defined as estimated dose relative to added dose ($E/A \times 100\%$).

2.3. Data analysis of the plasma profiles

A Kolmogorov Smirnov test showed that, even after transformation to natural logarithms, the plasma profile data were not normally distributed. Hence the untransformed plasma profile data for FSH and LH were compared per group by the nonparametric Friedman test, followed by a multiple comparisons test using Dunnett's procedure. This test evaluated the changes in the GnRH stimulation test compared with the mean of the two pretreatment values. Differences in the pretreatment values, the peak concentration, the relative peak compared with the pretreatment value (i.e., peak value/basal value) and the area under the curve (AUC) above the pretreatment concentration between the two groups were assessed for statistical significance by the nonparametric Wilcoxon–Mann–Whitney test. The AUC of the hormone concentrations after the GnRH challenge test was calculated by the trapezoidal method after subtraction of the mean pretreatment level per dog. Results are presented as median and range or mean $\pm$ S.E.M. The level of significance was $P < 0.05$.

2.4. Ethics of experimentation

This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University.

3. Results

The basal (pre-GnRH) plasma concentrations of FSH and LH were significantly higher in the OVX dogs than in the anestrous dogs (Table 2). In the anestrous dogs, plasma concentrations of both FSH and LH increased after GnRH administration. The plasma FSH concentration was significantly elevated at 10, 20, and 30 min, whereas the plasma LH concentration was significantly higher than the basal level at 10 and 20 min (Fig. 1). In the anestrous dogs, GnRH-induced plasma profiles of both FSH and LH were characterized by an abrupt and rapid rise followed by a slow decline; slower for FSH than for LH. The GnRH-induced increase in plasma LH concentration above basal levels was much higher than that of FSH. In the OVX bitches, plasma FSH concentration remained unchanged after GnRH administration. In contrast,

Table 2

Characteristics of the GnRH-induced surges of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in four anestrous and four ovariectomized (OVX) bitches (median and range)

	Anestrous bitches ($n = 4$)	Ovariectomized bitches ($n = 4$)
FSH pre-GnRH concentration (μg/L)	5.6[a] 2.8–13.9	81.7[a] 40.7–107.5
FSH peak amplitude (μg/L)	25.4 18.4–27.0	–
FSH peak amplitude relative to basal	3.9 2.0–9.5	–
FSH time of peak amplitude (min)	20 10–20	–
FSH AUC above baseline (μg min/L)	1475 333–1952	–
LH pre-GnRH concentration (μg/L)	1.02[a] 0.81–5.4	31.4[a] 6.4–64.3
LH peak amplitude (μg/L)	34.9[a] 26.5–38.4	60.7[a] 48.3–359
LH peak amplitude relative to basal	30.7[a] 9.7–41.4	3.8[a] 1.3–6.4
LH time of peak amplitude (min)	10 10–10	10 10–10
LH AUC above baseline (μg min/L)	1038 862–1237	2032 688–5546

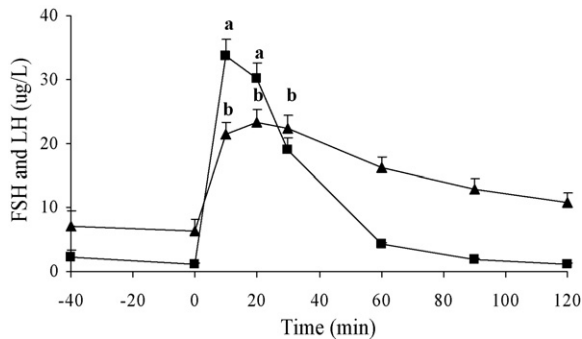[a] Indicates significant difference between anestrous and OVX dogs.

Fig. 1. Mean (± S.E.M.) plasma concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in four intact anestrous bitches after administration of synthetic gonadotropin releasing hormone (GnRH) (10 µg/kg) via the cephalic vein at $t = 0$ min. (▲) FSH, (■) LH. The letters (a and b) indicate significant difference compared with the mean of the pre-GnRH values for the plasma concentrations of LH and FSH, respectively. The bars indicating the S.E.M. are shown only when they exceed the size of the symbols.

plasma LH concentration was significantly increased at 10 and 20 min (Fig. 2). The median peak concentration of LH was significantly higher in the OVX dogs than in the anestrous dogs, but the relative increase in LH was significantly greater in the anestrous dogs than in the OVX dogs. The median AUC of the LH response did not differ between the anestrous and the OVX dogs (Table 2). The maximal GnRH-induced plasma FSH concentration in the anestrous bitches did not exceed the lowest plasma FSH concentration in the OVX bitches, whereas the GnRH-induced plasma LH concentrations in the anestrous bitches overlapped with the basal plasma LH concentrations in the OVX bitches (Table 2).
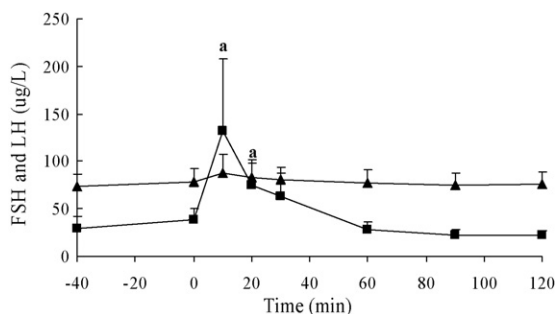

Fig. 2. Mean (± S.E.M.) plasma concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in four ovariectomized bitches after administration of synthetic gonadotropin releasing hormone (GnRH) (10 µg/kg) via the cephalic vein at $t = 0$ min. (▲) FSH, (■) LH. Letter (a) indicates significant difference compared with the mean of the pre-GnRH values for the plasma LH concentration. The bars indicating the S.E.M. are shown only when they exceed the size of the symbols.

4. Discussion

Pituitary gonadotropin release plays a central role in the development and function of the canine reproductive system. The measurement of plasma LH and FSH concentrations before and after the administration of a GnRH analogue permits assessment of the capacity of the pituitary to secrete gonadotropins under different reproductive conditions. In agreement with the findings of others, GnRH administration elicited a distinct release of LH [4,7–10] and FSH [10]. The course of the GnRH-induced surges in FSH and LH in the anestrous bitches was comparable to what has been reported previously for spontaneous FSH and LH pulses in anestrous bitches [1]. The GnRH-induced plasma LH increments above the pre-GnRH levels were much greater than the corresponding FSH increments. Subsequently, plasma FSH concentration declined more slowly than plasma LH concentration in the anestrous bitches. As in other mammals [24], this may be explained by a different pattern of glycosylation of FSH and LH, resulting in a longer half-life for FSH than LH.

The loss of ovarian feedback after ovariectomy resulted in increased plasma concentrations of FSH and LH in all OVX bitches, as has been reported earlier [11–16]. In the OVX bitches, GnRH only elicited a significant response in the plasma LH concentration. Since there was no significant rise in plasma FSH, it can be concluded that the pituitary response to GnRH in OVX bitches differs from that in anestrous bitches. It may be hypothesized that this is the consequence of different intracellular mechanisms for storage and release for FSH and LH. This view is supported by in vitro studies with cells of other mammalian species, which have shown that although FSH and LH are produced in the same cell type, they are stored in different secretory granules [25], newly synthesized FSH is secreted at a greater rate than LH [26], and that the magnitude of the FSH response to secretagogues is smaller than that of LH [26,27].

Occasionally, it can be difficult to verify the neuter status of dogs with an unknown reproductive status [7]. Although all pre-GnRH plasma LH concentrations were higher in OVX dogs than in sexually intact dogs, measurement of circulating LH concentration in a single sample has limited potential for distinguishing between OVX and sexually intact bitches. Taking into account the strong pulsatile nature of LH release [1,13], an overlap in the plasma LH concentration between larger groups of intact and OVX bitches may be expected. Indeed, in the current study GnRH-induced LH increments in the intact bitches overlapped pre-GnRH plasma LH concentrations

in the OVX bitches. A single high LH value measured by a commercial test kit was not reliable in indicating whether a bitch was intact [15]. In contrast, the results reported here indicate that measurement of plasma FSH concentration in a single sample may well prove reliable for determination of neuter status. Even after GnRH administration the maximum plasma FSH level in the intact bitches did not exceed the lowest plasma FSH level during the entire GnRH-stimulation test in the OVX bitches. Other investigators have also suggested that the pituitary capacity to secrete FSH in the absence of ovarian negative feedback is far greater than that ever observed in the intact bitch [12,13]. Such a difference could be expected if there is a specific inhibition of FSH as opposed to LH secretion in intact bitches, i.e., due to ovarian derived inhibin [28]. Furthermore, plasma FSH levels in women usually increase more than do LH levels when the ovaries are functionally inactive or surgically removed [29,30]. This indicates that differences in circulating FSH concentration between intact and OVX bitches could be greater than within-group variability. Another study with a greater number of animals is needed to evaluate the measurement of plasma FSH concentration as a potential diagnostic marker of neuter status.

In conclusion, the results of the present study indicate that in anestrous bitches GnRH challenge results in increased plasma levels of both FSH and LH, whereas in the OVX bitches, in which the basal plasma FSH and LH concentrations are higher, only a rise in the plasma LH concentration is present after GnRH stimulation. The results also suggest that a test to measure plasma concentration of FSH in single samples appears to have potential in verification of neuter status in bitches.

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