A rapid radioimmunoassay for determining plasma concentrations of LH in dogs

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Summary. A heterologous dog LH radioimmunoassay was modified to provide accurate results for LH concentrations in blood plasma of dogs within 3–4 h. This assay utilizes radioiodinated ovine LH (LER-1056-C2), antiserum against ovine LH (GDN-15) at a final dilution of 1:48 000 and dog LH (LER-1685-1) as standard. A 60-min incubation, including a 30-min delay in the addition of tracer, was carried out at 37°C. The free and antibody-bound hormone were separated by addition of a Micro Sepharose bead suspension containing anti-gamma-globulin, followed by incubation at room temperature for 30 min. The minimum detectable concentration in this assay, calculated from the precision profile, was 1.5 µg/l. The amount of dog LH needed to cause 50% reduction of the initial binding was 1.57 ± 0.13 ng/tube (15.7 µg/l for 100-µl samples). Daily blood samples were collected in heparinized tubes from the cephalic vein of 5 pointer and 7 beagle bitches from the onset of pro-oestrus until 3–4 days after either the last mating or artificial insemination with frozen semen or until metoestrus. Samples were assayed for LH content by the short and normal incubations as well as for progesterone and oestradiol-17β content. In all bitches plasma concentrations of progesterone increased rapidly within 1 week after the LH peak which indicates that they had ovulated. Comparison of the short (1.5 h) with the normal (24 h) incubation system resulted in a regression equation: $y = 1.0 + 0.7x$ ($r = 0.95$, $n = 153$ samples). The preovulatory LH peak averaged 10.4 ± 1.1 and 11.9 ± 1.7 µg/l in the short and normal incubation assay, respectively. We suggest that this rapid LH assay can be used in practice to define the optimum time for mating and artificial insemination of the bitch or to study physiological aspects of the ovulatory period.

Keywords: LH; radioimmunoassay; dog

Introduction

The dog pro-oestrous period ranges in length from 3 to 27 days (Tsutsui & Shimizu, 1975) with an average of 9 days. The length of the oestrous period, i.e. the period during which the bitch will stand for the male and allow mating, is also highly variable; it can be as short as 2–3 days or as long as 21 days (Concannon *et al.*, 1989). Because of the long oestrous period in the bitch and the great individual variation in reproductive events, methods to define the optimum time for achieving conception are in great demand among veterinary clinicians and dog breeders. The preovulatory LH peak precedes the onset of ovulation by 36–50 h (Phemister *et al.*, 1973; Concannon *et al.*, 1977). The ova are shed as primary oocytes and need another 2–3 days to mature before becoming fertilizable (Holst & Phemister, 1971; Phemister *et al.*, 1973; Tsutsui, 1989). Hence the optimum time for mating or artificial insemination should be 4 or 5 days after the LH surge. Irrespective of individual variation in total length of oestrus, identification of the LH peak would allow the period of highest fertility to be reliably predicted. In addition, a rapid method for determining LH concentrations would have the practical advantage of supplying the necessary information several days

before the mating or insemination would have to be performed. One possible way to establish a rapid LH radioimmunoassay would be to shorten the incubation time and improve the separation system. Bernard *et al.* (1983) used this approach to establish a rapid LH radioimmunoassay for predicting the time of ovulation in cows.

The aim of this study was to establish a rapid assay for LH for use in determining the timing of ovulation in the bitch.

Materials and Methods

Animals and sampling procedure. Five Pointer and 7 Beagle bitches were housed individually under natural light conditions, fed commercial rations and allowed free access to water. Daily blood samples were collected into heparinized tubes from the cephalic vein. Sampling was initiated at the onset of pro-oestrus and continued for 3–4 days after either the last mating or artificial insemination with frozen semen or until metoestrus. The blood samples were immediately centrifuged and the plasma obtained was kept at -20°C until assay.

Hormone assays. A heterologous dog LH radioimmunoassay (Smith & McDonald, 1974; DePalatis *et al.*, 1978) with certain modifications was used. The short incubation assay utilized radioiodinated ovine LH (LER-1056-C2), antiserum against ovine LH (GDN-15) at final dilution of 1:48 000 and dog LH (LER-1685-1) as standard. Ovine LH was iodinated by the chloramine-T method (Greenwood *et al.*, 1963) as described by Forsberg *et al.* (1989) using 8 μg chloramine T/2 μg glycoprotein and an exposure time of 40 sec. A 60-min incubation, including a 30-min delay in the addition of tracer, was carried out at 37°C . The free and antibody-bound hormone were separated by adding a suspension of Micro Sepharose beads (Pharmacia AB, Uppsala, Sweden) containing anti-gamma-globulin followed by incubation at room temperature for 30 min.

In the normal incubation assay ovine LH antiserum was diluted 1:150 000. Overnight incubation, including a 2-h delay in the addition of ^{125}I -labelled sheep LH, was carried out at room temperature. Free and antibody-bound LH were separated as described above. The sensitivity of the normal incubation assay, calculated from the precision profile, was 0.4 $\mu\text{g/l}$. The amount of dog LH needed to cause 50% reduction of the initial binding was 0.55 ± 0.05 ng/tube (5.5 $\mu\text{g/l}$ for 100 μl plasma). The intra-assay coefficient of variation for LH concentrations between 1.5 and 17.7 $\mu\text{g/l}$ was below 12%. The antiserum cross-reacted with dog FSH (LER-1685-3A) at 6%. All plasma samples were assayed using both the short and the normal LH incubation systems.

To reduce the time and the cost of analysis we have evaluated the possibility of performing the LH assay without the standard curve. The assay, called the 'binding test', consisted of tubes for non-specific binding, maximal binding, control samples with (positive) and without (negative) the preovulatory LH peak and unknowns. Fourteen plasma samples covering the range of the standard curve were analysed in 5 different binding tests.

Concentrations of progesterone were determined by radioimmunoassay using the antibody to an 11 α -hydroxyprogesterone-hemisuccinate-bovine serum albumin conjugate (Bosu *et al.*, 1976). The antiserum cross-reacted <1% for progestagens, oestrogens, androgens and corticoids, except for deoxycorticosterone (3.8%), pregnenolone (3.3%), and 5 β -pregnane-3,20-dione (11.0%). The intra-assay coefficient of variation varied between 5.5 and 18.5% within the range of the standard curve. The detection limit of the assay was 0.5 nmol/l.

Peripheral plasma concentrations of oestradiol-17 β were determined by radioimmunoassay, using an antiserum to 6-keto-oestradiol-17 β which cross-reacted 11.0% with oestrone (Boilert *et al.*, 1973; Lindberg *et al.*, 1974). The intra-assay coefficients of variation for oestradiol-17 β concentrations ranging between 57 and 735 pmol/l were below 15%. The detection limit of the assay was 25.0 pmol/l. Both the progesterone and the oestradiol-17 β methods had been applied earlier to dog plasma (Edqvist *et al.*, 1975; Linde & Karlsson, 1984; Linde-Forsberg & Forsberg, 1989).

Statistical methods. Data were subjected to an analysis of variance and Scheffe's test available in the statistical package offered by Statgraphics (STSC, Inc., Rockville, MD, USA). Means are expressed as $\pm$ s.e.m.

Results

In the short incubation assay, LH antiserum bound 15–25% of the ^{125}I -labelled sheep LH and the standard curve ranged from 0.2 to 6.4 ng. When attempting to optimize the short incubation assay it was found that a 30-min delay in the addition of tracer significantly increased (1.5-fold) the detection limit. The minimum detectable concentration in this assay, calculated from the precision profile, was 1.5 $\mu\text{g/l}$. The amount of dog LH needed to cause 50% reduction of the initial binding was 1.57 ± 0.13 ng/tube (15.7 $\mu\text{g/l}$ for 100- μl samples). The intra-assay coefficient of variation for LH concentrations in the range of 4.2 to 35.4 $\mu\text{g/l}$ was below 12%. The mean LH concentration in two pooled plasma samples and the coefficient of variation, estimated in 5 different assays, were 4.2

Fig. 1. Plasma oestradiol-17β (□---□), progesterone (*····*) and LH concentrations (+---+, normal assay; □—□, short assay) in a beagle bitch.

(CV 25.6%) and 10.9 (CV 11.6%) μg/l, respectively. The mean slope of the dose-response curve in 7 assays was -1.185 (CV 7.6%) after logit-log transformation. The LH antiserum cross-reacted with dog FSH at 6%. The time required to complete the test, including pipetting, incubation, counting and evaluation of results varied from 3 to 4 h. The radioiodinated sheep LH remained usable for 4–6 weeks provided the tracer was purified at weekly intervals by means of cellulose (Whatman CF11) adsorption.

The hormone profiles determined during pro-oestrus and oestrus in one Beagle bitch are shown in Fig. 1. Both the short assay and the normal assay indicated that the preovulatory peak of LH occurred on the same day. The basal concentrations of LH using the short and the normal incubation assay was around 1.5 and 0.5 μg/l, respectively. At 4 days before the LH surge there was an extra LH release, which was only detected by the normal incubation assay. Plasma concentrations of oestradiol-17β reached a maximum (160 pmol/l) 1 day before the preovulatory LH peak and had decreased to 30 pmol/l 8 days later. Plasma progesterone increased rapidly following the LH peak (Day 0) to a concentration of 77 nmol/l by Day 6 (Fig. 1). In other bitches, mean plasma progesterone concentrations significantly ($P < 0.05$) increased from 17 nmol/l by Day 2 (LH surge = Day 0) to 47 nmol/l by Day 4 and 83 nmol/l by Day 6.

Mean plasma LH concentrations during pro-oestrus and oestrus in Beagle bitches are presented in Fig. 2. The preovulatory LH peak averaged 10.4 ± 1.1 and 11.9 ± 1.7 μg/l according to the short and the normal incubation assay, respectively. Regardless of the assay used no difference was found in basal LH ranges or in the magnitude of the LH peak between Beagle and Pointer bitches. The basal concentrations of LH and the magnitude of the LH peak in Pointer bitches using the short incubation assay varied from 1.5 to 2.0 μg/l and from 6.1 to 8.9 μg/l, respectively.

Figure 3 depicts the degree of correlation between LH values obtained from the short (1.5 h) incubation assay and those obtained from the normal (24 h) assay. This relationship was described by the following linear regression equation: $y = 1.0 + 0.7x$ ($r = 0.95$, $n = 153$ samples).

Fig. 2. Plasma LH concentrations in 5 Beagle bitches as determined by the normal ($\square$ — $\square$) and the short ($\diamond$ — $\diamond$) assay. Values are mean $\pm$ s.e.m.

Fig. 3. Comparison between determinations of LH concentrations made on 153 plasma samples using the short assay and the normal assay.

Table 1. Characteristics of the binding test for plasma samples with and without the preovulatory LH peak, each assayed 5 times

No. of samples	LH ($\mu\text{g/l}$)	% Bound
1	1.7 ± 0.1^a	92.5 ± 0.8^a
2	4.2 ± 0.5^a	79.1 ± 2.3^a
3	6.9 ± 0.5^b	67.9 ± 1.7^b
4	10.9 ± 0.6^c	55.1 ± 1.5^c
5	8.7 ± 0.8^{bc}	62.0 ± 2.5^{bc}
6	2.9 ± 0.4^a	88.8 ± 2.4^a

Values are mean $\pm$ s.e.m.

^{a,b,c}Means in a row with different superscript letters are significantly different ($P < 0.05$).

Table 1 shows the characteristics of the binding test. Sample 3, in which there was an evident LH peak, caused a significant ($P < 0.05$) reduction of the initial binding (from 100% to 67.9%). Based on these results, samples causing more than a 27% reduction of the initial binding were classified as positive (the LH peak) and those causing less than a 27% reduction as negative. Overall, the incidence of true positive results in samples with the preovulatory LH peak was 100%. The incidence of true negative results without the preovulatory LH peak was 95%. The time required to complete the binding test, including pipetting, incubation, counting and evaluation of results varied from 1.5 to 2.5 h.

Discussion

The results of the present study demonstrate that the short-incubation LH assay provides reliable information about the preovulatory LH release in blood plasma of dogs. Basal LH concentrations recorded using the short-incubation assay were 3 times higher than those recorded after normal incubation. However, they were still in agreement with the previous studies (Concannon *et al.*, 1975; Chakraborty, 1987). The extremely high basal concentrations of LH presented in the study of Olson *et al.* (1982) have not been confirmed by others. The appearance of an extra LH release 4 days before the preovulatory LH peak found here in one bitch accords with a previous study (Chakraborty, 1987), although this extra LH peak was only detected with the normal incubation assay. Mean concentrations of preovulatory LH peaks were similar to those reported by Boyns *et al.* (1972), Concannon *et al.* (1975) and Fernandes *et al.* (1987), but were lower than those observed by Smith & McDonald (1974), Nett *et al.* (1975), Olson *et al.* (1982) and Chakraborty (1987). Because the duration of the preovulatory increase in LH (Nett *et al.*, 1975; Concannon *et al.*, 1975) is 18–48 h, it would appear that the collection of blood at 2-day intervals would be frequent enough to record the LH peaks. Results from the present study support these findings with regard to the normal incubation system. However, for the short-incubation assay presented here, as well as for the binding test, daily blood collection is recommended when the aim is to detect the preovulatory LH peak.

Since antiserum against ovine LH (GDN-15) is no longer available in large quantities it seems reasonable to evaluate other antisera which could be suitable for a rapid radioimmunoassay of dog LH. One possible antiserum might be a monoclonal antibody which reacts with LH from diverse mammalian species including dog LH (Matteri *et al.*, 1987).

With the present rapid radioimmunoassay of LH the best days for mating or artificial insemination of bitches can be predicted with great accuracy. This is particularly important when bitches are to be inseminated with frozen semen, because the post-thaw survival time of the spermatozoa is short. Although cytological studies of vaginal smears are easy to conduct, the findings are not specific enough to determine whether ovulation has taken place or when (Linde-Forsberg &

Forsberg, 1989). In practice, one progesterone result with concentration over 10 nmol/l during the oestrus allowed an approximation of the proper day for mating or insemination (C. Linde-Forsberg, unpublished results). Although the LH assay requires more frequent blood sampling than the progesterone assay, the possibility of predicting the day of ovulation in advance gives the breeder time to schedule the mating or insemination.

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