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NOTE

Theriogenology

Detection of urinary luteinizing hormone in Japanese black cows after administration of gonadotropin-releasing hormone

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ABSTRACT. The blood luteinizing hormone (LH) surge in cows is well studied. However, little is known about urinary LH in cows. This study examined urinary LH concentrations after administration of gonadotropin-releasing hormone (GnRH) in six Japanese black cows to induce LH secretion from the pituitary gland into the bloodstream. Abrupt rises in plasma and urinary LH were observed after GnRH administration. Plasma and urinary LH peaked at 2 and 5 hr, respectively. A positive correlation was observed between plasma LH concentrations and urinary LH amounts. Ovulation was confirmed in the cows after 48 hr of GnRH administration. These data strongly suggest that urinary LH is derived from plasma LH, which triggers ovulation in cows.

KEY WORDS: cow, luteinizing hormone (LH), ovulation, urine

The luteinizing hormone (LH) surge plays a dominant role in ovulation in mammals. It ends follicle-stimulating hormone (FSH)-dependent steroidogenesis and granulosa cell growth, promotes somatic cell differentiation into luteal cells, induces the expression of genes required for follicle rupture and ovulation, and activates multiple signaling cascades leading to oocyte maturation [3]. Monitoring the LH surge through frequent blood sampling provides the most direct evidence of approaching ovulation in cows. A recent study proposed detection of the serum LH surge as an alternative to frequent transrectal ultrasonography to monitor the disappearance of ovulatory follicles in estrous cows [1]. However, frequent blood sampling is impractical due to the need to restrain cows physically for series of invasive treatments.

Unlike blood sampling, it is easy to collect urine samples from cows. Urine is a convenient, valuable source for repetitive measurements of various biological markers. In medicine, urinary hormones and their metabolites, including LH, are commonly used to evaluate physiological state [2, 11, 14]. As well as the LH surge in blood, LH is detectable in the urine of women and has been already used as a biomarker to predict the onset of the fertile period [2, 11, 14]. Some studies have previously detected LH in urine samples of estrous Murrah buffaloes (*Bubalus bubalis*) [13] and Kangayam cattle (*Bos indicus*) [12]. However, no study has examined the urinary excretion of LH in other ungulates. Therefore, this study examined the temporal changes in urinary LH excretion in cows after administration of gonadotropin-releasing hormone (GnRH).

This experiment used six Japanese black cows, one primiparous and five multiparous (2.17 ± 0.31 parturitions), age range 2.6–4.8 (4.08 ± 0.37) years, days postpartum 85–353 (169.5 ± 40.6), and body weight (BW) 395–545 kg. The cows were housed with approximately 50 other cows in a dry-dirt lot with two covered shelters at Omyojin Farm of Iwate University. The study protocols were approved by the Animal Research Committee (A202023) and followed the guidelines for Animal Experiments of Iwate University.

The existence of a corpus luteum in the ovaries was confirmed by transrectal palpation and ultrasonography using a HS-101V ultrasonic system equipped with an HLV-155 5.0-MHz 50-mm linear transducer (Honda Electronics, Toyohashi, Japan), 3 days before GnRH administration. Next, 25 mg of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (dinoprost tromethamine; 5 ml Veterinary Pronalgon F Injection, Zoetis Japan, Tokyo, Japan) was administered to each cow by intramuscular injection to induce luteolysis for simultaneous GnRH administration. After confirmation of the existence of a dominant follicle with or without luteal regression in the ovaries by both transrectal palpation and ultrasonography, 100 µg of GnRH analogue (fertirelin acetate; 2 ml Conceral Injection, MSD Animal Health K. K, Tokyo, Japan) was administered to each cow by intramuscular injection. At 48 hr later, transrectal ultrasonography confirmed ovulation by the disappearance of the dominant follicle. The same researcher (T. H.) performed the transrectal palpation and ultrasonography.

Blood samples were collected from the jugular vein before and 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 24, and 48 hr after GnRH

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administration. The samples were placed on ice until centrifugation for plasma separation. Urine samples were collected from urination stimulated by rubbing and warming the genital region with the hands, cloth, or straw before and 1.5, 3, 4, 5, 6, 7, 8, 24, and 48 hr after the GnRH administration. The exact volume of each urine sample was measured using a measuring cylinder. Plasma and urine samples were aliquoted into tubes, frozen at −20°C until LH measurement, and analyzed within 1 week of sampling.

Plasma and urinary LH concentrations were measured using a Bovine LH ELISA Test kit (Endocrine Technologies, Newark, CA, USA) according to the manufacturer's instructions. The minimum detectable concentration of bovine LH with this assay is 0.25 ng/ml. A calibration curve was generated using bovine LH (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, USA) diluted to 10, 5, 1, 0.1, and 0.01 ng/ml using the diluent in the kit. Each sample was analyzed in duplicate, and this process was repeated until the coefficients of variation (CV) of duplicate absorbance values were within 10%. In each assay, 10 ng/ml bovine LH were measured and the intra- and inter-assay CV were 1.89% and 3.07%, respectively.

The ELISA kit used in this experiment was designed for the quantitative determination of serum and plasma LH in cows. Therefore, we first evaluated whether the kit could quantify the urinary LH concentration in a recovery test using pooled urine samples collected from the six cows before GnRH administration. Pooled urine spiked with LH standard at 1, 0.5, and 0.25 ng/ml, yielding 115.89, 108.30, and 97.32% recovery, respectively, which was within the reliable range of 80–120%.

To quantify the LH concentrations within the calibration curve, plasma samples obtained 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 hr after GnLH administration were diluted 10 times with the kit dilution buffer. Plasma samples obtained at 0.5, 1, and 2 hr from Cow A, and at 2 and 3 hr from Cow D were diluted to 20 times in the same manner. Urine samples obtained at 1.5, 3, 4, 5, 6, 7, and 8 hr from Cows B and C were concentrated using centrifugal filter units (Centriprep-10 K, Merck Millipore, Tullagreen, Carrigtwohill, County Cork, Ireland). The urinary excretion of LH was calculated from the LH concentration and the volume of each urine sample. Samples with LH concentrations under the detection limit of the ELISA kit were considered to be 0 ng/ml in the statistical analysis. Creatinine in the urine samples was measured using Folin's method [4] to normalize the urinary LH concentration.

Statistical analyses were performed in SPSS ver. 27 (IBM, Chicago, IL, USA). Temporal changes in plasma LH concentrations and the urinary LH to creatinine ratio were analyzed by the Friedman test as nonparametric repeated-measures analysis. Then comparison to the values before GnRH administration were examined by Wilcoxon matched-pair signed ranks test with exact P values calculated. A one-tailed test was used to assess whether urinary LH increased after GnRH administration. Plasma LH concentrations and urinary LH were compared using Spearman's rank correlation analysis. A P-value <0.05 was considered significant.

Corpus luteum whose diameters were over 15 mm were observed in the ovaries of all cows at $PGF_{2\alpha}$ administration, 3 days before GnRH administration. The existence of dominant follicles with diameters >11 mm in all cows treated with $PGF_{2\alpha}$ was confirmed, except for Cow F whose 17-mm-diameter follicle ruptured during the process of transrectal palpation at $PGF_{2\alpha}$ administration. The ovulation was confirmed by the disappearance of dominant follicles within 48 hr after GnRH administration.

All cows, including Cow F, exhibited increased plasma LH concentrations beginning 0.5 hr after GnRH administration. The highest plasma LH concentration was observed at 1 hr in two cows (A and B), at 2 hr in three cows (C, D, and E), and at 3 hr in one cow (F). Individual differences were observed among the cows in terms of the highest plasma LH concentration, from 17.2 (Cow C at 2 hr) to 83.2 (Cow D at 2 hr) ng/ml. Plasma LH concentrations then decreased in all cows to below 5 ng/ml by 7 hr after GnRH administration. Statistical analysis revealed significant temporal changes in plasma LH concentrations after GnRH administration in all six cows (Friedman test) and median concentrations at 0.5, 1, 2, 3, 4, and 5 hr were significantly higher than at 0 hr (Wilcoxon matched-pairs signed rank test, one-tailed P<0.05) (Fig. 1A). The median plasma LH concentration was the highest at 2 hr (43.9 ng/ml).

Urine samples were obtained from the six cows at all sampling points, except for Cow F, which did not urinate at 1.5, 3, 5, and 7 hr. Urine volumes ranged from 20–1,410 (median 390) ml. Urinary LH concentrations were under the detection limit in the six cows at 0 hr. Urinary LH was detectable until 4 hr after the GnRH administration in all six cows. The highest urinary LH concentrations varied between 0.21 and 1.56 ng/ml among the six cows. The urinary LH concentrations were normalized using urinary creatinine concentrations ranged from 0.5–5.2 (median 2.4) mg/ml. The highest urinary LH to creatinine ratio was observed at 3 hr in Cow C, at 4 hr in Cows A, B, and E, at 5 hr in Cow D, and at 6 hr in Cow F. The highest urinary LH-creatinine ratio in the six cows ranged from 0.066 (Cow F, at 6 hr) to 0.994 (Cow D, at 5 hr) ng/mg. The median of the ratio was the highest at 5 hr (0.131 ng/ml). The urinary LH-creatinine ratio in the six cows decreased until 24 hr after GnRH administration. Statistical analysis revealed significant temporal changes in the urinary LH-creatinine ratio after GnRH administration in the six cows (Friedman test). The medians of the ratio at 3, 4, 5, and 6 hr were significantly higher than that at 0 hr (Wilcoxon matched-pairs signed rank test, one-tailed P<0.05) (Fig. 1B). A correlation analysis between plasma LH concentration and urinary LH excretion using the highest and second-highest values revealed a significant correlation between plasma LH concentration and urinary LH (Spearman's rho 0.776; P=0.0030, Fig. 2).

This study demonstrated LH urinary excretion in cows after administration of GnRH. A transient increase was observed in urine LH levels following the increase in plasma LH concentrations in all cows tested, and ovulation was confirmed 48 hr after GnRH administration, as per previous reports [6–9]. In our experiment, the peak urinary LH-urinary creatinine ratio was observed between 3 and 6 hr (median 5 hr) after GnRH administration in each cow, which was approximately 3 hr later than that of the plasma LH concentration. Considering the positive correlation between the urinary LH amount and plasma LH concentration in cows, we strongly suggest that urinary LH is derived from plasma LH via the kidneys.

Some hormones excreted into the urine are considered potential biomarkers in cows and other ungulates. For example, urinary pregnanediol glucuronide, a common urinary metabolite of progesterone that reflects serum progesterone concentrations, is useful for discriminating between the presence and absence of a functional corpus luteum and monitoring the reproductive status, such as

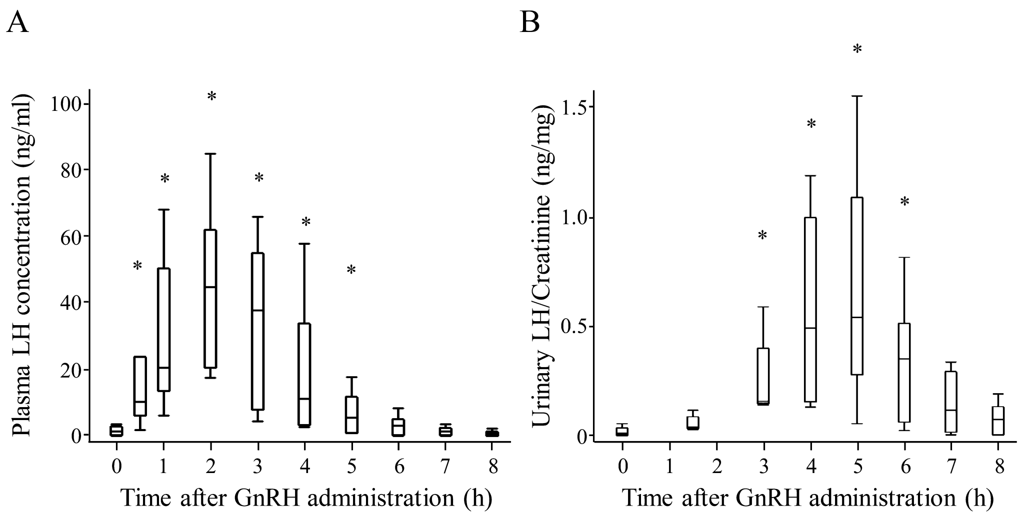

Fig. 1. Temporal changes in plasma luteinizing hormone (LH) concentrations (A) and urinary LH to creatinine ratio (B) in the six cows administered gonadotropin-releasing hormone (GnRH). Boxes show the median and interquartile range, and whiskers are the minimum and maximum. Temporal changes after GnRH administration were evaluated using the Wilcoxon matched-pairs signed rank test (one-tailed, $P<0.05$); asterisks indicate significantly higher values compared to the value before administration.

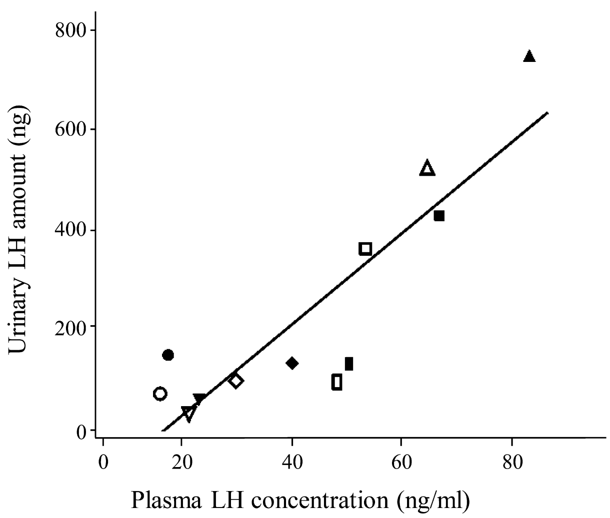

Fig. 2. The relationship between the highest and second-highest plasma luteinizing hormone (LH) concentrations and urinary LH amounts in the six cows. Y=9.03× −146.77 (R^2=0.77, P=0.0002). The highest and second-highest values are indicated by closed and open marks, respectively. The same marks indicate the same individuals.

estrus and pregnancy, in dairy cows [16]. Urinary LH is thought to predict ovulation in Murrah buffaloes, which naturally exhibit silent estrus [13]. Considering the transient increase in urinary LH levels before ovulation, we believe that urinary LH is a potential biomarker that predicts ovulation in cows. Urinary LH is detectable for only a few hours after the plasma LH increase, but is not detectable before or 24 hr after the GnRH administration. Therefore, the detection of LH in urine obtained by spot sampling from subject cows would be sufficient to predict ovulation, even if the peak of urinary LH excretion is missed.

One problem when deciding the appropriate period for artificial insemination is the decreased intensity of estrus behavior in high-milk-producing cows [15]. Heat stress also has a detrimental effect on fertility, including silent ovulation without estrus signs in cows under conditions of global warming [5]. Instead of behavioral observation, measurements of estrous expression using automated activity monitors have been applied to improve the pregnancy rates after artificial insemination [10]. In addition to such techniques, measuring urinary LH may also be useful for determining the optimum time for artificial insemination in cows with silent ovulation.

In conclusion, urinary LH increases following the plasma LH rise after GnRH administration in cows. In our experiment, the urinary LH peak was approximately 3 hr after the plasma LH peak. Our findings suggest that non-invasive monitoring of urinary LH excretion may predict ovulation in cows and thereby determine the optimal insemination time.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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