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—Original Article—

Relationship between bovine endometrial thickness and plasma progesterone and estradiol concentrations in natural and induced estrus

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Abstract. The objective of this study was to investigate cyclical changes in endometrial thickness in relation to progesterone (P_4) and estradiol-17β (E_2) concentrations during natural and induced estrus in 15 cows. In the prostaglandin (PG) $F_{2\alpha}$-induced estrus group, ultrasonography (USG) at 6-h intervals was used to determine endometrial thickness 48–24 h before the $PGF_{2\alpha}$ treatment until 24 h after ovulation (ovulation = Day 0). In the natural estrus group, USG was performed every 48 h from Day 3 to Days 15–18 after the first ovulation, and then every 6 h until 24 h after ovulation. Endometrial thickness was standardized using Day 13 as a reference day. Blood was collected during every USG examination and plasma P_4 and E_2 concentrations were determined. Endometrial thickness of the induced estrus group (n = 11) was greater than that of the natural estrus group (n = 9) between 60 and 12 h before ovulation ($P < 0.05$). In the natural estrus group, prior to an increase in endometrial thickness, a decrease in P_4 and an increase in E_2 were detected. In the induced estrus group, based on the time of ovulation, an increase in endometrial thickness was detected at the same time of a decrease in P_4 before an increase in E_2. These results suggest that decreases in P_4 concentrations may be a cue to changes in endometrial thickness, while increases in E_2 concentrations appear to sustain and/or enhance these changes.

Key words: Dairy cows, Edema, Endometrium, Estrous cycle, Ultrasonography

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The endometrium of mammals is essential for the establishment and maintenance of pregnancy. In women, the endometrium repeatedly grows and is lost during the menstrual cycle to maintain its function. Thus, endometrial thickness, which is typically assessed by ultrasonography (USG), may be indicative of fertility. Ultrasonography is often performed on patients undergoing *in vitro* fertilization and embryo transfer (IVF-ET) [1] and intrauterine insemination [2]. A thin [3, 4] or too thick [4, 5] endometrium has been linked to reduced fertility. Endometrial thickness has also been evaluated in mares [6] and cattle [7–10]. In cattle, studies focused on postpartum uterine involution [8, 10] and diagnoses of endometritis [8]. Other studies reported physiological changes in uterine morphology and endometrial thickness during the estrous cycle [7, 9]. Only one study [9], to the best of our knowledge, has examined the relationship between endometrial thickness and fertility in cows. More studies on the morphological changes in the endometrium including changes in endometrial thickness need to be conducted in relation to fertility and artificial breeding technologies in cattle since these changes may reflect the balance between ovarian steroid hormones and the capacity of the endometrium to respond to these hormones.

A histological examination of the bovine uterus during an induced estrous cycle revealed that an increase in endometrial thickness occurred because of hyperemia and edematous development during the follicular phase [11]. In a previous study [7] that characterized the ultrasonographic anatomy of the uterus during the estrous cycle in heifers, the thickness of the uterine wall was shown to increase during the luteal regression period and gradually decrease after ovulation. Another study [9] found a rapid increase in endometrial thickness in cows treated for timed artificial insemination (AI) within 24 h of induced luteolysis. Although conception rates are generally accepted to not significantly differ between natural and induced estrus in cattle [12–14], some studies described a higher conception rate in natural estrus groups than induced estrus groups following a treatment with prostaglandin (PG) $F_{2\alpha}$ [15, 16]. An extended period of follicular dominance [17] or the induction of ovulation in smaller dominant follicles [18–20] in the ovulation synchronization protocol have been attributed to decreased fertility and/or reduced embryonic development. These rapid changes in endometrial thickness in cows undergoing timed AI [9] may be another source or indicator of differences in fertility between natural and induced estrus groups. Therefore, changes in endometrial thickness between natural and induced estrus groups need to be examined in more detail.

Steroid hormones play a central role in the regulation of uterine functions including morphological changes in the endometrium during

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the estrous cycle [7, 21–24]. Although it is suggested that changes in the endometrium may correlate with peripheral progesterone (P_4) concentrations in cattle [9, 25] and estradiol-17β (E_2) concentrations in cattle [7, 9, 26, 27], ewes [28, 29], and humans [2, 30, 31], the relationship between endometrial thickness and steroid hormones has not yet been fully elucidated.

Furthermore, endometrial thickness may be affected by parity and, more importantly, individual differences. Therefore, some means of standardization needs to be incorporated when endometrial thickness is evaluated using a random group of cattle on commercial farms. Endometrial thickness was standardized in this study using a reference day during the luteal phase, and changes in endometrial thickness were analyzed in relation to those in P_4 and E_2 concentrations during natural and induced estrus.

Materials and Methods

Animals

This study was conducted according to the institutional guidelines for animal experiments of Rakuno Gakuen University (approval No. VH25C3). Fifteen lactating cows (eight primiparous and seven multiparous), between 35 and 86 days post-partum, from the Rakuno Gakuen University farm were used in this study. Cows were housed in freestall facilities. Lactating cows were milked twice daily and were fed a total mixed ration (TMR) formulated to meet or exceed dietary nutritional requirements for lactating dairy cows (NRC, 2001). None of the cows had been given any hormonal treatments to induce and/or synchronize estrus and ovulation prior to this study. Cows were found to be clinically normal with no abnormalities in their reproductive organs after examinations that included transrectal palpations, vaginoscopies [32], transrectal USGs of the genital tracts [33] and ovarian structures [34], and cytologies of the endometrium [35].

Induction and detection of estrus

Cows received a controlled internal drug release containing 1.9 g of progesterone (CIDR, Zoetis JAPAN, Tokyo, Japan), which was maintained for 5–7 days. Cows were then given $PGF_{2\alpha}$ (25 mg, dinoprost tromethamine, Pronalgon F, Zoetis JAPAN) at the time of the withdrawal of the CIDR device. During every examination, at least two individuals monitored the estrous behavior of the cows for 30 min. Estrus was determined by the detection of estrous behavior, such as sniffing the vagina of another cow, resting with the chin on another cow, and mounting.

Evaluation of endometrial thickness and the ovarian structure by USG

All USG examinations were conducted by one of three individuals. The ovaries and uteri of cows were examined transrectally using an ultrasonograph (HS-2100V, HONDA ELECTRONICS, Aichi, Japan) attached to a linear transducer (5–10 MHz, HLV-475M, HONDA ELECTRONICS). The transducer was inserted into the rectum to examine the ovaries and a cross-section of the uterus. All examinations were performed with the following settings: frequency 7.5 MHz, depth 3 cm, acoustic power 60%, and gain 86 dB. Scanning of the uterus was performed carefully and slowly along the dorsal surface of each uterine horn and care was taken to avoid deformation. Cross-sectional frozen images at the attachment of the intercornual ligament on each uterine horn were stored [36]. A moving USG image was also digitally recorded (VR570, TOSHIBA TELI CORPORATION, Tokyo, Japan) and used for the measurement of endometrial thickness when the frozen image taken on the farm was considered inappropriate. The endometrium, the inner region of the uterus surrounded by the myometrium, was characterized by an echoic appearance with dark and bright signals [24]. Endometrial thickness was assessed using ImageJ (Rasband, W.S., ImageJ, U.S. National Institute of Health, Bethesda, USA, http://imagej.nih.gov/ij/, 1997–2016). Since the image of the endometrial structure being scanned was often not circular, the diameter was estimated by averaging the major and minor axes of the endometrial area. If an intraluminal cavity was detected, the average of the major and minor axes of the cavity area was subtracted from endometrial thickness. Endometrial thickness was analyzed for each uterine horn in a separate frozen image and then averaged to establish the final measurement. In this study, no significant differences in endometrial thickness were observed between the left and right uterine horns. In the ovaries, the sizes of the corpus luteum and largest follicle were measured during every examination. The diameters of these ovarian structures were calculated by averaging the major and minor axes. The time of ovulation was defined as the time at which ovulation was first detected. Ovulation occurred in all cows within 48 h of the last detection of estrous behavior.

Blood collection

During every USG examination, approximately 20 ml of blood was collected from the jugular or caudal vein using a 20-ml syringe with an 18-gauge needle. Blood was transferred into EDTA-loaded vacuum tubes (sterile, Venoject II EDTA, TERUMO, Tokyo, Japan) and placed on ice for about 30 min before plasma was separated by centrifugation at 1530 × *g* for 15 min. Plasma samples were stored at –30°C until assayed.

Hormone assays

Plasma concentrations of P_4 and E_2 were evaluated using competitive double-antibody enzyme immunoassays (EIA) as previously described [37]. The primary antisera used for P_4 and E_2 assays were rabbit anti-progesterone-3-CMO-BSA serum (KZ-HS-P13, Cosmo Bio, Tokyo, Japan) and rabbit anti-estradiol-17β-6-CMO-BSA serum (FKA204, Cosmo Bio), respectively. Goat anti-rabbit IgG antiserum (111-005-003, Jackson ImmunoResearch, PA, USA) was used as the secondary antiserum. All samples were assayed in triplicates. The intra- and inter-assay coefficients of variation were 5.4 and 8.1% for P_4, and 5.0 and 14.6% for E_2, respectively.

Experimental design

Diagrammatic time lines of the experimental procedures used on the animals are shown in Fig. 1 and Table 1, respectively. First estrus was induced in all cows (n = 15) using a combination of a CIDR device and $PGF_{2\alpha}$. The presence of the corpus luteum and a large, presumptive dominant follicle were confirmed on the day of the first CIDR treatment by USG. Cows No. 1–9 (n = 9) were allowed to show natural estrus for the second estrous cycle (the natural estrus

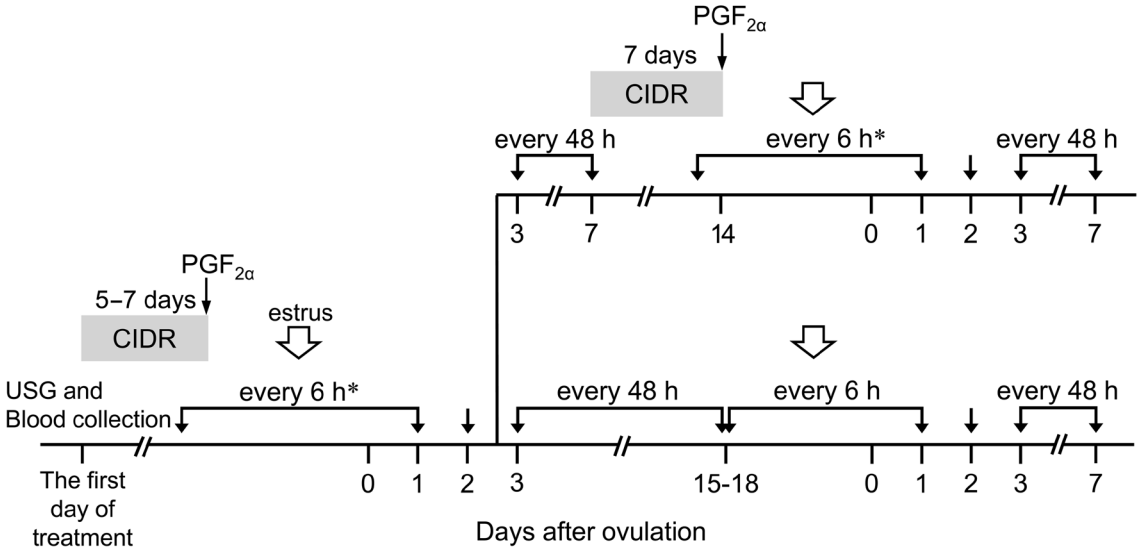

Fig. 1. Schedules of treatments and examinations of animals. Fifteen cows were treated with a combination of a controlled internal drug release (CIDR) device and prostaglandin (PG) $F_{2\alpha}$ (25 mg, dinoprost tromethamine) to induce estrus. Nine cows were allowed to show natural estrus (lower line, cows No. 1–9), while estrus was induced for the second time in 6 cows (upper line, cows No. 10–15). The presence of the corpus luteum and a large, presumptive dominant follicle were confirmed on the day of the first CIDR treatment by ultrasonography (USG). Day 0 = day of ovulation. See Table 1 for the use of individual cows. *USG at 6-h intervals was started 48–24 h before the $PGF_{2\alpha}$ treatment.

Table 1. Use of data from 30 estrous cycles in 15 cows

Cow No.	Parity	Days after parturition *	Selection of the reference date **	Experimental group	
			First estrous cycle	First estrous cycle	Second estrous cycle
1	5	73	+	–	Natural
2	1	61	+	–	Natural
3	1	68	+	–	Natural
4	1	40	+	–	Natural
5	1	39	+	–	Natural
6	1	36	+	–	Natural
7	6	80	+	Induced	Natural
8	2	40	+	Induced	Natural
9	2	35	+	Induced	Natural
10	2	35	–	Induced	Induced
11	2	36	–	Induced	Induced
12	3	86	–	–	Induced
13	1	52	–	–	Induced
14	1	43	–	–	Induced
15	1	37	–	–	Induced

* Days from parturition to initiation of the first CIDR treatment. ** Data from 9 cows (No. 1–9) were used to select a suitable date for standardization (Fig. 2). Estrus (first cycle) was induced in all 15 cows with a combination of a CIDR and $PGF_{2\alpha}$ (Fig. 1). Nine out of 15 cows were allowed to show natural estrus, while estrus was induced for the second time in 6 cows. Data from 9 cows in the second estrous cycle were used as the natural estrus group. Data from 5 and 6 cows in the first and second estrous cycles, respectively, were used as the induced estrus group. Data from 10 cows (No. 1–6 and No. 12–15) in the first estrous cycle were excluded from the analysis since USG at 6-h intervals was started after the $PGF_{2\alpha}$ treatment in these cows.

group), while estrus was induced for the second time in cows No. 10–15 (n = 6) (Fig. 1). Data from 11 out of 21 induced estruses were combined (the induced estrus group) (Table 1).

In the induced estrus group, USGs of endometrial thickness were conducted at 6-h intervals from 48 to 24 h before the $PGF_{2\alpha}$ treatment and continued until 24 h after ovulation (ovulation = Day 0). Thereafter, cows were examined on Days 2 and 3 after ovulation, then every 48 h until Day 7. All cows were examined on Day 13.

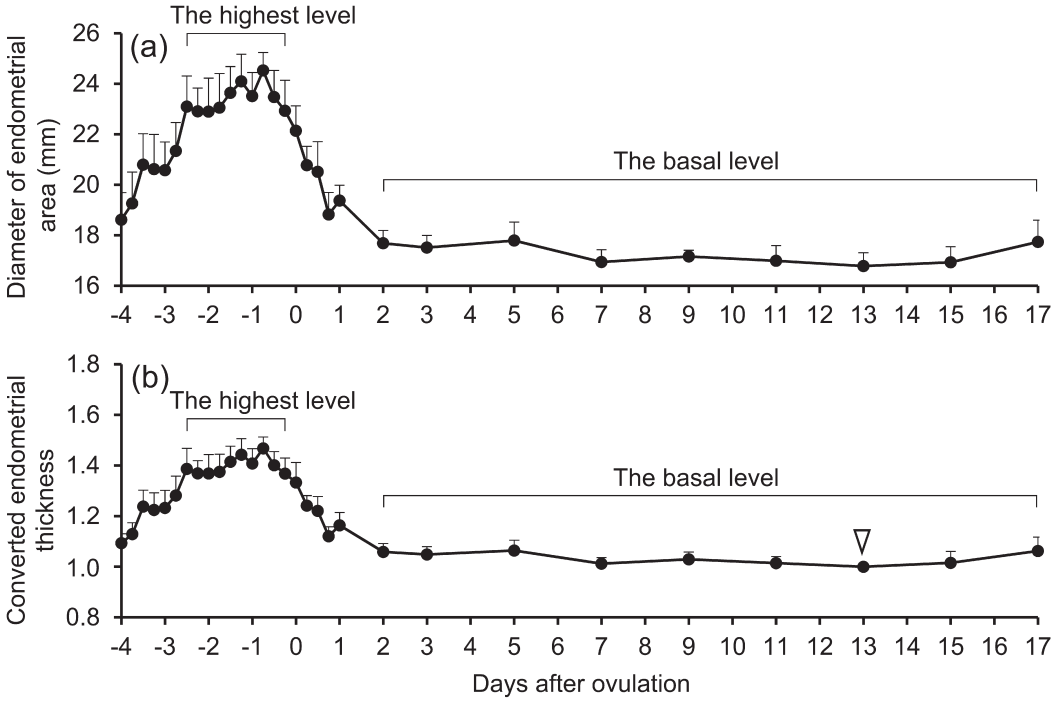

Fig. 2. Changes in endometrial thickness shown as actual measurements (a) and standardizations (b). Endometrial thickness was standardized by converting values to a ratio of endometrial thickness on the reference day (Day 13, white arrow head) using data obtained from nine cows (No. 1–9, Table 1) during first estrus and the subsequent luteal phase (mean ± SEM). Endometrial thickness was at the highest levels during the preovulatory period (60–6 h before ovulation) and at basal levels during the post-ovulatory period and luteal phase (Days 2–17) in both (a) and (b).

Data from 10 (No. 1–6 and No. 12–15, Table 1) out of 21 induced estruses during the first estrous cycle were excluded from the analysis since USG was conducted at 6-h intervals after the $PGF_{2\alpha}$ treatment in these cows.

In the natural estrus group, USG for 9 cows (No. 1–9, Fig. 1, lower line) was performed every 48 h from Day 3 to Days 15–18 after the first ovulation, and then every 6 h until 24 h after ovulation. USG examinations were conducted using the same schedule in the induced estrus group until Day 7.

Data analysis

The endometrial thickness, P_4 and E_2 concentrations data are represented as mean ± SEM. The endometrial thickness data of the first nine cows during the first estrous cycle (No. 1–9, Table 1) was used to set the most suitable reference day for the standardization of endometrial thickness during the estrous cycle. The endometrial thickness data from these nine cows in the first estrous cycle were compared using a one-way ANOVA followed by the Tukey-Kramer multiple-comparison test in Statcel (3rd ed., OMS, Saitama, Japan) [38]. The reference day of the estrous cycle was set on a day when minimum thickness and variations occurred. Endometrial thickness was then converted to a ratio on Day 13 (the reference day). The endometrial thickness, P_4, E_2 and E_2/P_4 ratio of the natural and induced estrus groups were compared using a two-way ANOVA with repeated measures followed by a post hoc analysis where appropriate. The two groups on the same days were compared using the Student's *t*-test. Values at different time points of endometrial thickness, P_4 and E_2 concentrations within the same group were compared using the Tukey-Kramer multiple-comparison test.

Results

Changes in endometrial thickness are shown in Fig. 2. Actual measurements of endometrial thickness (diameter of endometrial area) between 60 and 6 h before ovulation (follicular phase: 23.4 ± 0.3 mm, range: 16.9–29.6 mm, n = 9, Fig. 2a) was at the highest levels and greater ($P < 0.05$) than between Days 2 and 17 (post-ovulatory to luteal phase: 17.2 ± 0.2 mm, range: 13.4–22.8 mm, the basal levels). All data were converted to a ratio of endometrial thickness on Day 13 (16.8 ± 0.5 mm, range 13.4–18.7 mm, n = 9, Fig. 2b) to compensate for variations in endometrial thickness between individuals. Converted endometrial thickness was also highest and basal levels during the same periods.

Changes in endometrial thickness were converted to a ratio on Day 13 and the two groups were compared based on the time of ovulation (Fig. 3a). Endometrial thickness in the induced estrus group (closed circle) was greater than that in the natural estrus group (open circle) 60, 54, 36, 30, 18 and 12 h before ovulation ($P < 0.05$). However, it was similar in both estrus groups 6 h before ovulation. Peaks in endometrial thickness in the natural and induced estrus groups were 1.38- and 1.61-fold greater than basal levels (Day 13 as the reference day), respectively. Changes in P_4, E_2 and E_2/P_4 ratio were similar in

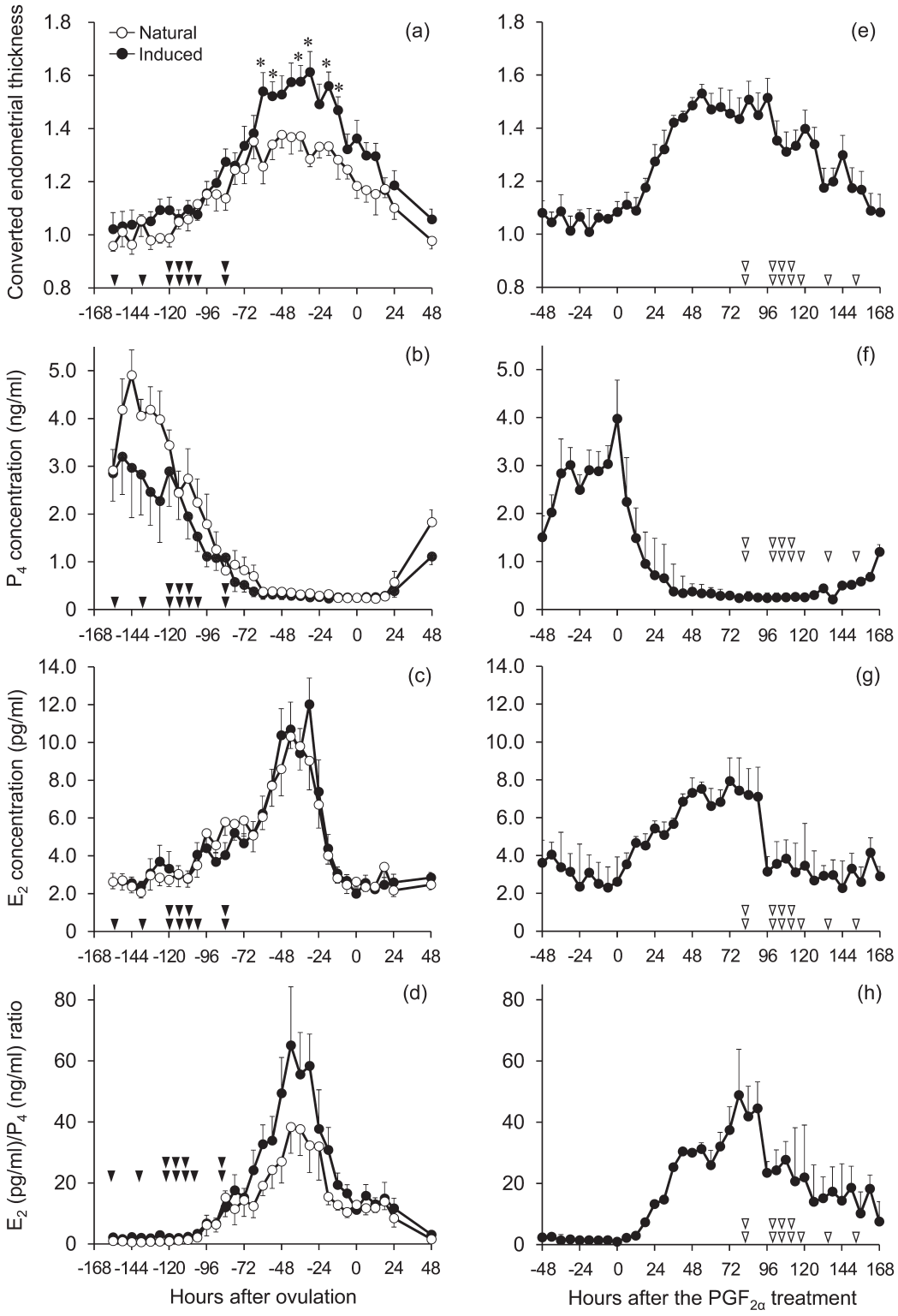

Fig. 3. Changes in endometrial thickness and progesterone (P_4) and estradiol-17β (E_2) concentrations in natural and prostaglandin (PG) $F_{2\alpha}$-induced estrus. Open and closed circles indicate natural (n = 9) and induced estrus (n = 11) groups, respectively. Data (mean ± SEM) were shown relative to the time of ovulation (a–d) and the $PGF_{2\alpha}$ treatment (e–h). Endometrial thickness was converted to a ratio based on the reference day (Day 13). The $PGF_{2\alpha}$ treatments (black arrow head) or ovulations (white arrow head) were shown in each panel. * Values differ from the natural estrus group at the same time points ($P < 0.05$).

Table 2. Numbers representing changes in endometrial thickness and plasma steroid hormones

Evaluation item	Natural estrus			Induced estrus		
	Endometrial thickness	P_4	E_2	Endometrial thickness	P_4	E_2
Durations between (h)						
the time an increase or decrease was first detected and ovulation	114	138	132	144 †	144 †	102 †
the $PGF_{2\alpha}$ treatment and the time an increase or decrease was first detected	–	–	–	6 #	6 #	6 #
the time the highest or basal levels were first reached and ovulation	96	66	54	66 †	78 †	48 †
the time an increase or decrease was first detected and the highest or basal levels were reached	18	72	78	18 # 78†	12 # 66 †	0 # 54 †
the time the highest level was first reached and a decrease was detected (= at the highest level)	102	–	24	54 † 102 #	–	18 † 84 #
the first time a decrease was detected and the basal level was reached	42	–	36	60 †	–	18 †
ovulation and the basal level	48	–	6	48 †	–	–6 †
Ratio to the basal value (Day 13 §)						
Highest point	1.38 (–48 h)	–	–	1.61†/1.53# (–30 h†/54 h#)	–	–
Means of the highest levels	1.27 (from 96 h before and 6 h after ovulation)	–	–	1.53†/1.42# (from 66 to 12 h before ovulation/ from 24 to 126 h after the $PGF_{2\alpha}$ treatment)	–	–

Values between different time points were compared by the Tukey-Kramer multiple comparison test. The time an increase/decrease was first detected and the time to the highest/basal levels were found in endometrial thickness and P_4 and E_2 concentrations, respectively. † Data were relative to the time of ovulation (Fig. 3a–c). # Data were relative to the time of the $PGF_{2\alpha}$ treatment (Fig. 3e–g). § Basal levels were found between Days 2 and 17, and Day 13 was taken as the reference day (Fig. 2).

both groups during the examination period.

Details of changes in endometrial thickness and steroid hormones over time are shown in Table 2. In the natural estrus group, endometrial thickness started to increase 114 h before ovulation and reached its highest levels 96 h before ovulation (Fig. 3a). Endometrial thickness remained at its highest levels until 6 h after ovulation and then started to decrease. Although endometrial thickness did not return to basal levels within 24 h of ovulation, during which USG was performed every 6 h, it returned to basal levels by 48 h after ovulation. Prior to changes in endometrial thickness, a decrease in P_4 concentrations and increase in E_2 concentrations were detected 138 and 132 h before ovulation, respectively (Fig. 3b and c). Progesterone concentrations reached their basal levels 66 h before ovulation. Estradiol-17β concentrations reached their highest levels 54 h before ovulation, remained at their highest levels until 30 h prior to ovulation, and then returned to basal levels by 6 h after ovulation. The duration between the time a decrease in P_4 concentrations was initially detected (138 h before ovulation) and the time endometrial thickness reached its highest levels (96 h before ovulation) was 42 h (Table 2).

In the induced estrus group, endometrial thickness and P_4 concentrations started to change 144 h (6 days) before ovulation (Fig. 3a and b). Estradiol-17β concentrations started to increase 102 h before ovulation (Fig. 3c, closed circle). Endometrial thickness reached its highest levels 66 h before ovulation, remained at its highest levels until 12 h before ovulation, and then decreased to basal levels by 48 h after ovulation. Progesterone concentrations reached their basal levels 78 h before ovulation. Estradiol-17β concentrations reached their highest levels 48 h before ovulation, remained at their highest levels until 30 h before ovulation, and then returned to basal levels by 6 h before ovulation.

When data were analyzed based on the time of the $PGF_{2\alpha}$ treatment (0 h, Fig. 3e–g, Table 2), endometrial thickness and P_4 and E_2 concentrations started to change immediately (6 h) after the $PGF_{2\alpha}$ treatment. Endometrial thickness increased rapidly 12 h after the $PGF_{2\alpha}$ treatment (Fig. 3e), and reached its highest levels 24 h after the $PGF_{2\alpha}$ treatment (endometrial thickness started to increase 6 h after the $PGF_{2\alpha}$ treatment and reached its highest levels 18 h after the initiation of an increase in endometrial thickness, Table 2). Endometrial thickness remained at its highest levels until 126 h after the $PGF_{2\alpha}$ treatment, and then started to decrease. Endometrial thickness did not return to basal levels by 168 h (Day 7) after the $PGF_{2\alpha}$ treatment. Progesterone concentrations reached their basal levels 18 h after the $PGF_{2\alpha}$ treatment (Fig. 3f). Estradiol-17β concentrations started to increase and reached their highest levels 6 h after the $PGF_{2\alpha}$ treatment, remained at their highest levels until 90 h after the $PGF_{2\alpha}$ treatment, and then started to decrease (Fig. 3g). Estradiol-17β concentrations had not returned to basal levels by 168 h (Day 7) after the $PGF_{2\alpha}$ treatment. The duration between the time a decrease in P_4 concentrations was initially detected (6 h after the $PGF_{2\alpha}$ treatment) and the time endometrial thickness reached its highest levels (24 h after the $PGF_{2\alpha}$ treatment) was 18 h (Table 2).

Discussion

In this study, endometrial thickness in the follicular phase (between 60 and 6 h before ovulation) of natural estrus was greater than that in the post-ovulatory to luteal phase (Days 2–17, Day 0 = ovulation, $P < 0.05$, Fig. 2a). These results were similar to previous findings that endometrial thickness varied between the stages of the estrous cycle [7, 9, 23, 24]; it started to increase approximately 3–4 days before ovulation [7, 9, 23, 24] and decreased approximately 1 day before to 3 days after ovulation [7, 9, 24, 25].

To the best of our knowledge, this study is the first to compare changes in endometrial thickness between cows that have undergone natural and induced estrus. Changes in endometrial thickness during the estrous cycle have been described in cattle that have experienced natural [7] and induced estrus [9]. However, these studies used animals in different categories (heifers *vs*. cows, respectively) and assessed uterine thickness in different sections of the uterus (uterine body *vs*. horns, respectively) with different measurement methods (uterine wall *vs.* endometrium, respectively). Our results showed that increases in endometrial thickness were more rapid and greater in induced than natural estrus groups (Fig. 3a and e). The duration between the time a decrease in P_4 concentrations was initially detected and endometrial thickness reached its highest levels was shorter in induced estrus (18 h, Table 2 and Fig. 3e and f) than natural estrus groups (42 h, Table 2 and Fig. 3a and b). Endometrial thickness increased to 1.38- and 1.61-fold of the basal levels in the natural and induced estrus groups, respectively (Table 2). The rapid change observed in endometrial thickness in the induced estrus group is consistent with that of previous studies [7, 9]. Endometrial thickness reached its maximal levels within 24 h of the induction of luteolysis [9], while it started to increase 3–4 days before ovulation and reached its maximal values the day before ovulation in natural estrus [7].

This study also examined changes (every 6 h) in endometrial thickness and P_4 and E_2 concentrations to determine the roles of two ovarian steroid hormones in the regulation of endometrial thickness. Previous studies suggested that steroid hormones play a central role in the regulation of functional and morphological changes in the endometrium [7, 21–24] including changes in endometrial thickness during the estrous cycle [7, 23, 24]. The relationship between endometrial thickness and E_2 concentrations was previously reported in humans [2, 30, 31], rats [39], and ewes [28]; however, information is limited in cattle [9]. Besides E_2, the results of a previous study [9] indicated that P_4 effected changes in endometrial thickness. Higher basal P_4 concentrations (≥ 0.5 ng/ml) during the periovulatory period were associated with reduced endometrial thickness (≤ 8 mm), while basal P_4 concentrations (≤ 0.5 ng/ml) were associated with increased endometrial thickness (≥ 8 mm). The results of this study were not sufficient to clarify which factor (i.e., P_4, E_2 concentrations or the E_2/P_4 ratio) is responsible for changes in endometrial thickness or how this regulation is coordinated. However, a detailed analysis of the timing of changes in P_4 and E_2 concentrations and endometrial thickness may provide an explanation (Table 2 and Fig. 3). Decreases in P_4 concentrations may be a cue to changes in endometrial thickness, while increases in E_2 concentrations may sustain and/or enhance these changes. According to these results, endometrial thickness started to increase as or after P_4 concentrations began to decrease with or without increasing E_2 concentrations (Table 2). In the induced estrus group, endometrial thickness reached its highest levels after P_4 concentrations returned to basal levels.

Endometrial thickness generally appeared to have the strongest correlation with the E_2/P_4 ratio in relation to timing and the extent of changes in the natural and induced estrus groups (Fig. 3a and d and 3e and h) indicating that E_2 plays a role in the maintenance and enhancement of endometrial changes. However, since endometrial thickness reached its highest levels earlier than E_2 concentrations (96 h and 54 h before ovulation, respectively, Table 2) in the natural estrus groups, E_2 concentrations at moderate levels may be sufficient to attain a maximal response in endometrial thickness.

In this study, $PGF_{2\alpha}$ was given, in the presence of a large, presumptive dominant follicle (Day 6–7 or Day 14), to induce estrus, thus, changes in P_4 and E_2 concentrations occurred more rapidly than in the natural estrus group (Table 2). As a result, E_2 concentrations reached the highest levels 18 h earlier than endometrial thickness. These hormonal changes following the $PGF_{2\alpha}$ treatment may be attributed to differences in changes in endometrial thickness between natural and induced estrus groups.

To determine the roles of P_4 and E_2 in the regulation of endometrial thickness, the molecular mechanisms underlying edema, such as the involvement of vascular epithelial growth factor (VEGF), a potent regulator of vascular permeability and angiogenesis, need to be examined. Previous studies reported that estrogen induces edema in the endometrium by increasing VEGF, which mediates vascular hyperpermeability in rodents [40–42] and humans [43]. Estradiol-17β is also associated with an increase in endometrial microvascular volume in ewes [28, 29] and cows [27]. In the bovine endometrium, VEGF mRNA and protein levels were highest at estrus and the early luteal stage, respectively [44]. Progestins regulate the expression of VEGF in the rodent uterus [41], monkey endometrium [45], and human endometrial stromal cells [46]; however, their effects are less potent and slower in onset than those of estrogen [41]. Details on VEGF and related molecules in the bovine endometrium between luteal regression and ovulation may provide new insight into the molecular mechanisms underlying edema in the endometrium.

In this study, we used cows in different parities and at different days after parturition (Table 1). Thus, it was necessary to compensate for variations in endometrial thickness between individuals resulting from different uterine sizes [9]. Although the use of heifers may be advantageous in that there are small individual differences in the size of the uterus [7], multiparous cows also need to be examined in order to establish a method that evaluates endometrial thickness objectively. In this study, all values for endometrial thickness were converted to a ratio of endometrial thickness on a reference day (Day 13) in the luteal phase (Fig. 2b) when endometrial thickness showed minimum variations due to the absence of content in the uterine lumen and uterine contractility [22]. Although day 13 was used as the reference day in this study, other days in the luteal phase (Days 2–17) could also have been used.

Endometrial thickness assessed by USG at 6-h intervals during the follicular phase showed marked variations between examinations. The reasons for this may include contractions of the uterus and rectum and other technical factors. Transrectal palpation or positioning of the USG probe on the uterus may stimulate uterine

contractions at the time of the examination. However, this may not have been the case in this study. In our preliminary study, in captured videos of USG, endometrial thickness showed negligible changes when the shapes of uterine cross-sections varied with contractions. Endometrial thickness as an average value of the major and minor axes of the endometrial area in a cross-section of the uterine horn was assessed. The uterine cavity was also measured in the same manner and subtracted from endometrial thickness. This method together with careful manipulation of the uterus during the USG examinations may prevent or minimize the effects of uterine contractions on the measurement of endometrial thickness.

A measurement site at which the intercornual ligament was attached was used in this study to improve the reproducibility of measurements [36]. However, it was difficult to position the probe at the exact same position and angle to the cross-sectional plane in every examination. Thus, the potential contribution of technical factors to variations in endometrial thickness cannot be ruled out.

Thus, endometrial thickness increased more rapidly and to a greater extent in the induced estrus group than natural estrus group. A detailed analysis of changes over time in P_4 and E_2 concentrations and endometrial thickness suggested that decreases in P_4 concentrations were cue to an increase in endometrial thickness and that increases in E_2 concentrations enhanced these endometrial changes.

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