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—Original Article—

Exposure to Estrogen Mimicking the Level of Late Pregnancy Suppresses Estrus Subsequently Induced by Estrogen at the Level of the Follicular Phase in Ovariectomized Shiba Goats

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Abstract. A high-estrogen environment during late pregnancy is suspected to cause postpartum silent ovulation, and progesterone (P_4) is suggested to recover estrus. However, few attempts have been undertaken to elucidate the influence of these steroids on estrus by analyzing hormonal profiles. We investigated estrus and luteinizing hormone (LH) surges in ovariectomized goats (n=6) assigned to three treatments in a cross-over design. In groups 1 and 2, 200 µg/kg body weight/day estradiol benzoate (Dose-200 E_2B) was administered for 14 days concurrent with P_4 for 11 days, while in the control, saline solution and P_4 were administered likewise. Ten days after the final administration of Dose-200 E_2B, group 2 was treated with P_4 for 8 days, and all groups were treated with 2 µg/kg body weight E_2B (Dose-2 E_2B) 20 days after the final administration of Dose-200 E_2B (or saline solution). The proportion of cases expressing estrus after the administration of Dose-2 E_2B was smaller ($P<0.01$) in group 1 than in the control (1/6, 3/6 and 6/6; groups 1 and 2 and the control, respectively). The proportions of cases generating LH surges did not differ ($P>0.1$) among the groups (5/6, 5/6 and 6/6; groups 1 and 2 and the control, respectively), but the peak concentrations in groups 1 and 2 (26.2 ± 14.7 and 11.3 ± 6.7 ng/ml) were lower ($P<0.01$) than those in the control (67.8 ± 19.4 ng/ml). These results demonstrated that elevation of plasma estrogen mimicking late pregnancy inhibits the subsequent estrus induced by estrogen simulating the follicular phase.

Key words: Estrus, High estrogen, LH surge, Progesterone, Silent ovulation

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Optimal reproductive management correlates directly or indirectly with the economic condition of dairy farms. For example, postpartum cows should be inseminated at appropriate periods depending on the physiological status of individual cows and the reproductive planning of individual farms. However, the high incidence of postpartum silent ovulation (silent estrus), ovarian quiescence or follicular cyst prevents inseminations in the appropriate period and prolongs calving intervals, resulting in economic loss. The mean intervals from parturition to ovulation in cows were reported to be approximately 2 weeks in the 1960s–70s [1–3], 3 weeks in the 1980s–1990s [4, 5] and 4 weeks or more in the 2000s [6, 7]. In postpartum dairy cows, 79–90% of the first ovulations were not accompanied by overt estrus, that is, silent ovulation; the rate of silent ovulation gradually decreased to 29–59% and 35–40% at the second and third ovulations, respectively [2, 8, 9]. Nevertheless, the presence of cows with repeated silent ovulations or that relapse back after resumption of normal ovulations accompanied by estrus [9] is a serious problem.

Estrus is induced by the action of estrogen upon the hypothalamus in the relative absence of progesterone (P_4) [10, 11]. On the other hand, Carrick and Shelton [12] demonstrated that repeated administration of 10 mg of estradiol benzoate (E_2B) brought about refractoriness in estrus expression to subsequent administration of 400 µg of E_2B in ovariectomized cows. Similar states were reported in ovariectomized ewes [13]. The refractoriness was eliminated and normal responsiveness in estrus expression to E_2B was restored by treatment with P_4 in both of those studies. It is hypothesized that a high concentration of estrogen in maternal circulation in the late gestation period may impair the estrus responsiveness to the subsequent concentration of estrogen secreted from postpartum preovulatory follicles in the course of resumption of ovarian cyclicity and that P_4 secreted from corpora lutea developed after postpartum ovulations may promote recovery of estrus responsiveness. However, in past studies using ovariectomized animals [12, 13], hormonal profiles were not described, although estrus expression was closely examined. Hence, for further understanding of the impaired responsiveness of estrus, it is necessary to clarify the actual concentrations of estrogen and P_4 in peripheral blood during treatment with E_2B and P_4 and to analyze them in reference to general endocrine events of intact animals in pregnancy and estrus. It is also important to evaluate the concentration of luteinizing hormone (LH) in order to elucidate whether exposure to a high-estrogen environment affects only estrus or both estrus and the LH surge.

In the present study, the Shiba goat was used as an experimental model. The endocrine and behavioral characteristics of normally cycling Shiba goats, which have many similarities to those of cows, were described previously in detail [14–17]. The neural mechanisms underlying the endocrine systems have also been studied well in

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this species [18, 19]. In this way, Shiba goats can contribute to investigation of the reproductive functions in cows as a suitable experimental model.

The objective of the present study was, firstly, to determine whether treatment with E_2B and P_4 mimicking the intense steroidal environment of late pregnancy influences the expression, times of onset and duration of estrus and vulval signs induced by subsequent treatment with E_2B, which was calculated to produce the moderate level of estrogen in the follicular phase. The second objective was to characterize the changes in the concentrations of estradiol-17β (E_2-17β), P_4 and LH and to consider them along with the aspects of estrus and vulva signs. The third objective was to assess the therapeutic effect of P_4 applied between the treatments simulating the endocrinal profile in late pregnancy and the follicular phase.

Materials and Methods

Animals

Six Shiba goats (8–10 years of age) ovariectomized at least 4 months before the experiment were used. Their body weights (bw) were 30.1 ± 2.4 (mean ± SD) kg. Shiba goats are annual breeders under natural daylight [14]. The goats were kept in an outside pen next to male goats, fed maintenance diets of alfalfa hay cubes (700 g/head/day) according to the dietary requirements of the National Research Council [20] and allowed free access to mineral blocks and water.

Experimental protocol

All procedures used in this experiment were approved by the university committee for the use and care of animals at the Tokyo University of Agriculture and Technology (Reception No. 24-41). The experiment was designed with an orthogonal Latin square [21] and conducted in three experimental periods. Each of the goats (n=6) was assigned to one of three different treatments (groups 1 and 2 and the control) in an experimental period and received all three treatments once in different sequences throughout the experiment (Table 1). The animals were left free from any treatment for 28 days between the experimental periods to wash out the residual effects of the treatment in the previous period by the time of the next period.

The experimental protocol is summarized in Fig. 1. One group of goats (group 1) initially received insertion of controlled internal drug-releasing devices containing 0.3 g of P_4 [EAZI-BREED (CIDR-G), Pfizer New Zealand, Auckland, New Zealand] for 10 days from the first day of the treatment (day 0) to day 10. They also received daily administration of E_2B (OVAHORMON, ASKA Pharmaceutical, Tokyo, Japan) at 200 µg/kg bw/day (Dose-200 E_2B) into the shoulder muscles for 14 days from day 0 to day 13. These treatments were intended to mimic the steroidal environment of late pregnancy. Then, they received a single intramuscular administration of 2 µg/kg bw E_2B (Dose-2 E_2B) on day 33. Dose-2 E_2B was calculated to simulate the increase in peripheral concentration of E_2-17β during the follicular phase. Another group (group 2) received a second insertion of CIDR-G for 8 days from days 23 to 31 in addition to the treatments of group 1 to assess the effect of progesterone neutralizing the influence of the high-estrogen environment. The other group of goats (control) was administered physiological saline daily from day 0 to 13 instead of Dose-200 E_2B, while the other treatments were carried out in the same way as in group 1. The administration of E_2B or physiological saline and the insertion and removal of the CIDR-G were all performed at 0900 h on each scheduled day.

Table 1. Orthogonal Latin square design for the three treatment groups (groups 1 and 2 and the control)

Goat ID	Experimental period		
	1	2	3
A	Group 1	Group 2	Control
B	Group 1	Control	Group 2
C	Group 2	Group 1	Control
D	Group 2	Control	Group 1
E	Control	Group 1	Group 2
F	Control	Group 2	Group 1

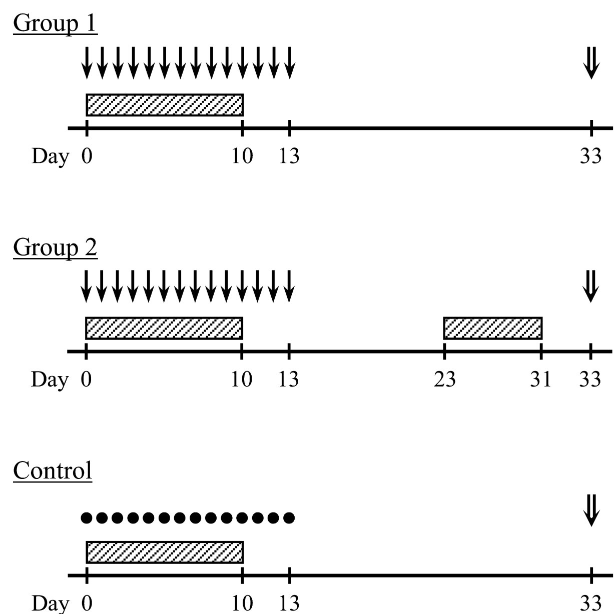

Fig. 1. Summary of the experimental protocols showing a schematic representation of the treatments in the three groups (groups 1 and 2 and the control). The numbers under the lines indicate the days after the initiation of the treatments. The hatched bars represent the period of insertion of controlled internal drug-releasing devices containing 0.3 g progesterone. The single- and double-lined arrows represent administration of 200 µg/kg body weight and 2 µg/kg body weight estradiol benzoate (Dose-200 E_2B and Dose-2 E_2B). The solid dots designate administration of saline solution.

Behavioral observation and vulval examination

All goats were visually observed for behavioral characteristics and examined for vulval signs at 6 h intervals for 48 h after administration of Dose-2 E_2B on Day 33. Each session of observation of the behaviors in the treated female goats was performed for 30 min using two males (15 min/head). One of the males, attached to a lead, was taken into the female pen and allowed to contact the females. The

responses of females to male mounting (standing or refusing) were recorded. The males were pulled down using the lead immediately after checking the female responses to their mounting to avoid copulation. The vulval signs, such as swelling and hyperemia, were examined after the behavioral observation.

Estrus was defined as when a female stood to be mounted by a male at least once in all observation sessions. The time of onset of estrus was defined as the time when estrus was observed for the first time. The duration of estrus was defined as the time from the first observed estrus to the first absence of estrus. The vulval signs were defined as when vulval swelling and/or hyperemia were observed at least once in all observation sessions. The time of onset of the vulval signs was defined as the time when vulval swelling and/or hyperemia appeared for the first time. The duration of the vulval signs was defined as the time from their first appearance to their disappearance.

Blood sampling

Blood samples were collected by jugular venipuncture twice a day (0900 h and 2100 h) from days 0 to 13, once a day (0900 h) from days 14 to 32 and at 3 h intervals from –3 to 48 h after administration of Dose-2 E_2B on day 33. Blood collection at 0900 h was carried out just before the hormone treatments. Blood samples were immediately heparinized and centrifuged at 1,740 × *g* for 20 min. Separated plasma was stored at –20 C until hormone assays.

Hormone assays

Plasma concentrations of E_2-17β were determined by radioimmunoassay as described previously [22]. The average sensitivity of the assay was 0.4 pg/ml, and the intra- and interassay coefficients of variation were 9.4 and 7.5%, respectively. Plasma concentrations of LH were measured by radioimmunoassay as described previously [23]. The average sensitivity of the assay was 0.1 ng/ml, and intra- and interassay coefficients of variation were 5.0 and 2.8%, respectively. Plasma concentrations of P_4 were evaluated using enzyme immunoassay as described previously [24], with minor modifications. Briefly, we applied heparin as an anticoagulant for collected blood samples, used diethyl ether extract of plasma for the assay and changed the total reaction volume. The sensitivity of the assay averaged 0.03 ng/ml, and the intra- and interassay coefficients of variation were 5.8 and 2.9%, respectively.

An LH surge was defined as an increase in LH concentrations over the level of the baseline plus at least two times the SD that subsequently remained elevated in at least two consecutive samples collected at 3 h intervals [25]. The baseline was obtained as the mean concentration of the samples collected for 48 h before administration of Dose-2 E_2B. The time of onset of the LH surge was defined as the time of the first of the consecutive samples composing the LH surge. The duration of the LH surge was defined as the time between onset of the LH surge and return to the baseline. The peak of the LH surge was defined as the maximum concentration observed during the surge.

Monitoring of clinical responses to the treatments

The physical states of all goats, including activity, appetite and excretion, were observed to monitor possible harmful effects of the treatment with E_2B and P_4. Furthermore, complete blood counts and serum biochemical tests were performed on days 0 and 35 of each experimental period. Inspection items of the serum biochemical tests were blood urea nitrogen, creatinine, glucose, total bilirubin, lactate dehydrogenase, glutamic-oxaloacetic transaminase, alkaline phosphatase, total protein and albumin.

Statistical analyses

Fisher's exact test was used to compare the discrete data for the treatment groups, including the occurrence of estrus and an LH surge. The continuous data, such as the time to onset, the duration or hormone concentrations, were compared using one-way analysis of variance followed by the Tukey-Kramer method or Student's *t*-test. Differences were considered significant when the P values were less than 0.05, and P values of less than 0.1 were considered to indicate tendencies for differences. Data are expressed as the mean ± SD.

Results

Clinical responses to the treatments

None of the goats showed any signs of abnormality in observation of the physiological states. The results of the clinical blood tests in the experiment also did not indicate any signs of disorder, referring to the normal ranges in the Shiba goat reported previously [26, 27].

Hormonal profiles from the initiation of the treatments to Dose-2 E_2B challenge (days 0–32)

Changes in the concentrations of E_2-17β, P_4 and LH from days 0 to 32 are shown in Fig. 2. The mean concentrations of E_2-17β from days 1 to 13 in groups 1 and 2, during repeated administration of Dose-200 E_2B, were higher ($P<0.01$) than that in the control (3308.5 ± 1350.2 and 3112.7 ± 1309.0 *vs.* 0.4 ± 0.6 pg/ml), whereas they did not differ between groups 1 and 2 ($P>0.1$). In groups 1 and 2, the elevated concentrations of E_2-17β declined rapidly from day 14.

The mean concentrations of P_4 during the first treatments with a CIDR-G in all groups (days 1–10) were 5.26 ± 2.50, 5.30 ± 2.25 and 5.30 ± 2.42 ng/ml in groups 1 and 2 and the control, respectively, and no differences were detected between the groups ($P>0.1$). The mean concentration of P_4 for the period of the second treatment with a CIDR-G in group 2 (days 24–31) was 4.50 ± 1.84 ng/ml, which was higher than those for the corresponding period in group 1 and the control ($P<0.01$; 0.06 ± 0.04 and 0.06 ± 0.04 ng/ml, respectively). The elevated concentrations of P_4 caused by the insertion of a CIDR-G decreased abruptly to under 0.5 ng/ml on the day following removal.

The concentrations of LH at 2100 h on day 0 in groups 1 and 2 were higher than in the control ($P<0.01$) because some cases showed marked increases like LH surges in groups 1 and 2. The mean concentrations of LH in groups 1 and 2 from days 1 to 23 were lower ($P<0.01$) than in the control (0.6 ± 0.5 and 0.6 ± 0.3 *vs.* 1.7 ± 1.3 ng/ml). The mean concentrations of LH from days 24 to 31 were different ($P<0.01$) among groups 1 and 2 and the control (1.6 ± 1.0, 0.5 ± 0.4 and 2.8 ± 1.3 ng/ml, respectively).

Estrus and vulval signs after Dose-2 E_2B challenge (days 33–35)

The results of observations of estrus and the vulval signs after administration of Dose-2 E_2B on day 33 are summarized in Table

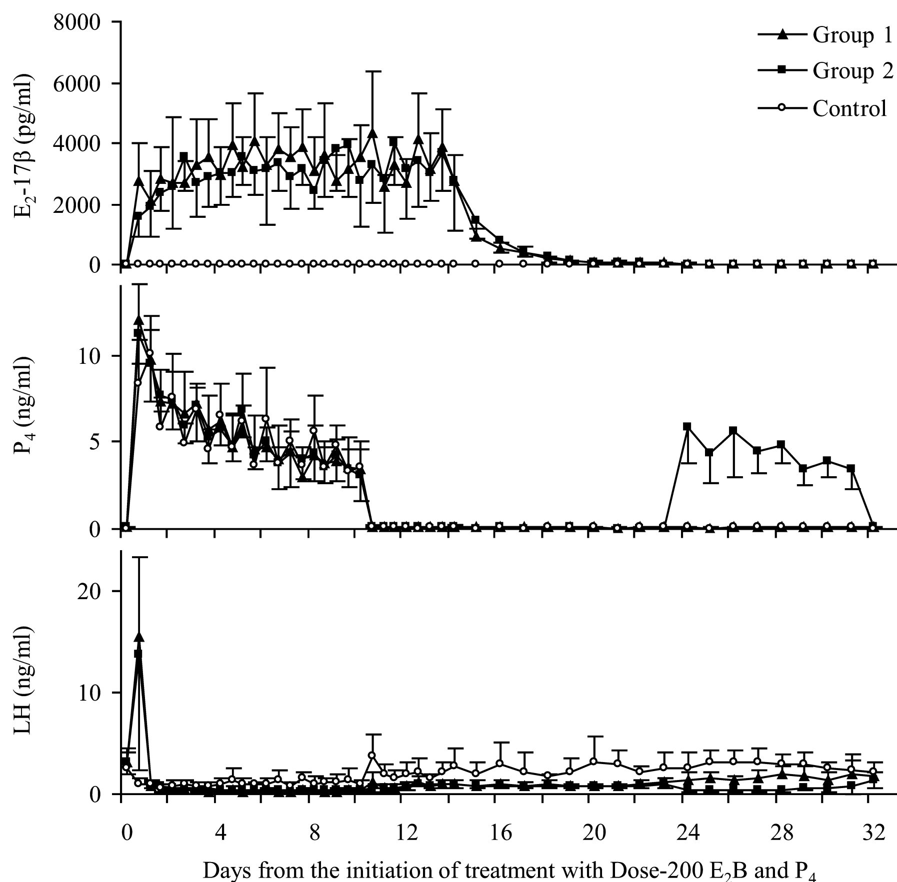

Fig. 2. The peripheral concentrations of estradiol-17β (E_2-17β; top panel), progesterone (P_4; middle panel) and luteinizing hormone (LH; bottom panel) from the initiation of treatment with 200 µg/kg body weight estradiol benzoate (Dose-200 E_2B) or saline solution, and P_4 (day 0) to day 32, one day before treatment with 2 µg/kg body weight estradiol benzoate (Dose-2 E_2B) in group 1 (n=6), group 2 (n=6) and the control (n=6).

2. The proportions of cases expressing estrus were 1/6 (16.7%), 3/6 (50%) and 6/6 (100%) in groups 1 and 2 and the control, respectively. The proportion was smaller in group 1 than in the control (P<0.01) and tended to be smaller in group 2 than in the control (P=0.09), while there was no difference between groups 1 and 2 (P>0.1). In groups 1 and 2 and the control, the times of onset of estrus were 18.0, 16.0 ± 2.8 and 17.0 ± 2.2 h, and the durations of estrus were 6.0, 16.0 ± 7.5 and 20.0 ± 2.8 h, respectively. The times of onset and the durations of estrus were not different between group 2 and the control (P>0.1). In groups 1 and 2 and the control, the proportions of cases expressing vulval signs were 0/6 (0%), 3/6 (50%) and 6/6 (100%), respectively; a difference was detected between group 1 and the control (P<0.01), and tendencies of differences were found between group 2 and the other two groups (P=0.09). The times of onset of the vulval signs were not different (P>0.1) between group 2 and the control (10.0 ± 2.8 *vs.* 11.0 ± 2.2 h). The duration of the vulval signs was shorter (P<0.05) in group 2 than in the control (18.0 ± 8.5 *vs.* 29.0 ± 2.2).

Hormonal profiles after Dose-2 E_2B challenge (days 33–35)

Profiles of E_2-17β, P_4 and LH for 48 h after administration of Dose-2 E_2B on day 33 are presented in Fig. 3. The P_4 concentrations in all treatment groups were under 0.2 ng/ml from –3 to 48 h after administration of Dose-2 E_2B.

The concentrations of E_2-17β just before administration of Dose-2 E_2B were higher (P<0.01) in groups 1 and 2 than in the control (1.7 ± 1.1 and 2.1 ± 1.3 *vs.* 0.5 ± 0.6 pg/ml), while they were not different between groups 1 and 2 (P>0.1). In groups 1 and 2 and the control, the concentrations of E_2-17β reached their maximums of 67.6 ± 7.8, 65.7 ± 11.7 and 59.4 ± 13.4 pg/ml, at 6.0 ± 0.0, 7.0 ± 2.8 and 10.5 ± 2.3 h after administration of Dose-2 E_2B, respectively. The maximum concentrations were not different among the treatment groups (P>0.1), but the times to the maximums were shorter in groups 1 and 2 than in the control (P<0.05). The E_2-17β concentrations in all groups returned to the pretreatment levels by 36 h after administration. There were no differences (P>0.1) in the maximum concentrations of E_2-17β between the cases expressing (n=10) and not expressing (n=8) estrus (61.2 ± 14.2 *vs.* 68.0 ± 8.0) or, as shown below, between the cases generating (n=16) and not

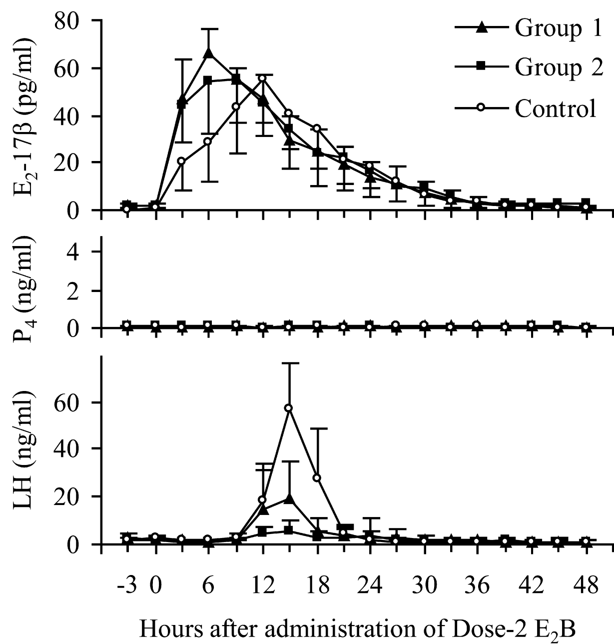

Fig. 3. Profiles of estradiol-17β (E_2-17β; top panel), progesterone (P_4; middle panel) and luteinizing hormone (LH; bottom panel) for 48 h after administration of 2 μg/kg body weight estradiol benzoate (Dose-2 E_2B) on day 33 in group 1 (n=6), group 2 (n=6) and the control (n=6).

generating (n=2) LH surges (63.2 ± 12.3 *vs.* 72.1 ± 7.0).

In all groups, the mean LH concentrations increased markedly from 12 to 18 h after administration of Dose-2 E_2B (Fig. 3). This is because LH surges were generated at that time in 5/6 (83.3%), 5/6 (83.3%) and 6/6 (100%) cases in groups 1 and 2 and the control, respectively (Table 3). The proportions of cases generating LH surges were not different among the groups (P>0.1). The times of onset and durations of the LH surges were not different among groups 1 and 2 and the control (P>0.1; 12.0 ± 0.0, 12.0 ± 3.3 and 13.0 ± 1.4 h; 10.8 ± 4.5, 9.6 ± 1.2 and 10.5 ± 2.3 h, respectively). The peak concentrations of the LH surges in groups 1 and 2 were lower (P<0.01) than in the control (26.2 ± 14.7 and 11.3 ± 6.7 *vs.* 67.8 ± 19.4 ng/ml), but there was no difference between groups 1 and 2 (P>0.1).

Relationship between the expression of estrus and the generation of an LH surge

The relationship between the expression of estrus and the generation of an LH surge after administration of Dose-2 E_2B is presented in Table 4. The proportions of cases showing both estrus and LH surges were 1/6 (16.7%), 3/6 (50%) and 6/6 (100%) in groups 1 and 2 and the control, respectively. The proportion was smaller in group 1 than in the control (P<0.01) and tended to be smaller in group 2 than in the control (P=0.09), while no difference was detected between groups 1 and 2 (P>0.1). The proportions of cases generating LH surges without expressing estrus were 4/6 (66.7%), 2/6 (33.3%) and 0/6 (0%) in groups 1 and 2 and the control, respectively. The proportion was greater in group 1 than in the control (P<0.05), but no difference was detected between group 2 and the control or between groups 1 and 2 (P>0.1). Neither estrus nor an LH surge was induced in 1/6 of the cases (16.7%) in each of groups 1 and 2, and the proportions of cases were not different among groups 1 and 2 and the control (P>0.1). There was no case showing estrus without an LH surge.

Discussion

In the present study, repeated administration of Dose-200 E_2B mimicking the intense estrogen environment in maternal circulation of late pregnancy reduced the proportion of cases expressing estrus following the single administration of Dose-2 E_2B simulating the level of E_2-17β in the follicular phase. Dose-200 E_2B did not decrease the proportion of cases generating LH surges induced by Dose-2 E_2B but lowered the peak concentration of the LH surges. The second P_4 treatment preceding the administration of Dose-2 E_2B seemed to slightly restore the proportion of cases expressing estrus but did not recover the lowered peak of the LH surges. Furthermore, half of the cases treated with Dose-200 E_2B generated LH surges without the expression of estrus after the subsequent administration of Dose-2 E_2B.

In this experiment, repeated administration of Dose-200 E_2B produced peripheral E_2-17β concentrations of 2000–3000 pg/ml in groups 1 and 2 and seemed to have appropriately mimicked the high-estrogen environment during late pregnancy in the sense of total biological activity of various estrogens for the following reasons. In normally cycling Shiba goats, the concentrations of E_2-17β at

Table 2. Expression [number and proportion (%) of cases], time of onset (h) and duration (h) of estrus and external vulval signs after administration of 2 μg/kg body weight estradiol benzoate (Dose-2 E_2B) on day 33 in groups 1 and 2 and the control

Group	Estrus				Vulval signs			
	Expression		Onset (h)*	Duration (h)	Expression		Onset (h)*	Duration (h)
	n	%			n	%		
Group 1 (n=6)	1[a]	16.7	18.0	6.0	0[d]	0	–	–
Group 2 (n=6)	3[ab]	50.0	16.0 ± 2.8	16.0 ± 7.5	3[e]	50.0	10.0 ± 2.8	18.0 ± 8.5[g]
Control (n=6)	6[c]	100.0	17.0 ± 2.2	20.0 ± 2.8	6[f]	100.0	11.0 ± 2.2	29.0 ± 2.2[h]

n=number of cases. * The time from administration of Dose-2 E_2B to onset of estrus and external vulval signs. [ac, df] The different superscripts represent significant differences (P<0.01) among the treatment groups in the same column. [gh] The different superscripts represent significant differences (P<0.05) among the treatment groups in the same column. [bc, de, ef] The different superscripts represent tendencies of differences (0.05≤P<0.1) among the treatment groups in the same column.

Table 3. Generation [number and proportion (%) of cases], time of onset (h), peak concentration (ng/ml) and duration (h) of an LH surge after administration of 2 μg/kg body weight estradiol benzoate (Dose-2 E_2B) on day 33 in groups 1 and 2 and the control

Group	LH surge				
	Generation		Onset (h)*	Peak (ng/ml)	Duration (h)
	n	%			
Group 1 (n=6)	5	83.3	12.0 ± 0.0	26.2 ± 14.7^{a}	10.8 ± 4.5
Group 2 (n=6)	5	83.3	12.0 ± 3.3	11.3 ± 6.7^{a}	9.6 ± 1.2
Control (n=6)	6	100.0	13.0 ± 1.4	67.8 ± 19.4^{b}	10.5 ± 2.3

n=number of cases. * The time from administration of Dose-2 E_2B to the LH surge. ^{ab} The different superscripts represent significant differences ($P<0.01$) among the treatment groups in the same column.

estrus [14, 15, 17] were reported to be approximately 20–30 pg/ml. In pregnant goats approaching parturition, it was reported that the concentration of E_2-17β in maternal circulation reaches maximally 10-fold as much as that at estrus, while estrone and estradiol-17α become 10- to 15-fold higher than E_2-17β [28–34]. Studies in sheep [35], humans [36, 37] and rats [36] suggested that estrone and estradiol-17α have about 20–60% binding affinities to estrogen receptors relative to E_2-17β. In turn, regarding the concentrations of E_2-17β after the single administration of Dose-2 E_2B, the maximum concentrations were approximately 60–65 pg/ml in all groups, which appeared to be comparable to the physiological level in the follicular phase and to be sufficient for eliciting estrus and LH surges in the goats with normal responsiveness to E_2-17β. Although the E_2-17β concentrations in groups 1 and 2 just before the administration of Dose-2 E_2B were higher than those in the control, they were at similar levels to the basal concentration of E_2-17β (2 pg/ml) measured on the day of ovulation in intact goats [15]. The mean concentration of P_4 during the treatment in our study achieved the level of the mid-luteal phase of the estrous cycle in Shiba goats [14, 15, 17].

We found that treatment with Dose-200 E_2B markedly suppressed the expression of estrus induced by the subsequent administration of Dose-2 E_2B by comparing the proportion of cases expressing estrus in group 1 with that in the control. These results indicate that the responsiveness of the hypothalamic center controlling the expression of estrus would be reduced by exposure to a high level of estrogen like that exhibited in late pregnancy and confirm previous studies in cows [12] and ewes [13, 38]. On the other hand, the cases in group 2, which received treatment with P_4 between the treatments mimicking late pregnancy and the subsequent follicular phase, were likely to express estrus in a proportion intermediate between those of group 1 and the control, although statistical differences were not detected among the groups. The present results are consistent with the studies suggesting the restorative effect of P_4 on estrus responsiveness impaired by high estrogen in ewes and cows [12, 13] and the facilitating effect for the complete expression of estrus in ewes and does [39–43]. However, these effects of P_4 were not as clear in the present study as in previous studies. This may be because the differences in the protocol to produce a high-estrogen environment in circulation would affect the degree of suppression of estrus. The 14 daily administrations of Dose-200 E_2B performed in our study may have brought about a more intense estrogen environment and suppressed the expression of estrus strongly compared with the previous studies mentioned above [12, 13], in which smaller numbers of repeated administrations of estrogen at various doses were performed at longer intervals. Thus, rigid suppression of estrus is assumed not to be neutralized by the effect of P_4, at least in some cases, and this may be the reason why some cows have repeated silent ovulations while others recover normal estrus gradually with the increase in the number of ovulations during the postpartum period. Regarding the vulval signs, repeated administration of Dose-200 E_2B definitely

Table 4. Relationship between the expression of estrus and generation of an LH surge [number and proportion (%) of cases] after administration of 2 μg/kg body weight estradiol benzoate (Dose-2 E_2B) on day 33 in groups 1 and 2 and the control

Group	Estrus / LH surge							
	+ / +		– / +		+ / –		– / –	
	n	%	n	%	n	%	n	%
Group 1 (n=6)	1^{a}	16.7	4^{d}	66.7	0	0	1	16.7
Group 2 (n=6)	3^{ab}	50.0	2de	33.3	0	0	1	16.7
Control (n=6)	6^{c}	100.0	0^{e}	0	0	0	0	0

n=number of cases. + / + Both estrus and an LH surge were positive. – / + Estrus was negative and an LH surge was positive. + / – Estrus was positive and an LH surge was negative. – / – Both estrus and an LH surge were negative. $^{ac,\ de}$ The different superscripts represent significant differences ($P<0.01$) among the treatment groups in the same column. ^{bc} The different superscripts represent a tendency of difference ($0.05 \leq P<0.1$) among the treatment groups in the same column.

suppressed their appearance after the subsequent administration of Dose-2 E_2B. Treatment with P_4 tended to be effective in restoring the responsiveness of the vulva to estrogen.

In our experiment, the proportions of cases generating LH surges did not differ among the groups. It has been demonstrated in cows [44–46] and ewes [47–49] that LH secretion in response to GnRH was suppressed until about 7 days postpartum and was regained gradually by 10 to 14 days, while the suppressed responsiveness of LH secretion to E_2-17β recovered in 3 to 4 weeks after parturition. In the present study, Dose-2 E_2B was administered 20 days after the end of repeated administration of Dose-200 E_2B. Therefore, we reasoned that the responsiveness of LH secretion to E_2-17β would have recovered to some extent at the time of administration of Dose-2 E_2B. However, the peak concentrations of the LH surges were smaller in the cases treated with Dose-200 E_2B than in those not treated. A candidate for the changes responsible for the lowered peak concentration of the LH surges is the reduced secretion of GnRH induced by estrogen. The storage of GnRH in the hypothalamus has been reported not to change during the postpartum period [50], and consequently, it is suspected that the reduction in GnRH secretion might be caused by the decreased estrogen receptors in the hypothalamus [51], where the center controlling the generation of a GnRH/LH surge appears to be located. Other candidates are the decreases in pituitary LH storage [47, 52], GnRH receptors [50] or estrogen receptors [50, 51] occurring after parturition or treatment with a high dose of estrogen. In the present study, the treatment with P_4 (days 23–31) in group 2 did not restore the peak concentration of the LH surges suppressed by Dose-200 E_2B. In ovariectomized ewes, it was reported that pretreatment with P_4 did not alter the peak concentration and duration of the LH surges induced by E_2-17β, although it delayed the onset [53]. These data are similar to our present results in the respect that the peak concentration and duration of the LH surges were not altered by the preceding exposure to P_4.

Half of groups 1 and 2, which were treated with Dose-200 E_2B, generated LH surges without the expression of estrus. Such a condition as observed in this experiment may correspond to silent ovulation in postpartum cows. Interestingly, Reames *et al.* [54] reported similar responses observed in a proportion of ovariectomized cows infused with various doses of E_2-17β. In their study, LH surges were induced without estrus in some cows infused with doses of E_2-17β calculated to produce a peripheral concentration of 3 to 9 pg/ml, while both LH surges and estrus were induced in all cows infused with a dose calculated to produce 12 pg/ml. These results suggested that the hypothalamic center controlling the generation of an LH surge has higher sensitivity to E_2-17β than the hypothalamic center controlling the expression of estrus. In contrast, our present results imply that the center of an LH surge suffers less inhibitory effects as a result of a high-estrogen environment and/or recovers spontaneously from the inhibition earlier than the center of estrus. Thus, the center of an LH surge seems to have higher tolerance than the center of estrus toward the intense estrogen environment in late pregnancy. This tolerance of the center of an LH surge may explain the high incidence of silent ovulations in postpartum cows [2, 8, 9].

There was no case expressing estrus without generating an LH surge in the present study. Although a large number of studies have been performed on postpartum cows and other domestic animals, we could not find evidence of estrus without an LH surge observed in the first preovulatory period postpartum. However, the obvious expression of estrus [55] and failure to generate LH surges [56, 57], that is, anovulatory estrus, were reported in cows with follicular cysts, which might include cases that occurred not only in the postpartum period but also in other periods. Comparative investigation of the endocrine and neural changes causing silent ovulation and anovulatory estrus may provide new insight into the research on these disorders.

In conclusion, the present study demonstrated that exposure to a high-estrogen environment can result in a condition corresponding to postpartum silent ovulation, which was characterized by the generation of an LH surge without the expression of estrus, by suppressing the center of estrus and the LH surge to different extents. It is also suggested that the restorative and/or facilitating effect of P_4 on estrus expression would not definitely neutralize the suppression caused by a high-estrogen environment, at least in some cases. This may be the reason for the presence of cows repeating silent ovulations in the postpartum period.

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