Response of Plasma Cortisol and Progesterone after ACTH Challenge in Ovariectomized Lactating Dairy Cows

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Abstract. Shortened and weakened estrous expressions could be one of the causes of poor heat detection rate. Non-specific acute stresses are assumed to depress expression of estrus by an increase of plasma progesterone which may originate from the adrenal cortex. The objective of the present study was to examine whether the adrenal cortex can secrete significant amounts of progesterone in response to exogenous adrenocorticotropic hormone (ACTH) in lactating cows. Four cows had estrus synchronized and were ovariectomized in the luteal phase. The cows were given 25 IU ACTH through an indwelling catheter 5 h after catheterization. Blood samples were collected at an interval of 30 min. In 3 of the 4 cows, plasma progesterone concentrations increased significantly 0.5–1.5 h after the first ACTH challenge with a mean peak value of 4.2 ± 0.4 (S.D.) ng/ml. A similar response was also observed after the second ACTH challenge. Peak plasma progesterone concentrations in the 3 cows after first ACTH challenge were comparable with the progesterone values in the luteal phase of each cow. The results suggest that lactating cows have the capability to secrete a significant amount of progesterone from the adrenal cortex.

Key words: Adrenocorticotropic hormone (ACTH), Adrenal cortex, Cortisol, Progesterone, Weakened estrus

A higher milk yield and a larger herd size are general trends in modern dairy farming, which have made heat detection of cows more difficult. Difficulty in detecting heat is the most important cause of decreased reproductive efficiency. Generally, standing to be mounted is recognized as the primary estrous symptom and duration of standing estrus was previously known to be 17.8 h [1]. Recent studies, however, have shown shortened estrous durations of less than 10 h [2–4]. It has also been reported that 50% [5] or 37% [6] of cows in estrus did not show standing estrus. The shortened duration of estrus as well as weakened estrous signs have been suggested to be major causes of the failure to detect estrus [6, 7]. In Japan, where the conception rate in high producing dairy cows has been recently declining, no study has been carried out to show the duration and intensity of estrus in cows.

Causes of weakened estrous signs have not been well described. The floor material or ground conditions in the paddock are known to affect intensity and duration of estrus in cattle [8–10]. Non-specific stresses caused by high milk production, claw diseases and a high ambient temperature in summer have also been reported to be possible causes of weakened estrus [11–14]. However, the mechanism by which the stresses...
cause depression of estrous intensity is as yet unknown.  

Endocrinological factors may cause decreased intensity of estrous signs in cows with silent heat [15–17]. Estrus is expressed under the influence of estrogen produced by the Graafian follicle. Estrogen plays an important role in the development and function of the secondary sex organs, the onset of estrus and the period of sexual receptivity. On the other hand, progesterone suppresses the maturation of dominant follicles and secretion of estrogen [18]. The female does not come into heat while progesterone is being produced. Plasma progesterone concentrations during periestrus in cows with silent estrus were significantly elevated compared to the values in cows showing observed estrus [16]. After adrenocorticotropic hormone (ACTH) administrations, cows showed a decrease in secretion of estrogen and luteinizing hormone and an increase in progesterone concentration [19, 20]. It is known that the adrenal cortex produces progesterone in cattle [21]. Therefore, it is presumed that cows under stress may increase adrenal progesterone, depressing the action of estrogen and suppressing estrous expressions. In a previous study, Wagner et al. [21] reported that treatment of 5 ovarioctomized heifers with 100 IU ACTH resulted in elevated plasma progesterone levels 1 hour after the treatment, while physiological saline-treated animals did not show such an increase (1.18 vs 0.09 ng/ml). In a more recent study by Bage et al. [22], 5 ovarioctomized virgin heifers and 5 ovarioctomized repeat breeder heifers responded to 48 IU ACTH showing peak plasma progesterone concentrations of 1.1 ng/ml and 0.7 ng/ml, respectively. Thus, it has been demonstrated that heifers are capable of secreting progesterone from the adrenal cortex in response to ACTH.

However, recent problems with decreased estrous activities are reported mostly in lactating cows rather than in heifers [23], and it is presumed that lactating cows can also secrete progesterone from the adrenal cortex as much as heifers do, since lactating cows are exposed to more stresses than heifers.

Practically it is difficult to use normal lactating cows which are due to be culled for various reasons, poor production, poor body condition, chronic lameness and sub-clinical mastitis, provided that the cows are generally healthy and lactating normally and being managed with normal herd mates, and have normal adrenocortical responses to ACTH as previously reported [24].

The objective of the present study was to investigate the possibility that the adrenal cortex can secrete an amount of progesterone sufficient to suppress estrus in lactating cows.

Materials and Methods

Animals and housing

This study was carried out at Hiroshima University Experimental Farm, Higashi-Hiroshima, Hiroshima Prefecture, south-eastern region of Japan, during a period from 4th June 2002–14th July 2002. Four Holstein Friesian lactating cows in their 2nd–6th lactations were used. The cows were from 211–269 days in milk. Body weights ranged between 540 and 670 kg. Prior to the experiment, they were showing normal estrous cycles. Three of the 4 animals had not been inseminated since the last calving, for they were due to be culled in August 2002 because of age, poor milk production and chronic mastitis or lameness. Two cows (Nos. 4 and 17) had longstanding lameness since the previous lactation, and one cow (No. 9) had sub-clinical mastitis. The other cow (No. 11) had not been inseminated either, because the cow had not shown clear signs of estrus for a long period after calving, and had poor milk production and poor body condition. All the four cows were, otherwise, generally healthy and were being fed, milked and managed in the same group with other healthy herd mates. Clinically no indication of stresses possibly caused by the lameness and the sub-clinical mastitis was seen. The cows were kept in a free-stall barn with herd mates during the experiment, except for the time of the ACTH challenge test, when they were kept in another separate stall and tied with 3–4 m long ropes. They were milked twice daily. All handling of the animals was performed with a minimal disruption of daily routine and with minimal stress. The experimental protocols and the animal care during the experiment met The International Guiding
Principles for Biomedical Research Involving Animals (Council for International Organizations of Medical Sciences).

**Schedule of treatment and blood sampling**

The 4 animals were ovariectomized and used for an ACTH challenge test to examine the response of adrenal progesterone to ACTH. Prior to ovariectomy, the cows with normal estrous cycles were treated intramuscularly with 25 mg of PGF$_{2\alpha}$ (dinoprost tromethamine, Pronargon F®, Pharmacia, Tokyo, Japan) twice at an interval of 14 days to synchronize estrus. The cows were pre-treated with 100 µg GnRH analog, fertireline acetate (Conceral®, Takeda Schering-Plough Co. Ltd., Tokyo), 7 days before each of the PGF$_{2\alpha}$ administrations. Ovariectomy was conducted in all four cows through incision of the vaginal wall 9 days after the second PGF$_{2\alpha}$ treatment.

Two consecutive ACTH challenge tests at an interval of 48 h were carried out 8 days and 10 days after the ovariectomy. Indwelling jugular vein catheters were inserted 6 h prior to each of the ACTH challenges. Twenty-five IU ACTH (acetotetra sactid, Cortrosin®, Daiichi Pharmaceutical Co., Tokyo) were administrated intramuscularly at 0:00 h.

Blood samples were collected at 9:30–10:00 daily by tail venepuncture throughout the estrous cycle until the cows were ovariectomized. Blood was sampled twice a day for 2 days from the third day after PGF$_{2\alpha}$ treatment. For 24 h after the ovariectomy, blood was sampled every 4 h, and then daily for 6 days until the cows were catheterized. Blood samples were collected through the indwelling jugular vein catheter every 30 min for 14 h to complete the ACTH challenge test (Fig. 1). Blood samples were immediately stored at 4 C and centrifuged (1,700 × g for 15 min) within 30 min after collection. Plasma was stored at −20 C until analysis for cortisol and progesterone.

**Hormone assay**

Plasma cortisol and progesterone concentrations were determined by enzyme immunoassay.

For the cortisol assay, 50 µl of plasma were extracted with dichloromethane for 5 min using a vortex mixer. The extracted solution was dried at 45 C for 50 min. After drying, the sample was dissolved with assay buffer, 0.05 M borate buffer containing 0.2% bovine serum albumin (Fraction V, Sigma Aldrich, Tokyo, Japan) and 0.1 mg/ml thimerosal (Sigma Aldrich). The standard solutions were prepared with assay buffer at concentrations of 0, 0.3, 1, 3, 10, 30 and 100 ng/ml. Duplicate plasma samples and standard solutions were used for the assay. Fifty microliters of standard solution or plasma samples were applied to the wells of plates that had been previously coated with goat antibody raised against rabbit IgG (ICN Biomedicals Inc., Ohio, USA). Cortisol antiserum (anti-cortisol-3-CMO-BSA IgG, COSMO Bio, Tokyo, Japan) diluted with assay buffer 40,000 times and horse radish peroxidase (HRP) cortisol conjugate (cortisol-3-CMO-HRP, COSMO Bio) solution (1:100,000) were added to the wells, 50 µl of each. The plate was incubated at room temperature for 2 h, and then washed three times with phosphate buffered saline (PBS). Substrate solution containing 0.5 mg/ml o-phenylenediamine, 0.2 M citric acid and 0.01% hydrogen peroxide (H$_2$O$_2$) was added to the wells.

![Fig. 1. Experimental schedule for blood sampling, estrus synchronization, ovariectomy, catheterization and ACTH challenge tests. d 0: the day of ovariectomy. Blood sampling interval: — Daily at 09:30–10:00, □ Twice daily, □ 4 h, □ 30 min.](image-url)
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(150 µl/well), followed by incubation for 30 min at room temperature. The reaction was stopped by addition of 50 µl of 6 N sulfuric acid (H₂SO₄). The optical density was measured for its absorbance at 492 nm, using a microplate reader (MPR-A4i, Tosoh, Tokyo, Japan). The intra-assay variation and inter-assay coefficients of variation in high and low cortisol pooled plasma samples were 7.2% and 23.4% (n=6), and 7.8% and 9.1% (n=5), respectively.

Plasma concentrations of progesterone were measured according to the method described by Isobe and Nakao [25]. The antiserum used was raised in rabbits against progesterone-3(E)-carboxymethylxoxime-BSA (Kambegawa Institute, Tokyo, Japan). Cross reactivity of the antibody with progesterone, 5α-progesterone, pregnenolone, 17α-hydroxyprogesterone, deoxycorticosterone and 20β-hydroxyprogesterone were 100, 5.8, 0.2, 0.05, 0.62 and 0.7 %, respectively [25]. The cross reactions of the progesterone antiserum with cortisol and corticosterone were 0.01 % for both. Duplicate samples of plasma were diluted 5 times with assay buffer and heated in a water bath at 70°C for 30 min. Horse radish peroxidase (HRP) labeled-progesterone (Sigma Aldrich) solution (1:10,000) and anti-progesterone serum solution (1:20,000), 50 µl of each, were added. The intra-assay variation and inter-assay coefficients of variation were 24.7% (n=5) and 13.7% (n=5), respectively.

The responses of plasma concentrations of cortisol and progesterone after ACTH administration were shown by the values before ACTH challenge, the peak value after ACTH and amount of cortisol and progesterone secreted after ACTH. Basal concentrations of both hormones were determined by the lowest value among the values of three samples collected at 1.0 h, 0.5 h and within 1 min before ACTH administration.

Peak plasma progesterone concentrations in the ovariectomized lactating cows after the ACTH challenge were compared with peak progesterone values of each cow in the luteal phase and in the follicular phase.

Area under the curve (AUC) values of plasma cortisol and progesterone up to 5 h after ACTH challenge were calculated. The correlation between

![Fig. 2.](image)

Fig. 2. Plasma cortisol and progesterone concentrations in 4 cows during synchronized estrous cycles before ovariectomy. d 0: the day of ovariectomy, Ovx.: the time of ovariectomy.
AUCs of plasma progesterone and cortisol after ACTH challenge was examined.

Statistical analysis

Significance of correlation between two variables was tested by using Pearson’s correlation coefficient.

Results

Plasma cortisol and progesterone concentrations during the estrous cycle synchronized by GnRH and PGF$_2$α before ovariectomy in each cow are presented in Fig. 2. In 3 animals (Nos. 4, 9 and 11) plasma progesterone concentrations dramatically decreased after PGF$_2$α treatment. Progesterone concentrations started to gradually increase 7 days after the first PGF$_2$α treatment and reached 4 ng/ml or above at 14 days after the treatment. In 3 cows plasma progesterone profiles indicated that estrus was successfully synchronized and that they were in the luteal phase on the day of ovariectomy. One cow (No. 17) did not respond to PGF$_2$α, but the cow was in the luteal phase on the day of ovariectomy. Cortisol concentrations in plasma remained below 10 ng/ml most of the time during the estrous cycle until ovariectomy.

The ovariectomy resulted in a marked fall in plasma progesterone concentrations to below 1.0 ng/ml (Fig. 3). In 3 of 4 cows (Nos. 4, 11 and 17), progesterone concentrations decreased to less than 1.0 ng/ml within 24 h after the operation. Progesterone concentrations in the other cow decreased to 0.3 ng/ml at 48 h after operation. A temporal rise of plasma cortisol was shown after ovariectomy as well as catheterization.

All the cows responded to ACTH with an apparent increase in plasma cortisol concentrations, indicating that the cows had normal adrenocortical function (Fig. 3).

Three of the 4 cows (Nos. 9, 11 and 17) showed high responsiveness of plasma progesterone concentrations to ACTH challenges, with increases
from 0.6 ng/ml or less to 2.2–4.5 ng/ml (Fig. 3). The peak plasma progesterone concentration after the second ACTH challenge in one cow (No. 11) was relatively lower than those of the other two (Nos. 9 and 17), but was still equivalent to the value in the luteal phase. Adrenocortical responses to the second ACTH challenge tended to be lower than the responses to the first challenge.

The column at the right side of each figure shows the mean of progesterone concentrations at 7 days after estrus of each animal in two cycles before ovariectomy. 0 h: the time of ACTH challenge.

The peak progesterone values in 3 cows after the first ACTH challenge were comparable with the values obtained in the luteal phase. The 3 cows also showed an increase of plasma progesterone after the second ACTH up to 2.0 ng/ml or higher (Fig. 4). Cow No. 4 also responded to ACTH challenges with a significant increase in plasma progesterone and cortisol. The peak progesterone values, however, were below 1.0 ng/ml.

Figure 5 shows the correlation between AUCs of plasma progesterone and cortisol in 8 ACTH challenge tests in the 4 cows. A significantly high positive correlation was observed between progesterone and cortisol AUCs (r=0.8, P<0.05).

Discussion

Basal plasma cortisol concentrations in cattle were reported to be about 5–10 ng/ml [26, 27], which may increase 5–10 fold from the basal level after administration of 25–50 IU of ACTH [22, 24]. In the present study 25 IU of ACTH were used for the challenge tests according to the dose used by Nakao and Grunert [27]. The basal plasma cortisol concentrations were 3.8–4.4 ng/ml and the peak values after ACTH administration were 33.2–46.5 ng/ml in this experiment. This result was in agreement with those of previous studies [19, 28].
Thus, all the cows used for this study were considered to have had normal adrenocortical function. For the present experiment, the university experimental farm allowed the authors to use only cows in the late lactation period which were due to be culled because of age, poor milk production, infertility and long standing chronic lameness or sub-clinical mastitis. All the cows were healthy and managed in the same group with other cows without difficulties. Besides the ACTH challenge tests, these cows showed that their adrenocortical functions were comparable with the adrenal function in cows without stresses as reported by Stoebel and Moberg [19] and Alam et al. [28].

Progesterone in peripheral blood in cattle is secreted mostly from the corpus luteum with a small quantity from the adrenal cortex [21]. Progesterone circulating in plasma in ovariectomized cows is, therefore, likely to be of adrenal cortex origin. In the present study ovariectomized cows showed a significant increase in plasma progesterone concentrations after ACTH administration. Peak progesterone levels after ACTH reached 3.1–3.7 ng/ml. This indicates that the adrenal cortex can secrete a considerable amount of progesterone in response to ACTH, and is supported by the finding of a significant ($P<0.05$) correlation ($r=0.8$) between AUCs of plasma cortisol and progesterone after ACTH challenges in the present study.

Plasma progesterone concentrations in cows in estrus are low, less than 1.0 ng/ml, with the average being 0.5 ng/ml [29]. After the formation of the corpus luteum, plasma progesterone concentrations increase to 1.0 ng/ml or higher. Generally, estrous expression in cows is suppressed when plasma progesterone concentrations are higher than 1.0 ng/ml [30]. Milk progesterone concentrations in cows showing weakened estrus were reported to be higher than the values in cows showing clear estrous signs [16].

Elevated plasma progesterone concentrations around the time of estrus could be due to acute stresses related to management, environmental and physiological factors [19, 31]. Delayed or incomplete luteolysis is an unlikely cause of increase in plasma progesterone during the follicular phase, since the maturation of a Graafian follicle occurs only after complete luteolysis.

In this study, plasma progesterone concentrations in ovariectomized cows increased to higher than 1.0 ng/ml after administration of ACTH. This means that the adrenal cortex can secrete sufficient progesterone to elevate plasma progesterone concentration up to 1.0 ng/ml or higher, a level which may suppress the expression of estrous signs. The elevation of plasma progesterone around the time of estrus may suppress LH pulse frequency by inhibiting GnRH pulse, which may in turn delay maturation of the Graafian follicle or decrease estradiol secretion from the follicle. A lack of estradiol surge results in a lack of LH surge and delays maturation of the follicle and ovum. However, the duration of the rise of plasma progesterone was less than 2 h in this study and it is not known whether or not this could have caused the suppression of estrus. Further studies may be needed to clarify the effects of duration of acute stress and rise of plasma progesterone on estrous signs. Timing of the elevation of plasma progesterone, in addition to the duration of the elevated plasma progesterone, is also crucial for suppression of estrus. It should be remembered that the cows used for the present experiment had chronic lameness and subclinical mastitis, although the diseases might not have significantly affected the adrenocortical response to ACTH. Further studies may be needed using healthy cows in early lactation to better demonstrate the role of adrenal progesterone in the depression of estrus.

Bage and colleagues [22] reported that in ovariectomized heifers, plasma progesterone concentrations elevated to 1.0 ng/ml after ACTH challenge. Compared with this result, the peak level of plasma progesterone after ACTH challenge in lactating cows in the present study was markedly higher. Lactating cows might have experienced more stress, due to parturition, lactation and under-nutrition, and accordingly might have higher responsiveness of the adrenal cortex to ACTH.

Some cows showed a rise of plasma progesterone and cortisol before catheterization. This might have been due to the frequent blood sampling at 4 h intervals.

Peak plasma cortisol concentrations in cows after 25 IU of ACTH challenge were 33.2–46.5 ng/ml in this study. Whether or not dairy cows are possibly exposed to equivalent stress in general management conditions has yet to be investigated.
Nakao and Grunert [27] reported that 25 IU ACTH-challenge test in cows after normal calving or dystocia showed peak plasma cortisol concentrations of 18–23 ng/ml and 15–45 ng/ml. Palpation per rectum also caused an elevation of plasma cortisol up to 12–14 ng/ml [26]. Cows with lameness were reported to show significantly elevated plasma cortisol compared to a control group [32]. Miyazawa [33] reported a significant increase of plasma cortisol up to 14.0–19.6 ng/ml in cows after milking. Administration of 25 IU ACTH caused increases in plasma cortisol much higher than the values in cows under acute stress. It may be interesting to use a dose of ACTH that would induce plasma cortisol responses equivalent to the values reported for cows under stress.

Further study is in progress at the authors’ laboratory to try to demonstrate the relationship among different doses of ACTH, plasma cortisol and progesterone concentrations in ovariectomized cows.

In conclusion, lactating cows may secrete a significant amount of progesterone from the adrenal cortex in response to acute stress, resulting in high plasma progesterone concentrations, which may be sufficient to suppress estrous expression.

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