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(乳牛における卵巣ホルモン濃度推移が次の発情周期での子宮内膜 EGF 濃度に及ぼす効果)

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Contents

Abbreviations

AI : Artificial insemination

ANOVA : Analysis of variance

BSA : Bovine serum albumin

CL : Corpus luteum

CIDR : Controlled internal drug release

CMO : Carboxymethiloxime

CV : Coefficient of variation

E_2 : Estradiol

EDTA : Ethylenediaminetetraacetic Acid

EIA : Enzyme immunoassay

EGF : Epidermal growth factor

GnRH : Gonadotropin-releasing hormone

g : Gram

h : Hour

IFNT : Interferon-τ

IgG : Immunoglobulin G

im : Intramuscular

LH : Luteinizing hormone

LPS : Lipopolysaccharide

M : Molar

mg : Milligram

min : Minute

mL : Milliliter

ng : Nanogram

P_4 : Progesterone

pg : Picogram

PG : Prostaglandin

PGE_2 : Prostaglandin E_2

$PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$

PR : Progesterone receptor

pH : Potential of hydrogen

RB : Repeat breeder

SD : Standard deviation

SEM : Standard error of the mean

SP : Seminal plasma

% : Percentage

µL : Microliter

µg : Microgram

USG : Ultrasonography

Notes

The contents of Chapter 1 have been published in the Theriogenology journal:

Vuongtuan Phong, Takeshi Fujikawa, Kohei Kawano, Yojiro Yanagawa, Seiji Katagiri. Effects of prostaglandin $F_{2\alpha}$ treatment at follicular wave emergence on endometrial epidermal growth factor concentration on day 3 of the next estrous cycle and on fertility in dairy cows. Theriogenology 2023; 212:189-196.

The contents of Chapter 2 have been prepared for publication in the journal of Reproduction and Development:

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Preface

Fertility plays a vital role in the profitability of dairy herds as it contributes to increased production and shorter calving intervals [13]. Over the past few decades, researchers have extensively documented reproductive inefficiencies in lactating dairy cattle; however, in recent years, the decline in fertility has become particularly alarming [45, 62]. Therefore, it has been recognized that high milk production is responsible for causing or at least being connected to the decrease in reproductive efficiency [78]. For example, conception rate was lower, pregnancy loss and twinning rate were higher for lactating cows compared to heifers [45, 61, 63, 65]. A reasonable explanation for the connection between milk production and fertility is the increased metabolism of ovarian steroid hormones as milk production increases [78]. The previous study demonstrated that the high-yielding cows need an elevated feed consumption to meet the high energy requirement, leading to increasing liver blood flow that, in turn, increases the clearance of both estradiol (E_2) and progesterone (P_4) in the circulation [64].

The two periods: (1) between luteolysis and estrus (or ovulation) and (2) early luteal phase may be essential to understand reduced fertility from the point of view of ovarian steroid hormones. The length from luteolysis to estrus or ovulation has been reported to be connected positively with preovulatory E_2 concentrations, which are necessary for uterine programming, early embryo development, and postovulatory P_4 concentrations, supporting the establishment of pregnancy [9, 12, 53]. In the postovulatory period, P_4 is crucial in establishing and maintaining pregnancy [20]. Increased postovulatory P_4 concentrations advance post-hatching expansion and elongation of conceptuses through the changes in endometrial gene expression, leading to changes in the composition of the histotroph [71]. The alterations of these steroid hormones have also been recognized in repeat breeder (RB) cows, although RB cows may not necessarily be high producers [29]. The reported alterations in the RB cows include a late postovulatory rise [4, 37, 68] and subsequent low concentrations of P_4 [4, 25, 37, 41]. The alterations also include decreased concentrations of E_2 before and after ovulation on the day of estrus [10]. These alterations have been linked to causes of early embryonic loss, including premature termination of the corpus luteum (CL) [51] and asynchrony between the uterus and embryonic development during the early luteal phase [22].

The alterations in the ovarian steroid hormone profile in high-yielding and RB cows could be amplified. They may become detectable in the endometrium as a growth factor and cytokine expression alteration since ovarian steroid hormones primarily regulate the timing and levels of expression of these local factors [10, 58]. Among growth factors and cytokines, epidermal

growth factor (EGF) seems to be one of the most critical regulatory components of uterine function and embryonic development [10, 58]. Estrogen stimulates EGF production in the mouse uterus [14, 18, 26] and human oviducts [1]. Drastic adverse effects on the inner cell mass [75], placenta formation, and viability of offspring [70, 75] have been reported in EGF receptor knockout mice. Epidermal growth factor replaces estrogen in the stimulation of uterine and vaginal growth and induction of lactoferrin (a major estrogen-inducible secretory protein) in mice [54] and a nidatory estrogen surge that initiates blastocyst attachment to the endometrium in rats [27]. The presence of EGF and its receptor in the endometrium have been reported in many farm species, including cattle [32, 38, 56], sheep [23, 24], goats [74] and pigs [36]. Further, EGF increases endometrial production of prostaglandin (PG) E_2 and the $PGE_2/PGF_{2\alpha}$ ratio in pigs [80] and rats [5]. These effects of EGF on endometrial PG production should enhance CL function in the cattle [2, 69]. Therefore, an altered endometrial EGF action may cause uterine dysfunction, which causes early embryonic loss in dairy cattle.

In fertile cows, the endometrial EGF concentrations peak twice, on days 2 - 4 and 13 - 14, with lesser concentrations of EGF around day 7 (day 0 = estrus) [33]. The decrease or loss of the peak has been linked to reduced fertility in RB cows and high-yielding cows [29, 30, 33, 55]. Conception rate in embryo recipient cows with the normal EGF concentrations on day 3 (4.70 - 13.50 ng/g tissue weight) [33] was higher than that of recipient cows with a low EGF concentration (< 4.70 ng/g tissue weight) (76.9% vs. 33.3%, respectively). By using treatments with exogenous hormones [34], seminal proteins [3], and osteopontin [39], the peak EGF concentration and, more importantly, fertility was restored in RB cows. Therefore, the relationship between the endometrial EGF profile and fertility in dairy cattle has been well demonstrated. Several risk factors for altering endometrial EGF profile have been reported in dairy cows. High level of milk production [55], elevated body temperature on the day of estrus [35], and elevated levels of endometrial leptin receptors [55] have been reported to increase the risk of EGF abnormality in dairy cows. However, the exact mechanism causing the loss of EGF peaks remains undefined.

As mentioned above, the synthesis of endometrial EGF is primarily regulated by E_2 and P_4 in dairy cattle [10, 14, 58]. In addition, low P_4 concentration in the early luteal phase has been demonstrated to cause global changes in the endometrial transcriptome [20]. Therefore, I hypothesized that an alteration of E_2 and/or P_4 in one of the critical periods from luteolysis to estrus or ovulation (Chapter 1) and in the early luteal phase (Chapter 2) may induce the loss or decrease of EGF peak concentrations and reduced fertility. In Chapter 1, I examined the effects

of the timing of luteolysis by administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in relation to the stage of follicular development on the profile of steroid hormones (E_2 and P_4) during the preovulatory period and EGF concentrations on day 3 (i.e., EGF profile) of the following estrous cycle (day 0 = estrus). Cows were treated with $PGF_{2\alpha}$ either on days 12 to 14 (selection phase group) or on days 16 to 17 of the estrous cycle (control group). Then, endometrial tissues were collected by biopsy on day 3 for EGF assays. In Chapter 2, I examined the effects of differing P_4 concentrations in the early luteal phase on the endometrial EGF concentration in the late luteal phase at the time of the second EGF peak and on day 3 of the following estrous cycle. Cows were either induced high P_4 concentrations during the early luteal phase by receiving an intravaginal P_4 device from day 5 to day 9 (day 0 = estrus) or low P_4 concentrations during the early luteal phase by receiving $PGF_{2\alpha}$ treatments on days 3, 3.5 and 4. Endometrial EGF concentrations were determined on day 14 of the cycle, and cows were allowed to show natural estrus. The endometrial tissue was obtained again on day 3 of the estrous cycle.

Chapter 1

Effects of prostaglandin $F_{2\alpha}$ treatment at follicular wave emergence on endometrial epidermal growth factor concentration on day 3 of the following estrous cycle and fertility in dairy cows

Introduction

Endometrial epidermal growth factor (EGF) concentration on day 3 of the estrous cycle has been shown as an indicator of endometrial function and fertility in dairy cattle [29, 32]. In normal cows, endometrial EGF concentration exhibits a cyclic change with two peaks on days 2 - 4 and 13 - 14 (day 0 = estrus) during the estrous cycle [31, 32]. The decrease or disappearance of the peak has been associated with reduced fertility in RB (repeat breeder) and high-yielding cows [29, 33, 55]. Conception rates were higher in embryo recipient cows with normal EGF concentrations on day 3 (4.7 - 13.5 ng/g tissue weight) [33] than in those with low EGF concentrations (< 4.70 ng/g tissue weight) (76.9% vs. 33.3%, respectively). Treatments with exogenous hormones [34], seminal proteins [3], and osteopontin [39] restored the peak of EGF concentrations and, more importantly, fertility in RB cows. This clearly demonstrates the association between endometrial EGF profile and fertility in dairy cattle. Furthermore, several risk factors for endometrial EGF profile alteration in dairy cows have been reported, including high level of milk production [55], elevated body temperature on the day of estrus [35], and elevated levels of endometrial leptin receptors [55]. However, the exact mechanism causing the loss of EGF peaks remains undefined.

The causes of reduced fertility in RB and high-yielding cows probably include environmental, management, and animal factors [43, 77]. Examples of animal factors are changes in ovarian steroid hormone concentrations: a slow increase in levels and low peak levels of estradiol (E_2) and progesterone (P_4), and the resulting alterations in growth factors and cytokines in the uterus [29, 44, 77]. High-yielding cows have lower concentrations of ovarian steroid hormones during the circulation than nonlactating dairy cows and heifers [64]. Greater feed intake increases liver blood flow, thus clearance of E_2 and P_4 from the circulation [76, 78]. Although RB cows may not necessarily be high producers, they exhibit similar alterations in ovarian steroid hormone concentrations to those observed in high-yielding cows [29].

These changes in the ovarian steroid hormone environment during the periovulatory period may cause a decrease in or loss of EGF peak levels in lactating dairy cows because EGF synthesis is mainly regulated by E_2 and P_4 in the endometrium [10, 14, 58].

Among the physiological changes reported in high-yielding cows [77], premature luteolysis [51], prolonged interluteal intervals [62], and delayed ovulation [40] extend the low P_4 period before estrus (i.e., a rise in E_2). Furthermore, the E_2 rise itself may be smaller in cows exhibiting these changes [44]. Therefore, this chapter examined the effects of the timing of luteolysis by administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in relation to the stage of follicular development on the profile of steroid hormones (E_2 and P_4) during the preovulatory period and EGF concentrations on day 3 (i.e., EGF profile) of the following estrous cycle.

Materials and Methods

Animals and eligibility for enrollment

All animal experiments were conducted from 2019 to 2021 in accordance with the Guide for the Care and Use of Laboratory Animals in Hokkaido University, Japan (experimental protocol #16-0071 and #19-0030). This chapter used 200 lactating Holstein cows that were housed in 14 commercial farms in Hokkaido, Japan. All cows were multiparous between two and six in parity and between 60 and 140 days postpartum at recruitment to the study. The cows were examined for health conditions, estrous cycle regularity, and subclinical inflammation of the uterus by cytology and oviductal patency, as previously described [3]. On day 3, they were examined for EGF concentration in the endometrial tissue obtained by biopsy. Cows without any abnormality in these examinations were used in this chapter.

Biopsy of the endometrial tissues

Uterine endometrial tissues were obtained using a biopsy instrument (3050100, Fujihira Industry, Tokyo, Japan) under caudal epidural anesthesia with 3 mL of 2% lidocaine (2% xylocaine, AstraZeneca, Osaka, Japan), as previously described [33]. Two pieces of uterine endometrial tissues from the intercaruncle region (25 - 50 mg) were obtained from the middle of three sections in the uterine horns, which were equally divided along the longitudinal axis. The caruncle region was distinguished from the intercaruncle region as fluffy cut surface due to its abundant blood vessels. If the biopsy sample contained a caruncle region, it was dissected

out, and the rest of the tissue was used [32, 39]. All tissue samples were obtained from the uterine horns on the contralateral side to corpus luteum (CL). Tissues were immediately frozen in liquid nitrogen and stored at -30°C for the EGF assay.

Measurement of EGF concentrations and judgment of the EGF profile

Uterine endometrial tissue samples were processed as previously described [28, 33] with a modification of changing the acetic acid concentration (01021-70, Kanto Chemical Co., Inc., Tokyo, Japan) for extraction solution from 1 to 0.1 M. The EGF concentrations in uterine endometrial tissue extracts were assessed using double-antibody sandwich enzyme immunoassay (EIA) with 96-well microtiter plates (Costar 3590, Corning, NY, USA) [33]. An anti-human EGF mouse monoclonal antibody (MAB636, R & D Systems, Inc., Minneapolis, MN, USA) was used as the solid-phase antibody and antihuman EGF rabbit antiserum (5022-100, Biogenesis, Poole, UK) for detection with a peroxidase-conjugated anti-rabbit IgG goat antibody (270335, Seikagaku, Tokyo, Japan). Neither of these antibodies exhibited significant cross-reactivity with other cytokines tested by the manufacturers. The assay system was verified using increasing concentrations of recombinant bovine EGF [3]. Linear regression analysis of recombinant bovine EGF concentrations and assay results gave $y = 0.96x + 0.39$, $r = 0.97$ [3]. The sensitivity of the assay was 10 pg/well. Intra- and interassay coefficient of variation (CV) were 5.8% and 6.3%, respectively.

Endometrial EGF concentrations were determined on day 3, and the EGF profile was considered normal when the EGF concentration was ≥ 4.70 ng/g tissue weight [31, 33].

Blood sampling and measurement of plasma E_2 and P_4 concentrations

Blood was collected daily from the day of $PGF_{2\alpha}$ treatment to ovulation. Blood sampling was conducted by puncture of the coccygeal vessels using ethylenediaminetetraacetric (EDTA)-loaded vacuum tubes (Venoject II EDTA, TERUMO, Tokyo, Japan, VJ-CA052L007) and placed on ice for about 30 min before separating the plasma via centrifugation at 4,500 × *g* for 15 min. Plasma samples were stored at -30°C until assayed.

Plasma E_2 and P_4 concentrations were determined using competitive double-antibody enzyme immunoassay, as previously described [79]. The primary antisera used for assay of E_2 and P_4 were anti-estradiol-17β-6-carboxymethiloxime-bovine serum albumin (CMO-BSA)

(FKA204; Cosmo Bio, Tokyo, Japan) and anti-progesterone-3-CMO-BSA (KZ-HS-P13; Cosmo Bio), respectively. The antiserum against E_2 cross-reacted with estrone (3.2%), estriol (1.7%), estrone sulfate (8.0%), and estradiol-17β (0.8%). In contrast, the antiserum against P_4 cross-reacted with 5α-dihydroxyprogesterone (12.6%), 11α-hydroxyprogesterone (5.3%), 20α-hydroxyprogesterone (0.2%), and pregnenolone (2.0%). Goat anti-rabbit serum (111-005-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as the secondary antibody. The intra- and interassay CV were 3.5% and 12.7% for E_2 and 4.4% and 6.9% for P_4, respectively.

Determination of luteolysis, estrus, and ovulation

Cows were examined for the ovarian morphology by ultrasonography using an ultrasound machine (HS-2100V, Honda Electronics, Aichi, Japan) attached to a linear transducer (5–10 MHz, HLV-475M, Honda Electronics). Corpus luteum regression was determined when the cows with plasma P_4 concentrations ≥ 1.0 ng/mL at the time of $PGF_{2\alpha}$ treatment exhibited P_4 concentrations < 1.0 ng/mL 48 h later [25]. Estrus was detected using an automated activity monitor (day 0 = estrus). True estrus alert was confirmed if the cow had at least one dominant follicle (> 15 mm in diameter) and an absence of CL (> 20 mm) either 7:00 - 8:00 or 19:00 by ultrasonography [26]. Animals confirmed to be in estrus were then monitored for ovulation by ultrasonography twice a day (7:00 - 8:00 and 19:00) for a maximum of six examinations (~72 h after the first examination) in study 1-1, whereas ovulation was confirmed by transrectal palpation every 24 h after AI (artificial insemination) in study 1-2. Ovulation was defined as the disappearance of a large follicle, followed by observation of a CL on the same approximate topographical location on the ovary. The time of ovulation was determined as the median time between the two ultrasound examinations (study 1-1) and transrectal palpations (study 1-2) wherein the ovulatory follicle disappeared.

Study design

Study 1-1

A total of 44 Holstein cows were randomly assigned to one of the following groups (Fig. 1-1): selection phase group, consisting of 20 cows treated with $PGF_{2\alpha}$ (dinoprost tromethamine 25 mg im, Pronalgon F, Zoetis Japan, Tokyo, Japan) on days 12 - 14 of the estrous cycle when

growing follicles first reached a diameter of 4 mm, and control group, consisting of 24 cows treated with $PGF_{2\alpha}$ on days 16 - 17. Cows were monitored for ovulation by ultrasonography twice a day after detection of estrus. Blood samples were collected immediately before $PGF_{2\alpha}$ treatment, 24 and 48 h after treatment, at estrus, and at the time of confirmation of ovulation to determine plasma E_2 and P_4 concentrations. The cows were examined for EGF concentrations on day 3 of the estrous cycle after the $PGF_{2\alpha}$ treatment.

Study 1-2

A total of 76 and 80 Holstein cows were treated with 25 mg of $PGF_{2\alpha}$ on day 12 (selection phase group) and day 16 (control group) of the estrous cycle, respectively (Fig. 1-2). All cows were subjected to AI at estrus (day 0) with frozen-thawed semen. The cows were subjected to a second AI if they failed to ovulate by 24 h after the first AI. Ovulation was monitored by transrectal palpation every 24 h starting from the time of the first AI until day 3 (at biopsy for EGF assay) and with an additional examination on day 12. A total of 20 randomly selected cows in each group were examined for plasma E_2 and P_4 concentrations at $PGF_{2\alpha}$ treatment, at estrus, and on days 3 and 7. Cows, in whom ovulation was confirmed by day 3, were examined for endometrial EGF concentration on day 3 to determine the EGF profile. Cows were diagnosed for pregnancy by ultrasonography between 30 and 35 days after the last AI.

Data analysis

Endometrial EGF concentrations between the groups were compared using Student's *t*-test. Changes in E_2 and P_4 concentrations were compared by analysis of variance with repeated measures. The proportion of cows showing different EGF profiles and the conception rate were compared using Fisher's exact test. *P* values less than 0.05 were considered different in all analyses. Data were expressed as mean ± SD in the text and tables and as means ± SEM in the figures. Data were analyzed using JMP Pro 17.0 (SAS Institute Inc., Tokyo, Japan).

Results

Study 1-1

The day of the follicular selection phase was 13.0 ± 0.8 days (days 12 - 14) after estrus in the selection phase group (Table 1-1). The intervals from $PGF_{2\alpha}$ treatment to estrus and from $PGF_{2\alpha}$ treatment to ovulation in this group were longer by about 48 h than those in the control group ($P < 0.05$). In contrast, the interval between estrus and ovulation in the selection phase group was longer by about 6 h than that in the control group.

Progesterone concentrations before and up to 48 h after $PGF_{2\alpha}$ treatment were similar in both groups ($P > 0.05$) (Fig. 1-3). No cows exhibited P_4 concentration below 1.0 ng/mL at $PGF_{2\alpha}$ treatment. All cows exhibited complete luteolysis within 2 days after $PGF_{2\alpha}$ treatment based on the progressive decrease in P_4 concentrations after treatment. Progesterone concentrations before $PGF_{2\alpha}$ treatment were at the same levels (control: 4.61 ± 1.46 ng/mL; selection phase group: 5.03 ± 1.48 ng/mL, $P > 0.05$). Furthermore, on the day of estrus and ovulation, P_4 concentrations were at the basal levels in both groups. On the day of estrus, estradiol concentrations were higher in the control group than in the selection phase group (12.34 ± 3.71 vs. 8.25 ± 2.71 pg/mL, $P < 0.05$). No differences were observed in E_2 concentrations between the two groups on other days ($P > 0.05$) (Fig. 1-3).

Endometrial EGF concentrations on day 3 of all cows ($P = 0.07$) showing both normal and altered EGF profiles (endometrial EGF concentrations lower than the lower limit of the normal range of 4.70 ng/g tissue weight) ($P < 0.05$) in the selection phase group were lower than those of cows in the control group (Table 1-2). As a result, the proportion of cows with normal EGF profile in the selection phase group (55.0%) was lower than that in the control group (79.2%) ($P < 0.05$). No significant difference was observed in the interval from estrus to ovulation between cows with normal and altered EGF profiles (Table 1-1).

Study 1-2

Of the 80 cows (88.8%) in the control group, 71 ovulated by 24 h after the first AI, and seven out of the remaining 9 cows ovulated in the next 24 h (Table 1-3). In contrast, 58 out of 76 cows (76.3%) in the selection phase group ovulated by 24 h after the first AI, and 11 out of the remaining 18 cows ovulated in the next 24 h. The proportion of cows that ovulated within 24 h after the first AI in the selection phase group was lower than that in the control group (*P*

< 0.05). All cows that had failed to ovulate by 24 h after the second AI in both groups did not ovulate by day 12, and a large (>1.5 cm) follicle and no CL was observed on the same day.

All cows in whom the P_4 concentration was examined ovulated by 24 h after the first AI. No cows exhibited P_4 concentrations below 1.0 ng/mL at $PGF_{2\alpha}$ treatment. Progesterone concentrations at $PGF_{2\alpha}$ treatment, at estrus, and on days 3 and 7 were at the same levels in both the control and selection phase groups (Fig. 1-4). Contrarily, E_2 concentrations on the day of estrus tended to be lower in the selection phase group than in the control group (15.61 ± 3.51 vs. 10.84 ± 4.62 pg/mL; $P = 0.08$) (Fig. 1-4). Estradiol concentrations on the other days were similar in the two groups (Fig. 1-4) ($P > 0.05$).

The EGF concentration in the selection phase group was lower than that in the control group ($P < 0.05$) (Table 1-3). As a result, the proportion of cows with EGF concentrations within the normal range on day 3 (i.e., the EGF profile) was lower in the selection phase group than in the control group ($P < 0.01$). Furthermore, the EGF concentrations of cows that had ovulated within 24 h after the first AI were compared, which were lower in the selection phase group than in the control group ($P < 0.05$).

The conception rate of cows that had ovulated within 24 h after the first AI (41.4%) and all cows (36.8%) was lower in the selection phase group than in the control group (57.7% and 55.0%, respectively) ($P < 0.05$) (Table 1-3).

Discussion

This chapter examined the effect of $PGF_{2\alpha}$ administration at the selection phase of follicular development on endometrial EGF concentration on day 3 of the following estrous cycle through changes in steroid hormone concentrations. Cows were treated with $PGF_{2\alpha}$ on the day when growing follicles first reached a diameter of 4 mm (between days 12 and 14) to extend the preovulatory period with low P_4 concentrations (selection phase group), whereas cows were treated with $PGF_{2\alpha}$ on days 16 to 17 in the control group. The selection phase group had lower endometrial EGF concentration on day 3, resulting in a lower proportion of cows with normal EGF profile in the following estrous cycle. Furthermore, the conception rate was lower in the selection phase group than in the control group. These results validate the hypothesis that the timing of $PGF_{2\alpha}$ administration in relation to the stage of follicular wave can affect the profile of steroid hormones (E_2 and P_4) during the preovulatory period. Consequently, this can potentially lead to an alteration of the endometrial EGF profile during the estrous cycle in cows.

The following three scenarios are known to prolong the follicular phase, and all have been associated with reduced fertility in dairy cows. First, clearance of circulating ovarian steroid hormone increases due to an increase in blood flow to the liver [64, 76] in association with increased dry matter intake to sustain high levels of lactation in high-yielding cows. As a result, an increase in plasma E_2 concentration slows down, and the peak level of E_2 decreases [64, 66]. These changes in E_2 cause a delay of LH surge and ovulation, thus prolonging the preovulatory period after CL regression. Although RB cows are not necessarily high producers, they exhibit the same changes in the circulating E_2 observed in high-yielding cows [29]. They also show a delay of preovulatory E_2 rise, lower E_2 peak [78], and delay of LH surge and ovulation [40].

Second, stress and lipopolysaccharide (LPS) have been associated with prolonged preovulatory period and anovulation in cows [67]. They both suppress the hypothalamus–pituitary–gonad axis and suppress ovulatory signals (i.e., GnRH and LH surge) [6, 59]. The uterus with inflammation caused by infection with gram-negative bacteria (e.g., *Escherichia coli*) [46], mastitis [15], and rumen with reduced pH [60] are typical source of LPS in modern dairy cows. Lipopolysaccharide also suppresses circulating E_2 levels by inhibiting its production in the follicle [47].

Lastly, $PGF_{2\alpha}$ treatment in cows in whom estrus was overlooked or cows that failed to become pregnant after AI could cause the same condition as the selection phase group, because

cows were often treated with $PGF_{2\alpha}$ without paying attention to the stage of follicular wave. This could be an iatrogenic cause of reduced fertility and may have occurred often in breeding management on a typical dairy farm.

The mechanism by which the prolonged preovulatory period lowers the peak EGF concentration on day 3 may be explained by the reduced sensitization effect before the increase of E_2 at estrus. Epidermal growth factor expression in the uterus is mainly regulated by P_4 sensitization and E_2 stimulus [10, 58]. The effect of P_4 sensitization may be weakened by extending the period from CL regression to estrus. The prolonged low P_4 environment also affects the capacity of preovulatory follicles to secrete E_2 [66]. This is consistent with the results of E_2 concentrations in this chapter. Although the frequency of E_2 measurements in this study may be insufficient to determine the peak timing or peak concentrations, the results of E_2 concentrations may indicate reduced estrogenic action in the selection phase group at estrus. The decrease in both P_4 sensitization and E_2 stimulus may reduce the EGF concentration in the endometrium.

As regards sensitization by P_4, the P_4 concentration in the previous cycle may affect the EGF profile. The duration of low P_4 concentration between luteolysis and estrus (or ovulation) were different between the two groups in studies 1-1 and 1-2. However, no difference was observed in P_4 concentrations at $PGF_{2\alpha}$ treatment between the two groups; thus, the level of P_4 in the late luteal phase may not have direct and/or significant impact on the EGF profile in the following estrous cycle. However, it is interesting to examine the effect of P_4 concentration in the early luteal phase on EGF expression in the following cycle because low P_4 concentration in this phase caused global changes in the endometrial transcriptome [21]. The endometrium that had been exposed to different levels of P_4 in the early luteal phase may respond differently to the preovulatory E_2 in EGF expression in the following cycle.

Reduction of fertility in the selection phase group occurred due to multiple factors, with decreased EGF concentration on day 3 being one of them. The low EGF on day 3 has been associated with reduced fertility mainly due to an increase in the early embryonic loss [33]. The prolonged low P_4 environment during the development and selection of dominant follicles may reduce fertility by impairing the oocyte quality and function of CL formed following ovulation. However, the latter may not be critical on fertility because the P_4 concentrations during the following estrous cycle (days 3 and 7 in study 1-2) were not different between the selection phase and control groups. The effect on the oocyte developmental competence may

be compromised [17], but the impact on fertility through the detrimental effect of oocyte quality still needs to be investigated.

This chapter demonstrated the association of prolonged low P_4 environment during the development of preovulatory follicles or before estrus with the EGF profile of the following estrous cycle and fertility. The selection phase group exhibited about 48 h longer periods in both $PGF_{2\alpha}$ treatment to estrus and ovulation than the control group. The period from estrus to ovulation in the selection phase group was 6 h longer than the control group. However, the extent to which prolonged low P_4 environment affects the EGF profile and fertility still needs to be investigated. Understanding the exact stage of follicle development or the follicle size at $PGF_{2\alpha}$ treatment, which affects the EGF concentration in the following cycle, is important information for improving the reproductive management of dairy herds.

In Chapter 1, I showed that the prolonged duration between CL regression and estrus (or ovulation) leading to changes in E_2 concentrations at estrus and the duration of low P_4 levels is one of the potential causes by which the EGF concentrations in the following estrous cycle decrease and reduce fertility in modern dairy cows. It is noteworthy that this condition may be caused by $PGF_{2\alpha}$ treatment to induce estrus, if treatment was given at the follicular selection phase.

Tables and Figures

Table 1-1. Interval from $PGF_{2\alpha}$ treatment to estrus and ovulation in cows that were treated with $PGF_{2\alpha}$ on days 16 to 17 of the estrous cycle (control group) and on the day of the follicular selection phase at the diestrus stage (selection phase group).

Group	Cycle day at $PGF_{2\alpha}$ treatment (n)		Interval from $PGF_{2\alpha}$ treatment to estrus (h)	Interval from $PGF_{2\alpha}$ treatment to ovulation (h)	Interval from estrus to ovulation (h)		
					Cows with normal EGF	Cows with altered EGF	Total
Control (24)	16 (21) 17 (3)	16.1 ± 0.3[a]	76.9 ± 15.4[a]	110.2 ± 17.7[a]	33.5 ± 7.0	32.4 ± 4.9	33.3 ± 6.5[a]
Selection phase* (20)	12 (6) 13 (9) 14 (5)	13.0 ± 0.8[b]	124.4 ± 19.4[b]	163.9 ± 21.8[b]	40.3 ± 9.4	38.5 ± 8.1	39.5 ± 8.7[b]

* $PGF_{2\alpha}$ was given between days 12 and 14 of the estrous cycle when developing follicles reached 4 mm.

[a,b] Values with different letters within the same column differ significantly ($P < 0.05$).

Values are presented as means ± SDs.

Table 1-2. Endometrial EGF concentrations on day 3 of the estrous cycle in cows received $PGF_{2\alpha}$ treatment on days 16 to 17 of the previous estrous cycle and on the day of the follicular selection phase at the diestrus stage.

Group	EGF profile	No. (%) of cows showing indicated profile	EGF concentrations (ng/g tissue weight)
Control	Normal	19 (79.2)[a]	6.18 ± 0.85[a]
	Altered	5 (20.8)[a]	2.96 ± 1.20[a]
	Subtotal	24 (100)	5.51 ± 1.61[A]
Selection phase*	Normal	11 (55.0)[b]	6.03 ± 0.99[b]
	Altered	9 (45.0)[b]	3.55 ± 1.01[b]
	Subtotal	20 (100)	4.80 ± 1.60[B]
Total	Normal	30 (68.2)	6.10 ± 0.90
	Altered	14 (31.8)	3.23 ± 0.99
	Subtotal	44 (100)	5.18 ± 1.63

* $PGF_{2\alpha}$ was given between days 12 and 14 of the estrous cycle when developing follicles reached 4 mm.

[ab] Values with different letters within the same EGF profiles between different groups differ significantly ($P < 0.05$).

[AB] Values with different letters between subtotals of the different groups tended to be different ($P = 0.07$).

EGF concentrations are presented as means ± SDs.

EGF profile was determined by the endometrial EGF concentration on day 3. EGF concentration of between 4.7 and 13.5 ng/g tissue weight (normal range) was considered to be normal [31, 33].

Table 1-3. Comparison of the effect of $PGF_{2\alpha}$ treatments on day 12 of the estrous cycle (control group) vs. day 16 of the estrous cycle (selection phase group) on the ovulation rate, EGF profile on day 3 of the following estrous cycle and conception rate in lactating Holstein cows.

Group	No. (%) of cows ovulated in indicated period*		Proportion of cows with the normal EGF profile (%)	EGF concentrations (ng/g tissue weight)	Conception (%)
Control	< 24 h after AI	71 (88.8)[a]	56/71 (78.9)	6.41 ± 0.75[a]	41/71 (57.7)[a]
	24h after AI <	7 (8.8)	4/7 (57.1)	5.31 ± 1.30	3/7 (42.9)
	No ovulation	2 (2.5)	-	-	0/2 (0)
	Subtotal	80 (100)	60/78 (76.9)[A]	6.31 ± 1.18[a]	44/80 (55.0)[a]
Selection phase	< 24 h after AI	58 (76.3)[b]	33/58 (56.9)	5.92 ± 1.08[b]	24/58 (41.4)[b]
	24h after AI <	11 (14.5)	4/11 (36.4)	4.27 ± 1.20	4/11 (36.4)
	No ovulation	7 (9.2)	-	-	0/7 (0)
	Subtotal	76 (100)	37/69 (53.6)[B]	5.66 ± 0.92[b]	28/76 (36.8)[b]
Total	< 24 h after AI	129 (82.7)	89/129 (69.0)	6.19 ± 0.68	65/129 (50.4)
	24h after AI <	18 (11.5)	8/18 (44.4)	4.67 ± 0.67	7/18 (38.9)
	No ovulation	9 (5.8)	-	-	0/9 (0)
	Subtotal	156 (100)	97/147 (66.0)	6.00 ± 0.58	72/156 (46.2)

* Time after the first AI.

[ab] Values with different letters between the same category of different groups differ ($P < 0.05$).

[AB] Values with different letters between different groups differ ($P < 0.01$).

EGF concentrations are presented as means ± SDs.

EGF profile was determined by the endometrial EGF concentration on day 3. EGF concentration of between 4.7 and 13.5 ng/g tissue weight (normal range) was considered to be normal [31, 33].

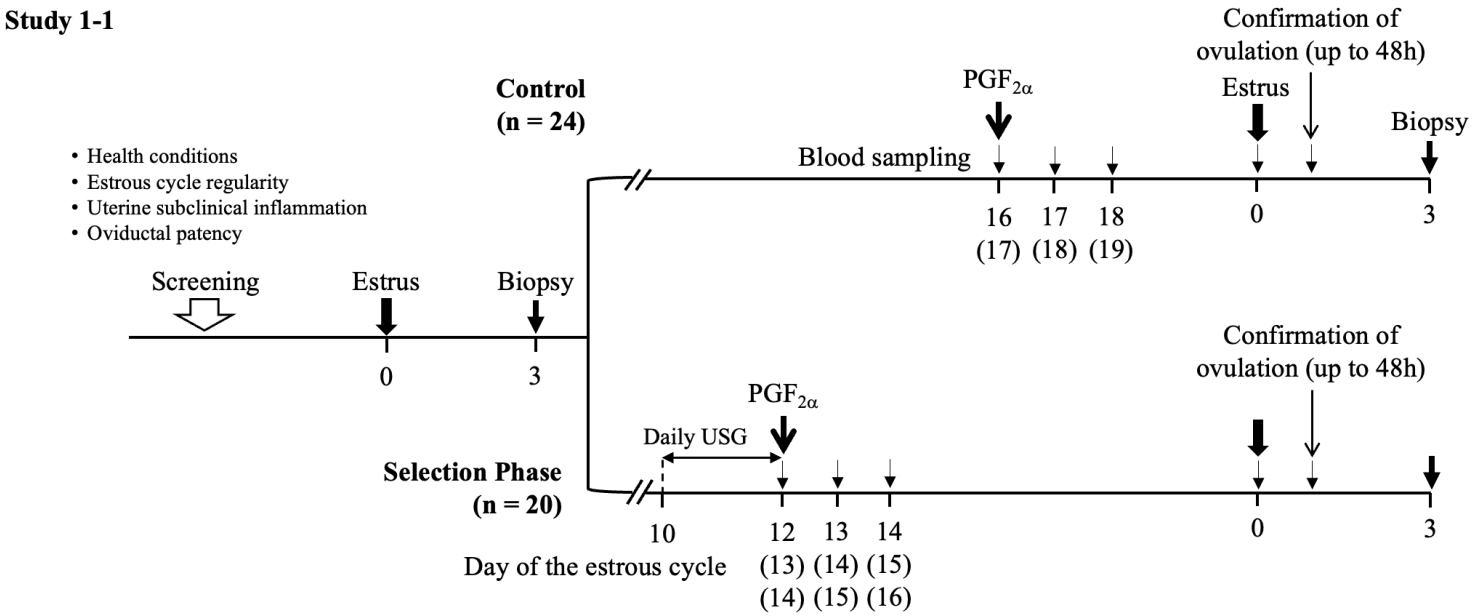

Fig. 1-1. Diagram of experimental procedures in study 1-1.

Cows used for the study after screening by one of veterinarian in the study group. Cows were assigned into one of the two groups. In the control group, $PGF_{2\alpha}$ was given on day 16 (n = 21) or day 17 (n =3) of the estrous cycle (estrus = day 0), while cows in the selection phase group were received $PGF_{2\alpha}$ treatment on the day growing follicles first reached 4 mm in diameter (day 12, day 13 and day 14; n = 6, n = 9 and n = 5; respectively) that was determined by daily transrectal ultrasonography, starting from day 10. Blood sampling for plasma E_2 and P_4 assays was conducted at $PGF_{2\alpha}$ treatment, 2 consecutive days after $PGF_{2\alpha}$ treatment, estrus and the confirmation of ovulation. The endometrial tissues were obtained by biopsy on day 3 and EGF concentrations were examined. $PGF_{2\alpha}$ = treatment with 25 mg of prostaglandin $F_{2\alpha}$; USG = Ultrasonography.

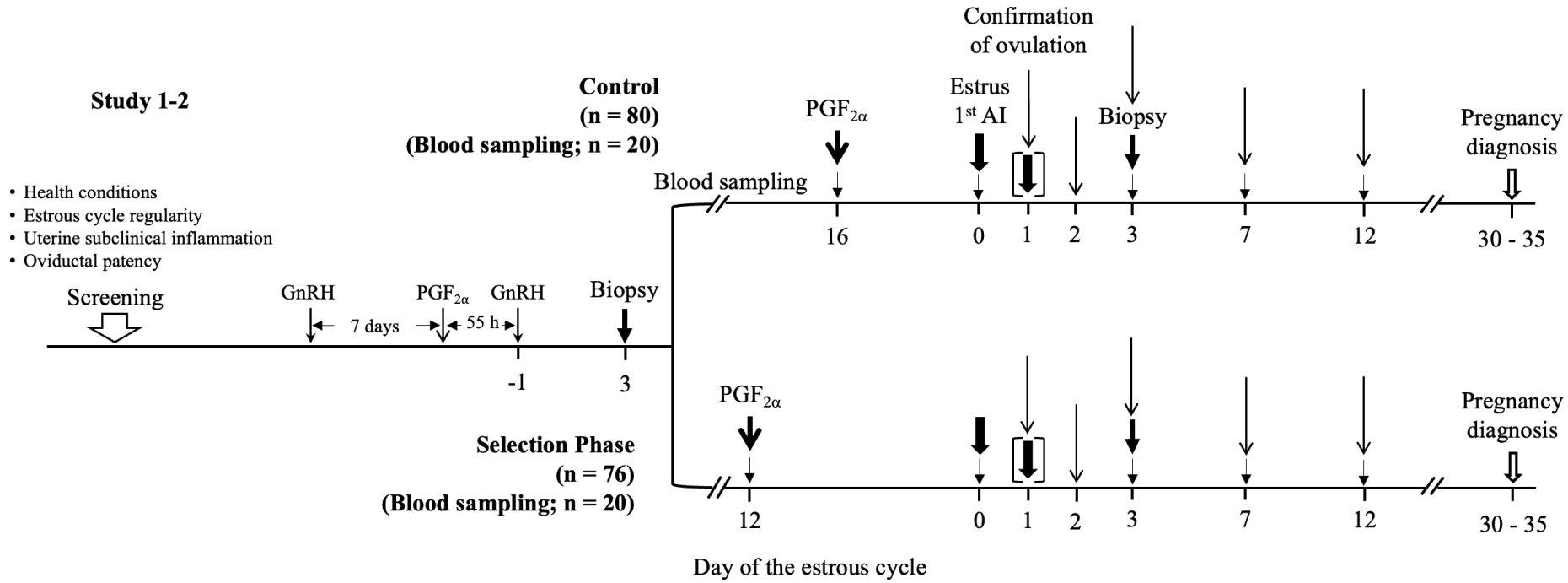

Fig. 1-2. Diagram of experimental procedures in study 1-2.

Cows used for the study after screening by one of veterinarian in the study group. Cows were assigned into one of the two groups. In the control group, $PGF_{2\alpha}$ was given on day 16 of the estrous cycle (n = 80; estrus = day 0), while cows in the selection phase group (n = 76) were received $PGF_{2\alpha}$ treatment on day 12 of the estrous cycle. All cows were subjected to AI at estrus. Cows were subjected to AI for the second time if they failed to ovulate by 24 h after the first AI. Ovulation was monitored by transrectal palpation at every 24 h starting from the time of the first AI until day 3 (at biopsy for EGF assay) and with an additional examination on day 12. Randomly selected 20 cows in each group were examined for plasma E_2 and P_4 concentrations at $PGF_{2\alpha}$ treatment, estrus, on day 3 and day 7. Cows, in which ovulation was confirmed by day 3, were determined

the endometrial EGF concentration on day 3 to determine the EGF profile. Cows were diagnosed for pregnancy by ultrasonography between 30 and 35 days after the last AI. $PGF_{2\alpha}$ = treatment with 25 mg of prostaglandin $F_{2\alpha}$.

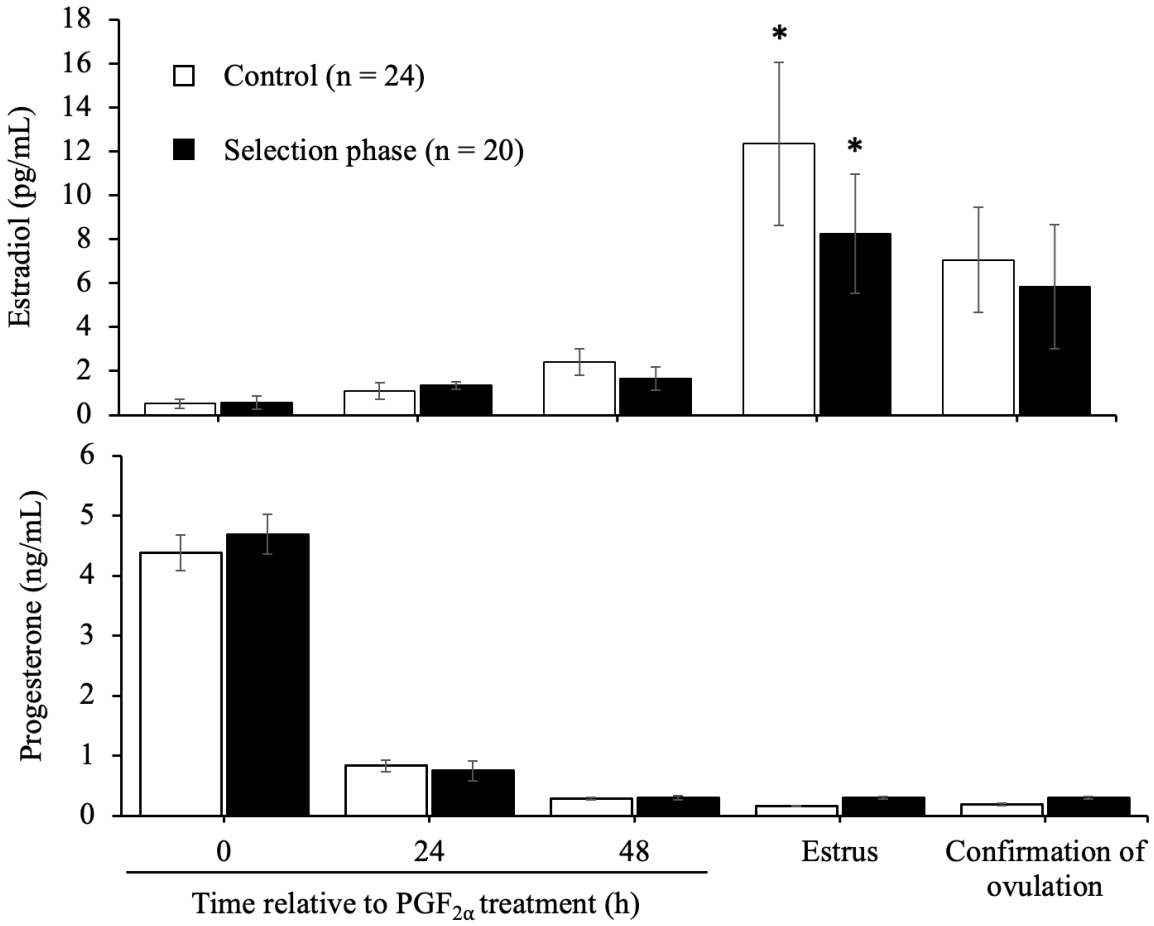

Fig. 1-3. Plasma E_2 and P_4 concentrations at $PGF_{2\alpha}$ treatments and on the next 2 consecutive days, at estrus, and at confirmation of ovulation in study 1-1. Cows were treated with $PGF_{2\alpha}$ on day 16 or day 17 of the estrous cycle in the control group (open bar □) and on the day of the follicular selection phase (days 12 to 14) in the selection phase group (closed bar ■). There were no differences in the circulating P_4 concentrations at all time points between two groups ($P > 0.05$). (*) Estradiol concentration was lower in the selection phase group compared to the control group on the day of estrus ($P < 0.05$), while there were no differences on the other days ($P > 0.05$).

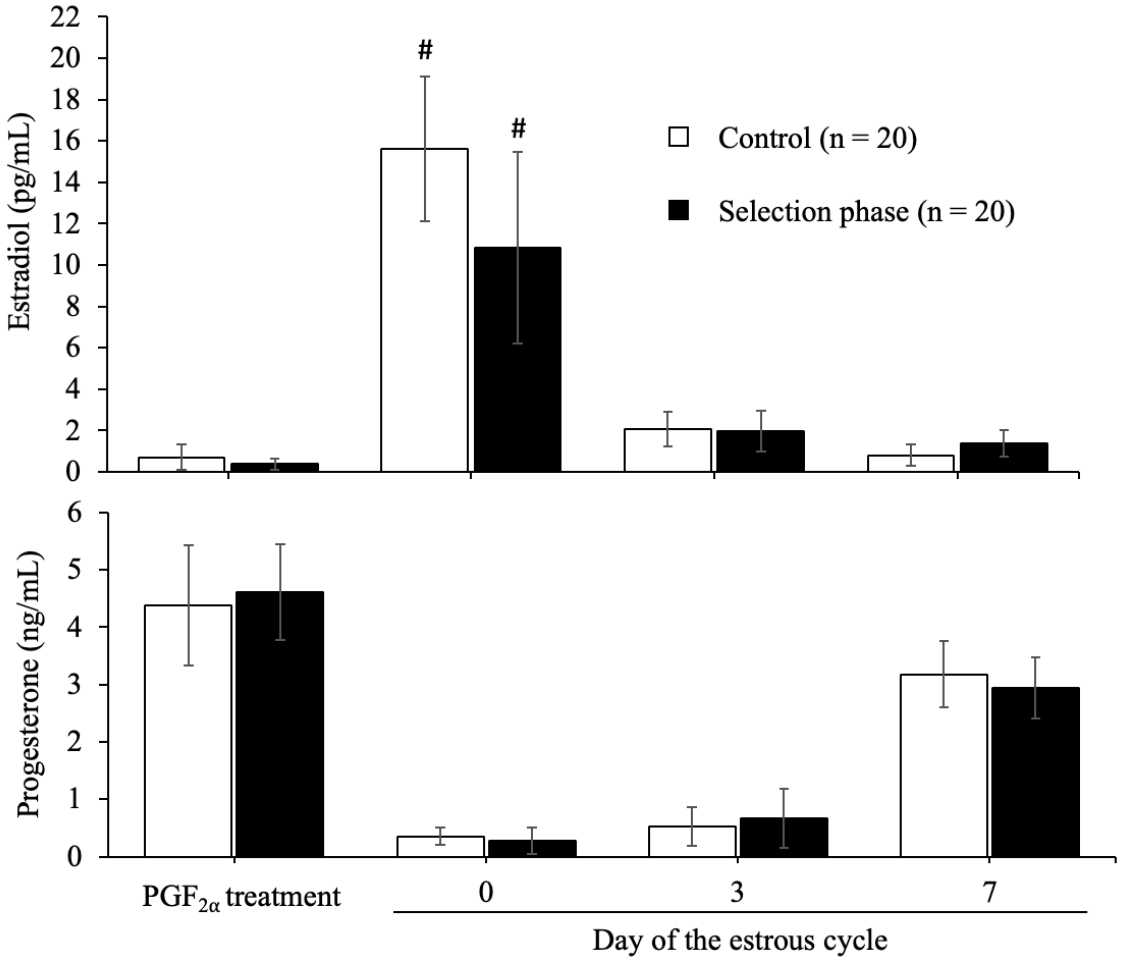

Fig. 1-4. Plasma E_2 and P_4 concentrations at $PGF_{2\alpha}$ treatment, at estrus, on day 3 and day 7 of the estrous cycle in study 1-2.

Cows were treated with $PGF_{2\alpha}$ on day 16 of the estrous cycle in the control group (open bar □) and on the day of the follicular selection phase (day 12) in the selection phase group (closed bar ■). There were no differences in the circulating P_4 concentrations at all time points between two groups ($P > 0.05$). (#) Estradiol concentration on the day of estrus tended to be lower in the selection phase group compared to the control group ($P = 0.08$), while there were no differences on the other days ($P > 0.05$).

Summary

Inadequate exposure to E_2 and P_4 may be the main cause of altered endometrial epidermal growth factor profile, leading to reduced fertility in dairy cows. I hypothesized that $PGF_{2\alpha}$ administration at different timings of the estrous cycle and stages of follicular development could change the profile of steroid hormones between luteolysis and estrus. This results in reduction in the peak concentration of endometrial EGF on day 3 (day 0 = estrus) in the following estrous cycle.

In study 1-1, lactating Holstein cows were treated with $PGF_{2\alpha}$ either on days 12 - 14 (selection phase group, n = 20) or on days 16 - 17 (control group, n = 24) of the estrous cycle. Blood samples were obtained before $PGF_{2\alpha}$ treatment, 24 and 48 h after treatment, and on the day of estrus and ovulation for E_2 and P_4 assays. Endometrial tissues were collected by biopsy on day 3 for EGF assays. The duration from $PGF_{2\alpha}$ treatment to both estrus and ovulation was longer in the selection phase group than in the control group ($P < 0.05$). The time between estrus and ovulation was longer in the selection phase group ($P < 0.05$). Estradiol concentrations were higher in the control group on the day of estrus ($P < 0.05$). Progesterone concentrations were not different between the groups. Endometrial EGF concentrations were lower in the selection phase group than in the control group ($P < 0.05$).

In study 1-2, lactating Holstein cows were treated with $PGF_{2\alpha}$ either on day 12 (selection phase group, n = 76) or day 16 (control group, n = 80). They were subjected to artificial insemination (AI) at estrus, and those that failed to ovulate by 24 h after the first AI were subjected to second AI. On day 3, endometrial EGF concentration was determined. At estrus and on days 3 and 7, blood was collected for E_2 and P_4 assay from 20 randomly selected cows in each group before $PGF_{2\alpha}$ treatment. The cows in the selection phase group exhibited lower EGF concentration, proportion of cows with normal EGF profile, and conception rate than cows in the control group ($P < 0.05$). On the day of estrus, E_2 concentrations tended to be lower in the selection phase group than in the control group ($P = 0.08$). Progesterone concentrations were not different between the groups; however the duration of low P_4 between luteolysis and estrus (or ovulation) was longer in Selection phase group compared to Control group. These findings suggest that low E_2 concentrations at estrus and low P_4 concentrations for a prolonged period due to premature termination of CL in the absence of a dominant follicle are potential causes of altered endometrial EGF profile in dairy cows.

Chapter 2

Effects of progesterone concentration during the early luteal phase on the endometrial epidermal growth factor concentrations in dairy cows

Introduction

An increase of progesterone (P_4) concentration after artificial insemination (AI) showed positive correlations to the conceptus survival and establishment of pregnancy in dairy cows [49, 50, 52]. While, low P_4 concentrations on day 5 post-ovulation [42, 68] or a delay in the normal rise in P_4 between days 4 and 5 post-ovulation have been associated with reduced fertility [42, 72]. One of the underlying mechanisms for the impact of P_4 on fertility is that low or suboptimal concentrations delay establishment of uterine receptivity to implantation [19]. The same authors described the advanced endometrial gene expression, particularly genes associated with energy sources or contributors to histotroph, which may contribute to advanced conceptus development on days 13 and 16 in pregnant and cyclic cattle under elevated P_4 [20].

In normal cows, the endometrial epidermal growth factor (EGF) concentrations shows a cyclic change with two peaks on days 2 - 4 and 13 - 14 (day 0 = estrus) during the estrous cycle [31, 32]. The decrease or disappearance of the peaks has been linked to reduced fertility in repeat breeder (RB) cows and high-yielding cows [29, 33, 55]. Lactating dairy cows typically show delayed increases in P_4 in the early luteal phase and lower concentrations of estradiol (E_2) at estrus [78]. Therefore, it may cause a decrease or loss of the EGF peak levels in lactating dairy cows since the synthesis of EGF is primarily regulated by E_2 and P_4 in the endometrium [10, 14, 58].

In Chapter 1, I demonstrated that the prolonged low P_4 period between corpus luteum (CL) regression and estrus (or ovulation) induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) treatment at the time of follicular wave or before selection of dominant follicle reduced EGF peak concentrations on day 3 of the following estrous cycle and reduced fertility in dairy cows. However, the effect of a delayed increase and low P_4 concentrations during the early luteal phase remained to be examined. The objective of the present chapter was to evaluate the effect of P_4 concentrations during the early luteal phase on the endometrial EGF concentrations on the following estrous cycle.

Materials and Methods

Animals and eligibility for enrollment

All animal experiments were performed from 2019 to 2023, in accordance with the Guidelines for Care and Use of the Experimental Animal Protocol of Hokkaido University, Japan (Experimental protocol #16-0071 and #19-0030). Lactating Holstein cows from commercial farms located in Hokkaido province, Japan were used in the study. Cows were examined for health conditions, regularity of the estrous cycle, subclinical inflammation of the uterus by cytology and oviductal patency as described previously [3]. Cows without any abnormality in these examinations were used in this chapter.

Biopsy of the endometrial tissues

Uterine endometrial tissues were obtained using a biopsy instrument (3050100, Fujihira Industry, Tokyo, Japan) under caudal epidural anesthesia with 3 mL of 2% lidocaine (2% xylocaine, AstraZeneca, Osaka, Japan), as described previously [33]. Two pieces of uterine endometrial tissues from the intercaruncle region (25 - 50 mg) were obtained from the middle of three sections in the uterine horns, which were equally divided along the longitudinal axis. The caruncle region was distinguished from the intercaruncle region as fluffy cut surface due to rich blood vessels. If the biopsy sample contained caruncle region, it was dissected out and the rest of the tissue was used [32, 39]. All tissue samples were obtained from the uterine horns on the contralateral side to CL. Tissues were immediately frozen in liquid nitrogen and stored below -30°C for the EGF assay.

Measurement of EGF concentrations and judgement of the EGF profile

Uterine endometrial tissue samples were processed as previously described [28, 33] with a modification of changing the concentration of acetic acid (01021-70, Kanto Chemical Co., Inc., Tokyo, Japan) for extraction solution from 1 M to 0.1 M. Epidermal growth factor concentrations in uterine endometrial tissue extracts were assessed using double-antibody sandwich enzyme immunoassay (EIA) with 96-well microtiter plates (Costar 3590, Corning, NY, USA) [33]. An anti-human EGF mouse monoclonal antibody (MAB636, R & D Systems, Inc., Minneapolis, MN, USA) was used as the solid-phase antibody and anti-human EGF rabbit antiserum (5022-100, Biogenesis, Poole, UK) for detection with a peroxidase-conjugated anti-

rabbit IgG goat antibody (270335, Seikagaku, Tokyo, Japan). Neither of these antibodies showed significant cross-reactivity with other cytokines tested by the manufacturers. The assay system was verified for bovine uterine tissue using increasing concentrations of recombinant bovine EGF ($y = 0.96x + 0.39$, $r = 0.97$) [3]. The sensitivity of the assay was 10 pg/well. Intra- and inter-assay coefficient of variation (CV) at 10 pg/well were 5.8 and 6.3%, respectively.

Endometrial EGF concentrations were determined on day 3, and the EGF profile was considered normal when the EGF concentration was ≥ 4.70 ng/g tissue weight [31, 33].

Blood sampling and measurement of plasma P_4 concentrations

Blood was collected daily from the day of $PGF_{2\alpha}$ treatment to ovulation. Blood sampling was conducted by puncture of the coccygeal vessels using ethylenediaminetetraacetric (EDTA)-loaded vacuum tubes (Venoject II EDTA, TERUMO, Tokyo, Japan, VJ-CA052L007) and placed on ice for about 30 min before plasma was separated by centrifugation at 4500 × g for 15 min. Plasma samples were stored at -30°C until assayed.

Plasma P_4 concentrations were determined using competitive double-antibody enzyme immunoassay, as described previously [79]. The primary antibody used for the P_4 assay was antiprogesterone-3-carboxymethiloxime-bovine serum albumin (CMO-BSA) (KZ-HS-P13; Cosmo Bio, Tokyo, Japan). Goat anti-rabbit serum (111-005003; Jacson Immuno Research Laboratories, West Grove, PA, USA) was used as the secondary antibody. The inter- and intra-assay CV were 4.4 and 6.9%.

Study design

Study 2-1

Lactating Holstein cows were observed for estrus (day 0) and the endometrial tissue was obtained by biopsy for EGF assay. Cows with the normal EGF profile were randomly assigned to one of three following groups: High P_4 (n = 10), Low P_4 (n = 10), and Control groups (n = 10) (Fig. 2-1). Cows in High P_4 group were received an intravaginal P_4 releasing device (CIDR 1900, Zoetis Japan, Tokyo, Japan) on day 5 and removed on day 9. Cows in Low P_4 group were treated with three doses of $PGF_{2\alpha}$ (Dinoprost tromethamine 25 mg im, Pronalgon F, Zoetis Japan, Tokyo, Japan) on days 3, 3.5, and 4. Cows in the control group were remained untreated. Daily blood samples were collected from days 3 to 9 for P_4 assay. The endometrial tissue

sample was obtained for EGF assay on day 14 of the cycle, and cows were allowed to show natural estrus. The endometrial tissue was obtained again on day 3 of the estrous cycle.

Study 2-2

Thirty cows were treated same as study 2-1, but they were induced estrus with $PGF_{2\alpha}$ on day 9. Cows were observed for estrus and the endometrial tissue was obtained for the second time on day 3 (Fig. 2-1).

Data analysis

Concentrations of progesterone were analyzed by ANOVA for repeated measures. The effects of treatment group, day and the interactions between group and day were included in the model as independent variables. Analysis of the endometrial EGF concentrations was performed by ANOVA using a model that included the effects of treatment and time. $P \leq 0.05$ was considered different in all analyses, and from $0.05 < P \leq 0.10$ was designated as a tendency. Data were presented as the mean ± SD in the text and tables while the mean ± SEM in the figures. Data were analyzed using the computer software JMP Pro 17.0 (SAS Institute Inc., Tokyo, Japan).

Results

Study 2-1

Concentrations of P_4 in plasma during early luteal phase differed among treatments (High P_4 = 3.28 ± 2.62 ng/mL; Low P_4 = 0.71 ± 0.36; Control = 1.75 ± 1.43;) ($P < 0.05$) (Fig. 2-2). However, no difference in P_4 concentrations on day 3 (High P_4 = 0.39 ± 0.10 ng/mL; Low P_4 = 0.38 ± 0.12; Control = 0.39 ± 0.16) and day 4 (High P_4 = 0.44 ± 0.13 ng/mL; Low P_4 = 0.40 ± 0.08; Control = 0.46 ± 0.14) was observed among three treatments ($P > 0.05$). On day 5, insertion of CIDR (controlled internal drug release) device increased ($P < 0.05$) concentrations of P_4 in High P_4 group that reached a plateau on day 9, whereas cows in Low P_4 and Control groups had a continuous increase in P_4 concentrations. From days 5 to 9, P_4 concentrations were lesser ($P < 0.05$) for Low P_4 group compared with Control and High P_4 groups (High P_4 = 4.4 ± 2.13 ng/mL; Low P_4 = 0.8 ± 0.35; Control = 2.3 ± 1.35) and were significantly different between High P_4 and Control groups ($P < 0.05$).

Endometrial EGF concentrations on day 3 of the initial estrous cycle did not differ among groups ($P > 0.05$), and all cows showed normal EGF profile (the endometrial EGF concentrations higher than the lower limit of the normal range of 4.70 ng/g tissue weight) (Table 2-1).

On day 14, endometrial EGF concentrations were greater ($P < 0.05$) for High P_4 group compared with Low P_4 group and tended to be higher ($P = 0.06$) for High P_4 group compared with Control group (High P_4 = 6.65 ± 0.94; Low P_4 = 5.17 ± 1.07; Control = 5.78 ± 1.03 ng/g tissue weight) (Table 2-1). The number of cows showed normal endometrial EGF concentrations on day 14 was 6 out of 10 cows in the Low P_4 group, compared to 9 out of 10 cows in the Control group (Table 2-1). None of the cows in the High P_4 group showed a low endometrial EGF concentration on day 14 (Table 2-1).

There was interaction between the effect of time and treatment of P_4 on EGF concentrations ($P < 0.05$). On day 3 of the following estrous cycle, endometrial EGF concentrations in Low P_4 group were lower than that in High P_4 and Control groups ($P < 0.05$). There were no differences in EGF concentrations between High P_4 and Control groups ($P > 0.05$) (Table 2-1). The number of cows showed abnormal EGF profile in Low P_4 group on day 3 of the following estrous cycle was 5 out of 10 cows, compared to none of 10 cows in High P_4 and Control groups (Table 2-1).

Study 2-2

All cows in three groups showed normal EGF profile on day 3 of the initial cycle (Table 2-2). After treatment, 4 cows showed abnormal EGF profile in Low P_4 group; whereas one and none of cows showed an abnormal profile in High P_4 and Control groups, respectively (Table 2-2). As a result, the EGF concentrations were lower in Low P_4 group than High P_4 and Control groups (High P_4 = 6.65 ± 0.94; Low P_4 = 5.17 ± 1.07; Control = 5.78 ± 1.03 ng/g tissue weight) ($P < 0.05$) (Fig. 2-4). There was no interaction in EGF concentrations between time and treatments ($P > 0.05$).

Discussion

In the previous chapter, I induced the prolonged low P_4 environment between CL regression and estrus (or ovulation) by treatments of $PGF_{2\alpha}$ between days 12 - 14 (day 0 =

estrus). The results showed that lower endometrial EGF concentrations on day 3 of the following estrous cycle, and reduced conception rate in these cows compared to the cows in the control group. The results in this chapter demonstrated that induced low P_4 concentrations during the early luteal phase decreased the endometrial EGF concentrations on day 14 of the initial estrous cycle and day 3 of the following estrous cycle. This can be a mechanism by which an alteration of the EGF profile occurs in cows. In contrast, the supplementation of P_4 during the early estrous cycle did not result in an increase in EGF concentrations.

Administration of $PGF_{2\alpha}$ on days 3, 3.5 and 4 led to a significant reduction in circulating P_4 concentrations, due to a change in vascularization of the CL, rather than CL size per se [8, 19, 21]. On the other hand, insertion of a CIDR on day 5 (day 0 = estrus) significantly increased circulating P_4 concentrations [49], which is similar to the findings in this chapter. As a result, cows in Low P_4 group had lower P_4 concentrations from days 5 to 9 compared with High P_4 and Control cows. Numerous studies have connected low concentrations of P_4 during post-ovulatory period with reductions in conceptus growth and elongation [19, 21], decreased interferon-τ (IFNT) production [48], and lower pregnancy rates in cattle [50]. In contrast, elevated concentrations of circulating P_4 in the period immediately following conception have been associated with advancement of conceptus elongation [11], increased IFNT production [48, 50], and, in some cases, higher pregnancy rates in cattle [52, 73].

The effect of low P_4 on fertility in dairy cattle occurred by multiple causes, and decreased EGF concentrations on days 3 and/or 14 may be one of them. The low EGF on day 3 has been linked to reduced fertility mainly due to an increase in the early embryonic loss [33]. Although the mechanism for the influence of EGF concentrations on day 3 on embryo is unclear, it can be explained that the failure of the increase in EGF concentrations at this stage of the estrous cycle impairs the uterine environment, in turn, causes asynchrony between uterus and the blastocyst, as suggested by previous studies [16, 37, 68]. In contrast, the roles of EGF on day 14 in embryonic survival and development have been suggested. Development of elongating (days 14 - 16) bovine blastocysts seems to depend on the maternal supply that includes EGF [38]. Moreover, the effect of EGF to increase production ratio of $PGE_2/PGF_{2\alpha}$ may promote CL function [2, 69, 80].

Progesterone plays an important role for establishment and maintenance of pregnancy in cattle, paradoxically, the loss of P_4 receptors (PR) in uterine epithelia appears to be a prerequisite for maternal recognition of pregnancy and conceptus development in early

pregnancy [7]. By using the similar low P_4 model, the authors founded that the expression of a number of genes correlated with the down-regulation of PR was very low or no detectable on day 7 of the estrous cycle [19]. Conversely, elevated P_4 concentrations induce early down-regulation of PR [57] and is associated with an advancement in the normal temporal changes of the endometrial transcriptome [20]. Moreover, the synthesis of EGF is primarily regulated by E_2 and P_4 in the endometrium [10, 14, 58]. Therefore, it is reasonable to hypothesize that the low P_4 concentration during the early luteal phase lower the EGF concentration on day 14 and on day 3 of the following estrous cycle by the temporal changes in endometrial gene expression.

In conclusion, the Chapter 2 demonstrated that the low P_4 concentration during the early luteal phase is one of the potential causes by which the EGF concentrations on day 14 of the estrous cycle and day 3 of the following estrous cycle decrease in dairy cows.

Tables and Figures

Table 2-1. Effects of concentrations of progesterone during the early luteal phase on endometrial EGF concentrations in dairy cows in Study 2-1.

Group[1] (No. of cows)	EGF profile	Day 3 of the present cycle	Day 14 of the present cycle		Day 3 of the following cycle	
		EGF concentrations (ng/g tissue weight)	No. (%) of cows showing indicated profile	EGF concentrations (ng/g tissue weight)	No. (%) of cows showing indicated profile	EGF concentrations (ng/g tissue weight)
High P_4 (10)	Normal	5.65 ± 1.04	10 (100)	6.65 ± 0.94	10 (100)	6.36 ± 0.94
	Altered	-	0 (0)	-	0 (0)	-
	Subtotal	5.65 ± 1.04	10 (100)	6.65 ± 0.94^{ax}	10 (100)	6.36 ± 0.94^{a}
Low P_4 (10)	Normal	5.82 ± 0.72	6 (60)	5.76 ± 0.95	5 (50)	5.70 ± 1.28
	Altered	-	4 (40)	4.28 ± 0.46	5 (50)	3.68 ± 0.45
	Subtotal	5.82 ± 0.72^{A}	10 (100)	5.17 ± 1.07^{b}	10 (100)	4.69 ± 1.40^{bB}
Control (10)	Normal	5.81 ± 0.98	9 (90)	5.99 ± 0.82	10 (100)	6.04 ± 1.03
	Altered	-	1 (10)	3.84	0 (0)	-
	Subtotal	5.81 ± 0.98	10 (100)	5.78 ± 1.03^{y}	10 (100)	6.04 ± 1.03^{a}
Total (30)	Normal	5.76 ± 0.90	25 (83.33)	6.20 ± 0.95	25 (83.33)	6.10 ± 1.13
	Altered	-	5 (16.67)	4.19 ± 0.45	5 (16.67)	3.68 ± 0.45
	Subtotal	5.76 ± 0.90	30 (100)	5.87 ± 1.16	30 (100)	5.70 ± 1.39

[1] High P_4 = Cows were received a CIDR device on day 5 and removed on day 9; Low P_4 = Cows were treated with three doses of $PGF_{2\alpha}$ on days 3, 3.5 and 4; Control = Cows were remained untreated (day 0 = estrus).

[ab] Values with different letters within the same EGF profiles between different groups differ significantly ($P < 0.05$).

[xy] Values with different letters within the same EGF profiles between different groups tended to be different ($P = 0.06$).

[AB] Values with different letters within the same EGF profiles within the same groups differ significantly ($P < 0.05$).

EGF concentrations are presented as means ± SDs.

EGF profile was determined by the endometrial EGF concentration on day 3. EGF concentration of between 4.7 and 13.5 ng/g tissue weight (normal range) was considered to be normal [31, 33].

Table 2-2. Effects of concentrations of progesterone during the early luteal phase on endometrial EGF concentrations in dairy cows in Study 2-2.

Group[1] (No. of cows)	EGF profile	Day 3 of the present cycle	Day 3 of the following cycle	
		EGF concentrations (ng/g tissue weight)	No. (%) of cows showing indicated profile	EGF concentrations (ng/g tissue weight)
High P_4 (10)	Normal	5.77 ± 0.73	10 (100)	5.77 ± 0.74
	Altered	-	0 (0)	-
	Subtotal	5.77 ± 0.73	10 (100)	5.77 ± 0.74[a]
Low P_4 (10)	Normal	5.76 ± 0.65	6 (60)	5.88 ± 0.91
	Altered	-	4 (40)	4.04 ± 0.59
	Subtotal	5.76 ± 0.65[A]	10 (100)	4.88 ± 0.91[bB]
Control (10)	Normal	5.77 ± 0.73	9 (90)	5.86 ± 0.68
	Altered	-	1 (10)	4.53
	Subtotal	5.77 ± 0.73	10 (100)	5.73 ± 0.77[a]
Total (30)	Normal	5.77 ± 0.68	25 (83.33)	5.72 ± 0.68
	Altered	-	5 (16.67)	4.14 ± 0.55
	Subtotal	5.77 ± 0.68	30 (100)	5.46 ± 0.88

[1] High P_4 = Cows were received a CIDR device on day 5 and removed on day 9; Low P_4 = Cows were treated with three doses of $PGF_{2\alpha}$ on days 3, 3.5 and 4; Control = Cows were remained untreated (day 0 = estrus).

[ab] Values with different letters within the same EGF profiles between different groups differ significantly ($P < 0.05$).

[AB] Values with different letters within the same EGF profiles within the same groups differ significantly ($P < 0.05$).

EGF concentrations are presented as means ± SDs.

EGF profile was determined by the endometrial EGF concentration on day 3. EGF concentration of between 4.7 and 13.5 ng/g tissue weight (normal range) was considered to be normal [31, 33].

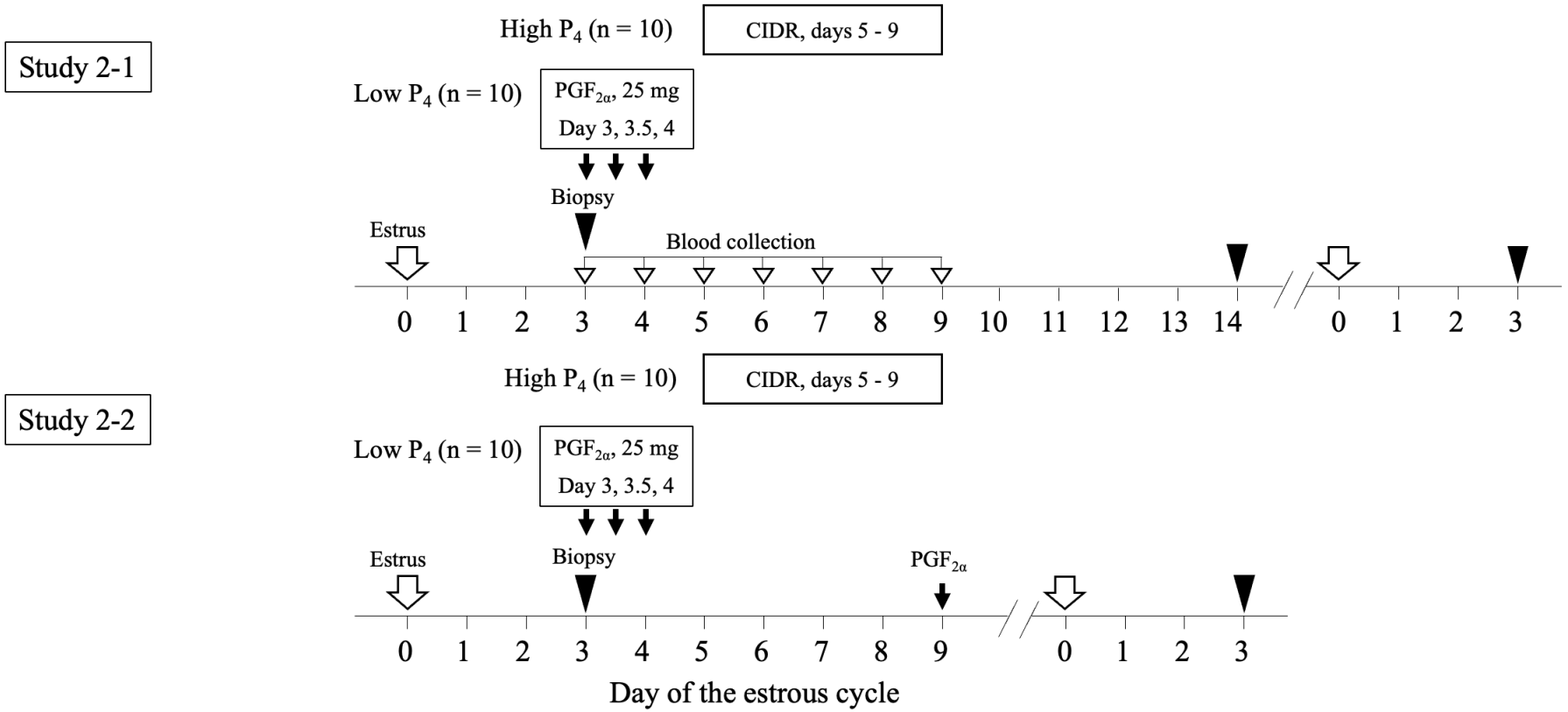

Fig. 2-1. Timeline of the treatment sequences for studies 2-1 and 2-2.

Cows were observed for estrus (day 0) and the endometrial tissue was obtained by biopsy for EGF assay on day 3 of the estrous cycle. Cows were assigned to High P_4 (n = 10), Low P_4 (n =10) and Control group (n = 10). Cows in High P_4 group were received a CIDR device on day 5 and removed on day 9; while cows in Low P_4 group were treated with three doses of $PGF_{2\alpha}$ on days 3, 3.5 and 4. Cows in Control group were remained untreated. In study 2-1, daily blood samples were collected from days 3 - 9 for P_4 assay. All cows were observed for natural estrus and the

endometrial tissue sample was obtained for the second time on day 3. In Study 2-2, cows were treated same as study 2-1 but treated with $PGF_{2\alpha}$ on day 9 to induce estrus. Cows were observed for natural estrus and the endometrial tissue sample was obtained for the second time on day 3.

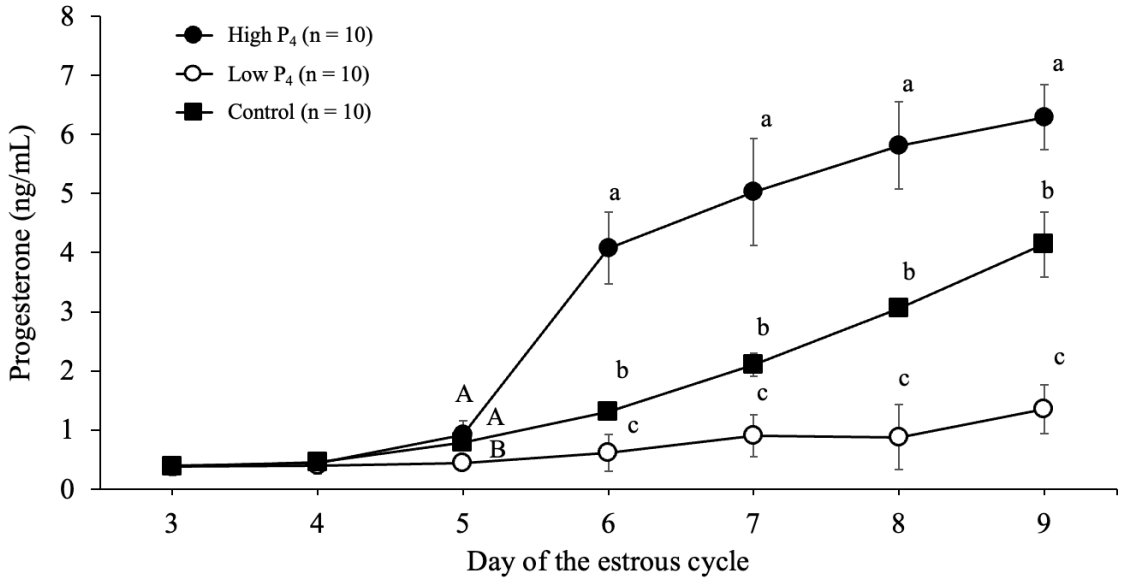

Figure 2-2. Plasma P_4 concentrations from days 3 - 9 in study 2-1.

Cows in High P_4 group (closed circle ●) were received a CIDR device on day 5 and removed on day 9; while cows in Low P_4 group (open circle ○) were treated with three doses of $PGF_{2\alpha}$ on days 3, 3.5 and 4. Cows in Control group (closed bar ■) were remained untreated. The mean concentrations of P_4 from days 3 - 9 were: High P_4 = 3.28 ± 2.62; Low P_4 = 0.71 ± 0.36; Control = 1.75 ± 1.43 ng/mL) ($P < 0.05$). Effects of treatment ($P < 0.05$); time ($P < 0.05$), interaction between treatment and time ($P < 0.05$). Errors bars represent SD.

^{a,b} indicates that within the day, P_4 concentrations differed among High P_4, Low P_4 and Control groups.

^{A,B} indicates that within the day, P_4 concentrations lesser for Low P_4 group compared to High P_4 and Control groups; but no difference between High P_4 and Control groups.

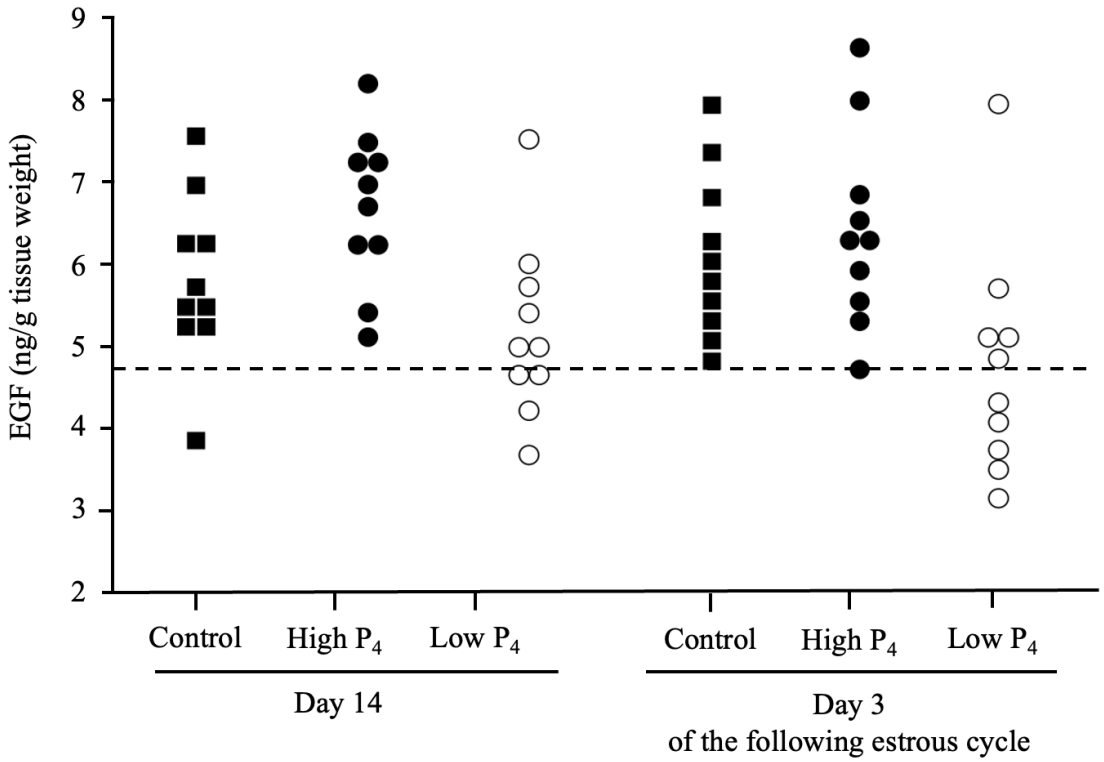

Figure 2-3. Endometrial EGF concentrations in study 2-1.

Cows in High P_4 group (closed circle ●) were received a CIDR device on day 5 and removed on day 9; while cows in Low P_4 group (open circle ○) were treated with three doses of $PGF_{2\alpha}$ on days 3, 3.5 and 4. Cows in Control group (closed bar ■) were remained untreated. Endometrial EGF concentrations on day 3 of the present estrous cycle did not differ among groups ($P > 0.05$). On day 14, endometrial EGF concentrations were greater ($P < 0.05$) for High P_4 compared with Low P_4 and tended to be higher ($P = 0.06$) for High P_4 compared with Control. Concentrations of EGF on day 3 of the following estrous cycle was affected ($P < 0.05$) by the interaction between time and treatment.

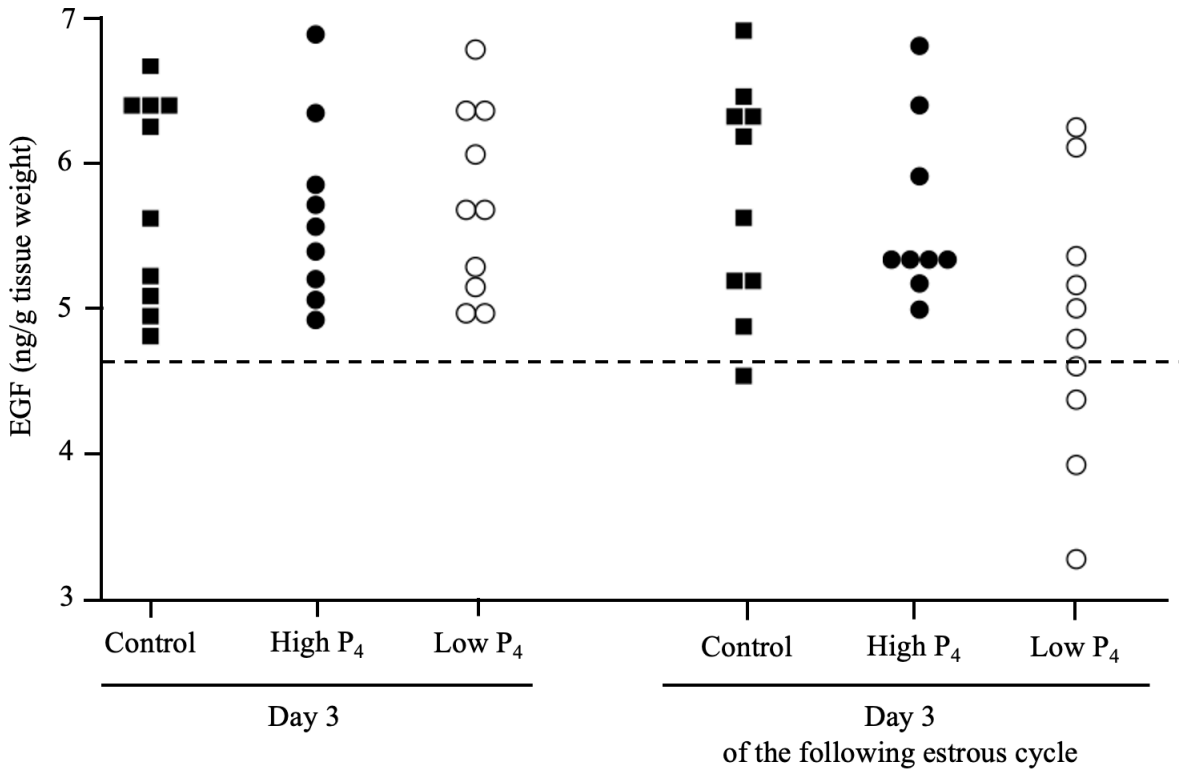

Fig. 2-4. Endometrial EGF concentrations in study 2-2.

Cows in High P_4 group (closed circle ●) were received a CIDR device on day 5 and removed on day 9; while cows in Low P_4 group (open circle ○) were treated with three doses of $PGF_{2\alpha}$ on days 3, 3.5 and 4. Cows in Control group (closed bar ■) were remained untreated. Endometrial EGF concentrations on day 3 of the present estrous cycle did not differ among groups ($P > 0.05$). On day 3 of the following estrous cycle, endometrial EGF concentrations were lesser ($P < 0.05$) for Low P_4 compared with High P_4 and Control. Treatment affected EGF concentration in Low P_4 group ($P < 0.05$). There was no interaction between time and treatments ($P > 0.05$).

Summary

Expression of endometrial EGF is primarily regulated by E_2 and P_4. Low P_4 concentrations in the early luteal phase has been demonstrated as a causative factor in the low pregnancy rates observed in dairy cows. Lower circulating concentrations of P_4 in this period altered the normal temporal changes in uterine gene expression, and these changes were associated with a reduced capacity of the uterus to support conceptus development. However, the action of low P_4 concentrations on the endometrial EGF profile are not known. Therefore, the studies in this chapter aimed to evaluate the effects of different P_4 concentrations during the early luteal phase on EGF concentrations.

In study 2-1, lactating Holstein cows were assigned randomly to one of three treatments: High P_4 (n = 10) received an intravaginal P_4 device from days 5 - 9 (day 0 = estrus); Low P_4 (n = 10) received $PGF_{2\alpha}$ injections on days 3, 3.5 and 4; Control (n = 10). They were allowed to show natural estrus. Endometrial biopsy for EGF assays was conducted on day 3 and day 14 of the estrous cycle, and day 3 of the following estrous cycle. On day 14, the number of cows showed normal endometrial EGF concentrations was 6 out of 10 cows in Low P_4 group, compared to 9 and 10 out of 10 cows in Control group and High P_4 group, respectively. Endometrial EGF concentrations in Low P_4 group were lower than that in High P_4 and Control groups ($P < 0.05$).

In study 2-2, cows were treated same as in study 2-1; however, all cows received $PGF_{2\alpha}$ treatment on day 9 of the estrous cycle to induce estrus. Cows were observed for estrus, and an endometrial tissue sample was obtained on day 3 of the initial and the following estrous cycle (day 0 = estrus). Epidermal growth factor concentrations were decreased after treatment only in Low P_4 group ($P < 0.05$). Four cows showed abnormal EGF concentrations in Low P_4 group; whereas one and none of cow showed abnormal EGF concentrations in Control group and High P_4 group, respectively. Reducing P_4 concentrations during the early luteal phase altered endometrial EGF concentrations.

Summary and Conclusions

Fertility reduced significantly in the modern high-yielding cows compared with traditional moderate-producing cows. Substantial evidence indicates that the difference in calving rate between the two groups of cows may reflect an increased frequency of early embryonic loss occurring before day 16 of pregnancy. A considerable proportion of early embryo loss could be attributed to changes in ovarian steroid hormones during the two periods: (1) between luteolysis and estrus (or ovulation) and (2) early luteal phase. Epidermal growth factor (EGF) profile in the uterine endometrium has been identified as an indicator of endometrial function and fertility. The loss or decrease of these peaks has been linked to reduced fertility in repeat breeder (RB) cows and high-producing cows. Since the expression of EGF in the endometrium is primarily regulated by estradiol (E_2) and progesterone (P_4), changes in circulating E_2 and P_4 concentrations may be amplified in the endometrium as an altered EGF profile. In Chapter 1 of the present study, I examined the effect of the timing of luteolysis induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administration in relation to the stage of follicular development on the profile of E_2 and P_4 between luteolysis and ovulation and EGF concentrations on day 3 of the following estrous cycle. In Chapter 2, I manipulated the P_4 concentrations during the early luteal phase and examined the effects of these alterations on the EGF peaks and fertility.

In Chapter 1, I examined the effects of $PGF_{2\alpha}$ administration at different timings of the estrous cycle on the profile of steroid hormones during the preovulatory period and the peak concentration of endometrial EGF on day 3 (day 0 = estrus) in the following estrous cycle. This chapter consists of two studies. All lactating Holstein cows were examined for health conditions, the regularity of estrous cycle, and the uterine cytology for subclinical inflammation and oviductal patency. Cows were also examined for the EGF concentration on day 3. Cows without any abnormality in these examinations were used. In the first study, cows were treated with $PGF_{2\alpha}$ either on the first day that follicles reached 4 mm (days 12 - 14, selection phase group, n = 20) or on days 16 or 17 in the presence of a dominant follicle and a corpus luteum (CL) (control group, n = 24). Endometrial tissues were collected to determine EGF profile, which was considered normal when the EGF concentration was ≥ 4.70 ng/g tissue weight. Endometrial EGF concentration of cows with both the normal and altered profiles was lower in the selection phase group than that in the control group ($P < 0.05$). Then, in the second study, lactating Holstein cows were treated with $PGF_{2\alpha}$ either on day 12 (selection phase group, n = 76) or on day 16 (control group, n = 80) to examine the effect of treatment on fertility. All cows were subjected to artificial insemination (AI) at estrus. The endometrial EGF profile was

determined on day 3. The cows of the selection phase group showed lower EGF concentration, proportion of cows with the normal EGF profile, and conception rate than those of the control group ($P < 0.05$).

The mechanism by which the prolonged preovulatory period lowers the peak endometrial EGF concentration on day 3 may be explained, at least in part, by the decrease in both P_4 sensitization and E_2 stimulus. This is consistent with the results of E_2 concentrations in this chapter. Estradiol concentrations were lower in the selection phase group than the control group on the day of estrus, while there were no differences on the other days. The duration of low P_4 concentrations between luteolysis and estrus (ovulation) in Selection phase group was longer than those in Control group. However, there was no difference in P_4 concentrations at $PGF_{2\alpha}$ treatment between the two groups, so the P_4 concentration in the late luteal phase may not have a direct and/or significant impact on the EGF profile in the following estrous cycle. This chapter demonstrated that the prolonged duration between CL regression and estrus (or ovulation) leading to changes in E_2 concentrations at estrus and the duration of low P_4 concentrations is one of the potential causes by which the EGF concentrations in the estrous cycle decreased and reduced fertility in the dairy cows.

In Chapter 2, I examined the effects of different P_4 levels during the early luteal phase on endometrial EGF concentrations. In the first study, 30 lactating Holstein cows were assigned randomly to one of the three groups: High P_4 group (n = 10) that received an intravaginal P_4 device from days 5 to 9 (day 0 = estrus); Low P_4 group (n = 10) that received $PGF_{2\alpha}$ treatments on days 3, 3.5 and 4; Control group (n = 10) received no further treatment. Endometrial tissue biopsy was conducted on day 3 and day 14 of the estrous cycle, and day 3 of the estrous cycle naturally occurring after treatment to determine the EGF concentrations. There was no difference in endometrial EGF concentrations at the first examination on day 3 ($P > 0.05$), thus all cows were considered to have normal EGF profile. On day 14, the proportion of cows that showed endometrial EGF concentrations lower than the lower limit of the normal range on day 14 was greater for Low P_4 compared with Control (40% vs 10%; Low P_4 vs. Control, 10 cows each]. While none of cows in High P_4 group showed alteration of endometrial EGF concentration on day 14. On day 3 of the following estrous cycle, EGF concentrations in Low P_4 group were lower than those in High P_4 and Control groups ($P < 0.05$). In the second study, 30 cows were treated as in the first study, but they were induced estrus with $PGF_{2\alpha}$ treatment on day 9. Cows were observed for estrus and the endometrial EGF profile was examined for the second time on day 3 of the following estrous cycle. Four cows showed abnormal EGF

concentrations in Low P_4 group; whereas one and none out of 10 cows showed abnormal EGF concentrations in High P_4 and Control groups, respectively. As a result, the EGF concentrations were lower for Low P_4 group than those of the High P_4 and Control groups ($P < 0.05$). The results suggested that low P_4 concentrations during the early luteal phase impair the endometrial EGF profile of the later stage of the same estrous cycle (i.e., day 14) and that in the following estrous cycle (i.e., day 3) in dairy cattle.

The present study indicated that the alterations of E_2 and/or P_4 during different timing of estrous cycle in relation to the follicular developmental stages is one of the potential causes by which EGF concentrations in the following estrous cycle decrease and reduce fertility in the dairy cow. Although further study is necessary in the future, the findings of the present study enhance our understanding of the etiology of this problem, providing more information to select potential markers that are easy and sensitive enough to detect an alteration in the EGF profile or a physiological change associated with the alteration of the EGF profile under field conditions.

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References

1. Adachi K, Kurachi H, Homma H, Adachi H, Imai T, Sakata M, Higashiguchi O, Yamaguchi M, Morishige K, Sakoyama Y, et al. Estrogen induces epidermal growth factor (EGF) receptor and its ligands in human fallopian tube: involvement of EGF but not transforming growth factor-alpha in estrogen-induced tubal cell growth in vitro. Endocrinology 136, 2110-2119, 1995.
2. Asselin E, Bazer FW, Fortier MA. Recombinant ovine and bovine interferons tau regulate prostaglandin production and oxytocin response in cultured bovine endometrial cells. Biol Reprod 56, 402-408, 1997.
3. Badrakh D, Yanagawa Y, Nagano M, Katagiri S. Effect of seminal plasma infusion into the vagina on the normalization of endometrial epidermal growth factor concentrations and fertility in repeat breeder dairy cows. J Reprod Dev 66, 149-154, 2020.
4. Bage R, Gustafsson H, Larsson B, Forsberg M, Rodriguez-Martinez H. Repeat breeding in dairy heifers: follicular dynamics and estrous cycle characteristics in relation to sexual hormone patterns. Theriogenology 57, 2257-2269, 2002.
5. Bany BM, Kennedy TG. Regulation by epidermal growth factor of prostaglandin production and cyclooxygenase activity in sensitized rat endometrial stromal cells in vitro. J Reprod Fertil 104, 57-62, 1995.
6. Battaglia DF, Krasa HB, Padmanabhan V, Viguié C, Karsch FJ. Endocrine alterations that underlie endotoxin-induced disruption of the follicular phase in ewes. Biol Reprod 62, 45-53, 2000.
7. Bazer FW, Spencer TE, Johnson GA. Interferons and uterine receptivity. Semin Reprod Med 27, 90-102, 2009.
8. Beltman ME, Roche JF, Lonergan P, Forde N, Crowe MA. Evaluation of models to induce low progesterone during the early luteal phase in cattle. Theriogenology 72, 986-992, 2009.
9. Bridges GA, Mussard ML, Pate JL, Ott TL, Hansen TR, Day ML. Impact of preovulatory estradiol concentrations on conceptus development and uterine gene expression. Anim Reprod Sci 133, 16-26, 2012.
10. Brigstock DR. Growth factors in the uterus: steroidal regulation and biological actions. Baillieres Clin Endocrinol Metab 5, 791-808, 1991.

11. Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans AC, Kenny DA, Roche JF, Lonergan P. Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. Reprod Fertil Dev 20, 368-375, 2008.

12. Dadarwal D, Mapletoft RJ, Adams GP, Pfeifer LF, Creelman C, Singh J. Effect of progesterone concentration and duration of proestrus on fertility in beef cattle after fixed-time artificial insemination. Theriogenology 79, 859-866, 2013.

13. De Vries A. Economic value of pregnancy in dairy cattle. J Dairy Sci 89, 3876-3885, 2006.

14. DiAugustine RP, Petrusz P, Bell GI, Brown CF, Korach KS, McLachlan JA, Teng CT. Influence of estrogens on mouse uterine epidermal growth factor precursor protein and messenger ribonucleic acid. Endocrinology 122, 2355-2363, 1988.

15. Dosogne H, Meyer E, Sturk A, van Loon J, Massart-Leën AM, Burvenich C. Effect of enrofloxacin treatment on plasma endotoxin during bovine Escherichia coli mastitis. Inflamm Res 51, 201-205, 2002.

16. Erb RE, Garverick HA, Randel RD, Brown BL, Callahan CJ. Profiles of reproductive hormones associated with fertile and nonfertile inseminations of dairy cows. Theriogenology 5, 227-242, 1976.

17. Fair T, Lonergan P. The role of progesterone in oocyte acquisition of developmental competence. Reprod Domest Anim 47 Suppl 4, 142-147, 2012.

18. Falck L, Forsberg JG. Immunohistochemical studies on the expression and estrogen dependency of EGF and its receptor and C-fos proto-oncogene in the uterus and vagina of normal and neonatally estrogen-treated mice. Anat Rec 245, 459-471, 1996.

19. Forde N, Beltman ME, Duffy GB, Duffy P, Mehta JP, O'Gaora P, Roche JF, Lonergan P, Crowe MA. Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation. Biol Reprod 84, 266-278, 2011.

20. Forde N, Carter F, Fair T, Crowe MA, Evans AC, Spencer TE, Bazer FW, McBride R, Boland MP, O'Gaora P, Lonergan P, Roche JF. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. Biol Reprod 81, 784-794, 2009.

21. Forde N, Mehta JP, Minten M, Crowe MA, Roche JF, Spencer TE, Lonergan P. Effects of low progesterone on the endometrial transcriptome in cattle. Biol Reprod 87, 124, 2012.

22. Geisert RD, Fox TC, Morgan GL, Wells ME, Wettemann RP, Zavy MT. Survival of bovine embryos transferred to progesterone-treated asynchronous recipients. Reproduction 92, 475-482, 1991.

23. Gharib-Hamrouche N, Chêne N, Guillomot M, Martal J. Localization and characterization of EGF/TGF-alpha receptors on peri-implantation trophoblast in sheep. J Reprod Fertil 98, 385-392, 1993.

24. Gharib-Hamrouche N, Chêne N, Martal J. Comparative expression of TGF-alpha and EGF genes in the ovine conceptus and uterine endometrium in the peri-implantation period. Reprod Nutr Dev 35, 291-303, 1995.

25. Hansel W. Plasma hormone concentrations associated with early embryo mortality in heifers. J Reprod Fertil Suppl 30, 231-239, 1981.

26. Huet-Hudson YM, Chakraborty C, De SK, Suzuki Y, Andrews GK, Dey SK. Estrogen regulates the synthesis of epidermal growth factor in mouse uterine epithelial cells. Mol Endocrinol 4, 510-523, 1990.

27. Johnson DC, Chatterjee S. Embryo implantation in the rat uterus induced by epidermal growth factor. J Reprod Fertil 99, 557-579, 1993.

28. Katagiri S, Moon YS, Yuen BH. The role for the uterine insulin-like growth factor I in early embryonic loss after superovulation in the rat. Presented in part at the 50th Annual Meeting of The American Fertility Society, San Antonio, Texas, November 5 to 10, 1994. Fertility and Sterility 65, 426-436, 1996.

29. Katagiri S, Moriyoshi M. Alteration of the endometrial EGF profile as a potential mechanism connecting the alterations in the ovarian steroid hormone profile to embryonic loss in repeat breeders and high-producing cows. J Reprod Dev 59, 415-420, 2013.

30. Katagiri S, Moriyoshi M, Takahashi Y. Low incidence of an altered endometrial epidermal growth factor (EGF) profile in repeat breeder Holstein heifers and differential effect of parity on the EGF profile between fertile Holstein (dairy) and Japanese black (beef) cattle. J Reprod Dev 59, 575-579, 2013.

31. Katagiri S, Moriyoshi M, Yanagawa Y. Endometrial epidermal growth factor profile and its abnormalities in dairy cows. J Reprod Dev 62, 465-470, 2016.

32. Katagiri S, Takahashi Y. Changes in EGF concentrations during estrous cycle in bovine endometrium and their alterations in repeat breeder cows. Theriogenology 62, 103-112, 2004.

33. Katagiri S, Takahashi Y. Potential relationship between normalization of endometrial epidermal growth factor profile and restoration of fertility in repeat breeder cows. Anim Reprod Sci 95, 54-66, 2006.

34. Katagiri S, Takahashi Y. A progestin-based treatment with a high dose of estradiol benzoate normalizes cyclic changes in endometrial EGF concentrations and restores fertility in repeat breeder cows. J Reprod Dev 54, 473-479, 2008.

35. Kawano K, Yanagawa Y, Nagano M, Katagiri S. Effects of heat stress on the endometrial epidermal growth factor profile and fertility in dairy cows. J Reprod Dev 68, 144-151, 2022.

36. Kennedy TG, Brown KD, Vaughan TJ. Expression of the genes for the epidermal growth factor receptor and its ligands in porcine oviduct and endometrium. Biol Reprod 50, 751-756, 1994.

37. Kimura M, Nakao T, Moriyoshi M, Kawata K. Luteal phase deficiency as a possible cause of repeat breeding in dairy cows. Br Vet J 143, 560-566, 1987.

38. Kliem A, Tetens F, Klonisch T, Grealy M, Fischer B. Epidermal growth factor receptor and ligands in elongating bovine blastocysts. Mol Reprod Dev 51, 402-412, 1998.

39. Kyaw HM, Sato H, Tagami T, Yanagawa Y, Nagano M, Katagiri S. Effects of milk osteopontin on the endometrial epidermal growth factor profile and restoration of fertility in repeat breeder dairy cows. Theriogenology 184, 26-33, 2022.

40. Lamming GE, Darwash AO. The use of milk progesterone profiles to characterise components of subfertility in milked dairy cows. Anim Reprod Sci 52, 175-190, 1998.

41. Lamming GE, Darwash AO, Back HL. Corpus luteum function in dairy cows and embryo mortality. J Reprod Fertil Suppl 37, 245-252, 1989.

42. Larson SF, Butler WR, Currie WB. Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cows. J Dairy Sci 80, 1288-1295, 1997.

43. Levine HD. The repeat breeder cow. Bov Pract 33, 97-105, 1999.

44. Lopez H, Satter LD, Wiltbank MC. Relationship between level of milk production and estrous behavior of lactating dairy cows. Anim Reprod Sci 81, 209-223, 2004.

45. Lucy MC. Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci 84, 1277-1293, 2001.

46. Magata F. Lipopolysaccharide-induced mechanisms of ovarian dysfunction in cows with uterine inflammatory diseases. J Reprod Dev 66, 311-317, 2020.

47. Magata F, Horiuchi M, Echizenya R, Miura R, Chiba S, Matsui M, Miyamoto A, Kobayashi Y, Shimizu T. Lipopolysaccharide in ovarian follicular fluid influences the steroid production in large follicles of dairy cows. Anim Reprod Sci 144, 6-13, 2014.

48. Mann G, Lamming G. The influence of progesterone during early pregnancy in cattle. Reprod Domes Anim 34, 269-274, 1999.

49. Mann GE, Fray MD, Lamming GE. Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. Vet J 171, 500-503, 2006.

50. Mann GE, Lamming GE. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction 121, 175-180, 2001.

51. Mann GE, Lamming GE. The role of sub-optimal preovulatory oestradiol secretion in the aetiology of premature luteolysis during the short oestrous cycle in the cow. Anim Reprod Sci 64, 171-180, 2000.

52. McNeill RE, Diskin MG, Sreenan JM, Morris DG. Associations between milk progesterone concentration on different days and with embryo survival during the early luteal phase in dairy cows. Theriogenology 65, 1435-1441, 2006.

53. Mesquita FS, Ramos RS, Pugliesi G, Andrade SCS, Van Hoeck V, Langbeen A, Oliveira ML, Gonella-Diaza AM, Gasparin G, Fukumasu H, Pulz LH, Membrive CM, Coutinho LL, Binelli M. The receptive endometrial transcriptomic signature indicates an earlier shift from proliferation to metabolism at early diestrus in the cow. Biol Reprod 93, 1-12, 2015.

54. Nelson KG, Takahashi T, Bossert NL, Walmer DK, McLachlan JA. Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth and differentiation. Proc Natl Acad Sci USA 88, 21-25, 1991.

55. Ninpetch N, Badrakh D, Hay Mar K, Kawano K, Yanagawa Y, Nagano M, Katagiri S. Leptin receptor expression and its change in association with the normalization of EGF profile after seminal plasma treatment in repeat breeder dairy cows. J Reprod Dev 68, 209-215, 2022.

56. Ohtani S, Okuda K, Ohtani M, Yamada J. Immunohistochemically-determined changes in the distribution of insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF) in the bovine endometrium during the estrous cycle. J Vet Med Sci 58, 1211-1217, 1996.

57. Okumu LA, Forde N, Fahey AG, Fitzpatrick E, Roche JF, Crowe MA, Lonergan P. The effect of elevated progesterone and pregnancy status on mRNA expression and localisation of progesterone and oestrogen receptors in the bovine uterus. Reproduction 140, 143-153, 2010.

58. Paria BC, Song H, Dey SK. Implantation: molecular basis of embryo-uterine dialogue. Int J Dev Biol 45, 597-605, 2001.

59. Peter AT, Bosu WT, DeDecker RJ. Suppression of preovulatory luteinizing hormone surges in heifers after intrauterine infusions of Escherichia coli endotoxin. Am J Vet Res 50, 368-373, 1989.

60. Plaizier JC, Khafipour E, Li S, Gozho GN, Krause DO. Subacute ruminal acidosis (SARA), endotoxins and health consequences. Anim Feed Sci Technol 172, 9-21, 2012.

61. Pursley JR, Kosorok MR, Wiltbank MC. Reproductive management of lactating dairy cows using synchronization of ovulation. J Dairy Sci 80, 301-306, 1997.

62. Royal MD, Darwash AO, Flint APF, Webb R, Woolliams JA, Lamming GE. Declining fertility in dairy cattle: changes in traditional and endocrine parameters of fertility. Anim Sci 70, 487-501, 2000.

63. Ryan DP, Boland MP. Frequency of twin births among Holstein-Friesian cows in a warm dry climate. Theriogenology 36, 1-10, 1991.

64. Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17beta in dairy cattle. J Dairy Sci 85, 2831-2842, 2002.

65. Santos JE, Thatcher WW, Chebel RC, Cerri RL, Galvão KN. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Anim Reprod Sci 82-83, 513-535, 2004.

66. Sartori R, Haughian JM, Shaver RD, Rosa GJ, Wiltbank MC. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. J Dairy Sci 87, 905-920, 2004.

67. Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth HJ. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. Biol Reprod 81, 1025-1032, 2009.

68. Shelton K, Gayerie De Abreu MF, Hunter MG, Parkinson TJ, Lamming GE. Luteal inadequacy during the early luteal phase of subfertile cows. J Reprod Fertil 90, 1-10, 1990.

69. Shelton K, Parkinson TJ, Hunter MG, Kelly RW, Lamming GE. Prostaglandin E-2 as a potential luteotrophic agent during early pregnancy in cattle. J Reprod Fertil 90, 11-17, 1990.

70. Sibilia M, Wagner EF. Strain-dependent epithelial defects in mice lacking the EGF receptor. Science 269, 234-238, 1995.

71. Spencer TE, Sandra O, Wolf E. Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. Reproduction 135, 165-179, 2008.

72. Starbuck GR, Darwash AO, Mann GE, Lamming GE. The detection and treatment of post insemination progesterone insufficiency in dairy cows. BSAP Occasional Publication 26, 447-450, 2001.

73. Stronge AJ, Sreenan JM, Diskin MG, Mee JF, Kenny DA, Morris DG. Post-insemination milk progesterone concentration and embryo survival in dairy cows. Theriogenology 64, 1212-1224, 2005.

74. Tamada H, Yoh C, Inaba T, Takano H, Kawate N, Sawada T. Epidermal growth factor (EGF) in the goat uterus: immunohistochemical localization of EGF and EGF receptor and effect of EGF on uterine activity in vivo. Theriogenology 54, 159-169, 2000.

75. Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC, et al. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science 269, 230-234, 1995.

76. Vasconcelos JL, Sangsritavong S, Tsai SJ, Wiltbank MC. Acute reduction in serum progesterone concentrations after feed intake in dairy cows. Theriogenology 60, 795-807, 2003.

77. Walsh SW, Williams EJ, Evans ACO. A review of the causes of poor fertility in high milk producing dairy cows. Anim Reprod Sci 123, 127-138, 2011.

78. Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gümen A. Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. Theriogenology 65, 17-29, 2006.

79. Yanagawa Y, Matsuura Y, Suzuki M, Saga S, Okuyama H, Fukui D, Bando G, Nagano M, Katagiri S, Takahashi Y, Tsubota T. Accessory corpora lutea formation in pregnant Hokkaido sika deer (*Cervus nippon yesoensis*) investigated by examination of ovarian dynamics and steroid hormone concentrations. J Reprod Dev 61, 61-66, 2015.

80. Zhang Z, Krause M, Davis DL. Epidermal growth factor receptors in porcine endometrium: binding characteristics and the regulation of prostaglandin E and F2 alpha production. Biol Reprod 46, 932-936, 1992.

Summary in Japanese

牛では子宮内膜中の上皮成長因子 (EGF) 濃度およびその変化は、子宮機能あるいは受胎性の指標とされ、低受胎牛の診断に活用されている。 正常な受胎性を示す乳牛の子宮内膜 EGF 濃度は、 発情周期中の 2〜4 日目および 13〜14 日目に一過性の濃度上昇を示し、そのピーク濃度の低下または濃度上昇の消失は、リピートブリーダー（RB）および高泌乳牛の受胎性低下の原因になることが知られている。子宮内膜における EGF の発現は、主にエストラジオール (E_2) とプロジェステロン (P_4) によって調節されるため、これらの低受胎牛に共通して報告されている血中 E_2 および P_4 濃度の変化は 、子宮内膜において EGF 濃度変化の異常として増幅されると考えられる。とくに、(1) 黄体退行と発情（または排卵）の間、(2) 黄体期初期の 2 つの期間は、血中卵巣ステロイドホルモン濃度の変化と受胎性の低下が関連付けられていることから、これらの卵巣ホルモンの異常が子宮での EGF 濃度異常を介して受胎性を低下させるという仮説は、低受胎牛の病態を理解する上で検討に値する。そこで本研究では、黄体退行から発情（または排卵）までの期間（第 1 章）および黄体期初期（第 2 章）における E_2 および P_4 濃度の変化が、その後の発情周期における周期的な EGF 濃度変化の喪失またはピーク濃度の低下を引き起こし、受胎性を低下させるとの仮説を検討した。

第 1 章では、発情周期中の異なるタイミングでプロスタグランジン（PG）$F_{2\alpha}$ を投与して黄体退行を誘起し、黄体退行から排卵までの 血中 E_2 および P_4 濃度変化と次の発情周期における 3 日目の EGF 濃度に及ぼす効果を検討した。まず、卵胞ウェーブの選抜期（卵胞群の直径が初めて 4 mm を超えた日）にあたる発情後 12〜14 日目（選抜期群、n=20）または黄体退行期にあたる 16〜17 日目（対照群、n=24）の乳牛に $PGF_{2\alpha}$ を投与して発情を誘起し、処置後の血中 E_2 および P_4 濃度を調べるとともに、発情後 3 日目に子宮内膜組織を生検により採取して EGF 濃度を測定した。その結果、両群の $PGF_{2\alpha}$ 投与時の P_4 濃度、およびその後排卵までの P_4 濃度変化には両群間に差異は認められなかった。しかし、黄体退行から発情と排卵までの期間が延長したことにより、P_4 濃度の低値が持続した期間が延長した。また、発情時の E_2 濃度は選抜期群で低下した（$P < 0.05$）。子宮 EGF 濃度が正常範囲の下限以下の低値を示した牛の割合は、対照群に比べ選抜期群の方が高かった（79.6% vs. 53.6%, P

< 0.05)。また、選抜期群の牛は対照群の牛よりも受胎率が低かった（36.8% vs. 55.0%, P < 0.05)。以上の結果より、選抜期群にみられた黄体退行から発情あるいは排卵までの期間の延長に伴う E_2 および P_4 濃度推移の変化が次の発情周期における子宮内膜 EGF 濃度の低下につながったと考えられた。

第 2 章では、黄体期前期の P_4 濃度を調節して、この時期の P_4 濃度の違いが黄体後期（発情後 13〜14 日目）および次の発情後 3 日目の子宮内膜 EGF 濃度に及ぼす効果を調べた。 まず、発情後 5〜9 日目まで膣内に P_4 徐放剤を留置することでこの時期の P_4 濃度を増加させる処置（高 P_4 群、n=10)、 発情後 3 日目、3.5 日目および 4 日目に $PGF_{2\alpha}$ を投与して黄体退行を抑制する処置（低 P_4 群、n=10)、あるいはいずれの処置も行わない群（対照群、n=10）を設定し、次の発情までの期間は無処置とした。すべての牛で処置した周期の発情後 14 日目および次の発情周期の 3 日目に子宮内膜の EGF 濃度を調べた。その結果、14 日目の子宮内膜 EGF 濃度は、低 P_4 群では高 P_4 群に比べ低値を示した（P < 0.05)。同様に、低 P_4 群の EGF 濃度は次の発情周期 3 日目においても高 P_4 群および対照群の EGF 濃度よりも低かった（P < 0.05)。次いで、同様に処置した 3 群に対して発情後 9 日目に $PGF_{2\alpha}$ を投与して発情を誘起し、発情後 3 日目の EGF 濃度を調べたところ、自然発情の場合と同様に低 P_4 群で EGF 濃度が低値を示した。これらの結果は、黄体期初期の P_4 濃度が次の発情周期の EGF 濃度変化に影響を及ぼすことを示している。また、自然発情および発情後 9 日目に発情を誘起した誘起発情の両方で同様の結果が得られたことから、異なる P_4 濃度に対する黄体期前期の子宮内膜の応答の違いがその原因となることが示唆された。

本研究により、低受胎牛において報告されている黄体退行と発情または排卵の間、および黄体期前期の 2 つの時期における血中卵巣ステロイドホルモン濃度の変化が子宮での EGF 濃度異常を介して受胎性を低下させることが明らかとなった。