Role of metabolic and endocrine factors in an alteration of the endometrial epidermal growth factor concentration in repeat breeder dairy cows

（乳用リピートブリーダー牛の子宮内膜上皮成長因子濃度異常における代謝および内分泌因子の役割）

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Abbreviations

AI: Artificial insemination

BCS: Body condition score

cc: Cubic centimeter

CV: Coefficient of variation

dpp: Days postpartum

DMI: Dry matter intake

E_2: Estradiol-17β

EGF: Epidermal growth factor

EIA: Enzyme Immunoassay

FCM: Fat corrected milk

g: Gram

GnRH: Gonadotropin-releasing hormone

h: Hour

Kg: Kilogram

LH: Luteinizing hormone

min: Minute

mg: Milligram

ml: Milliliter

mRNA: Messenger ribonucleic acid

NEB: Negative energy balance

ng: Nanogram

ob gene: Obese gene

Ob-R: Leptin receptor

PG: Prostaglandin

pg: Picogram

P_4: Progesterone

s: Second

SD: Standard deviation

SNPs: Single nucleotide polymorphism

SP: Seminal plasma

VWP: Voluntary waiting period

μg: Microgram

Contents

Note

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Preface

The performance of dairy herds depends on genetic selection for milk production and herd management. Milk production of dairy cattle has been increasing from 5,000 kg per year to 9,000 kg per year, from the mid-nineteenth century until the present day [1]. The percentage of animals that display standing estrus declined 80% to 50% and the first service pregnancy rate from 70% to 40%, suggesting that fertility has declined over the last 50 years while milk production is increasing [2,3]. In contrast, dairy cows have become delicate and fragile animals that require high levels of nutritional and cow comfort management to realize their genetic potential [4-6]. These changes inevitably result in the reduction of the fertility rate in modern dairy herds with the management of an average level.

Repeat breeder cows are defined as the cows which failed to conceive after several (usually three or more) inseminations without anatomical and infectious abnormalities, and with an apparently normal estrous cycle [7-11]. The incidence of repeat breeding in dairy cattle has been reported between 10% and 24% [10,12]. Repeat breeding syndrome has been a major cause of an economic loss of the dairy [13,14] and beef industries [15]. The causes of infertility in repeat breeding syndrome are unclear but include environmental, management, and animal factors [13]. Fertilization failure and embryonic death are thought to be major causes of repeat breeding, but mechanisms are not clearly understood [15]. The incidence of early embryonic loss increased during the last 40 years while fertilization rates were kept at a high level (about 90%) [16]. The incidence of high-quality embryos at 6-7 days after artificial insemination (AI) decreases in lactating cows (between 33% to 53%) compared with heifers (72%) and dry cows (83%) [17]. Together the retarded embryonic development might be common in high-yielding cows and become a major cause of infertility in repeat breeder cows [18]. Interestingly, a reciprocal embryo transfer study that exchanged embryos between fertile and repeat breeder cows showed that uterine environment, but not the quality of embryos, is the factor increasing embryonic loss in repeat breeder cows [19].

An increased level of milk production has a negative impact on the uterine environment and function due to decreased progesterone (P_4) and estradiol-17β (E_2) concentrations in the circulation [20]. The high-yielding cows need to intake a large amount of food to meet the high energy requirement, and this leads to increase liver blood flow [21] that, in turn, to increase the clearance of both P_4 and E_2 from the circulation [6]. Repeat breeder cows may not necessarily be high producers but exhibit similar alterations in the profiles of ovarian steroid hormones to those found in high producing cows [22].

The alterations of ovarian steroid hormones in high-yielding and repeat breeder cows could be amplified and become detectable in the endometrium as an alteration of growth factor and cytokine expression since ovarian steroid hormones regulate the expression of these local factors [22,23]. Epidermal growth factor (EGF) is one of the most important regulatory components of uterine function and embryonic development [23,24]. Estrogen stimulates EGF production in the uterus [25-27]. EGF replaces estrogen in the uterine, vaginal growth and lactoferrin (estrogen-inducible secretory protein) [28], and a nidatory estrogen surge that initiates blastocyst attachment to the endometrium in rodents [29,30]. Drastic negative effects on the number of inner cell mass [31], placenta formation, and viability of offspring [31,32] have been reported in mice.

The presence of EGF and its receptor in the endometrium have been reported in many farm animals, including cattle [13,33,34], sheep [35,36], goats [37], and pigs [38]. Moreover, EGF increases the production of prostaglandin (PG) E_2 in the endometrium and the PGE_2:$PGF_{2\alpha}$ ratio in pigs [39] and rats [40]. These effects of EGF on PG synthesis enhance corpus luteum function and support the survival of embryo in cattle [41,42]. Therefore, an alteration of EGF action in the endometrium may cause uterine dysfunction and increase early embryonic loss in cattle.

The endometrial EGF exhibits the peak twice on day 2 to 4 and day 13 to 14 with lesser EGF concentrations around day 7 of the estrous cycle in fertile cows [13,43,44]. Loss of the two peaks in the EGF profiles has been linked to reduced fertility in repeat breeder and high-yielding cows [13,15,43,44]. This alteration of EGF concentrations was found in about 70% of lactating dairy repeat breeder cows and 40% of high-yielding cows at 60 days postpartum (dpp) [22,43]. However, the etiology of the abnormality of the EGF profile in the endometrium is not well understood. It is of interest to examine the process of recovery of EGF cyclicity in the endometrium during the postpartum period.

In chapter 1, I aimed to examine the recovery of endometrial EGF cycle in lactating cows, in which the regular estrous cycle resumed. Firstly, EGF profiles of normal cyclic dairy cows were examined in every estrous cycle from calving to 90 dpp to determine the changes of EGF cycle. Then, parity, body condition score (BCS) at calving, day of the first ovulation, peak milk yield, and time of the peak were considered as factors that may affect the recovery of endometrial EGF cyclicity. The study confirmed a potential association of high levels of milk production with delay of the recovery in the endometrial EGF cyclic change in lactating dairy cows.

Results of the chapter 1 prompted me to examine the role of the leptin system in the etiology of abnormality in the EGF profile in dairy cows. Leptin is a peptide hormone, a product of the obese (*ob*) gene [45,46]. Leptin expression is influenced by energy storage status and

correlated to the degree of body fat mass in humans, rodents, and cattle [47-49]. More importantly, leptin plays an important role in the regulation of feed intake, energy expenditure, and endocrine function [50]. The leptin synthesis can be increased by glucocorticoids [51], lipid, and estrogen [52]. In contrast, testosterone [53], thyroid hormone [54], and β-adrenergic agonists [55] inhibit leptin synthesis. Exogenous leptin injection is found to restore fertility in infertile *ob/ob* mice [56]. Changes in leptin levels and mRNA expression in the adipose tissue were also associated with onset of puberty in cattle [57]. Leptin has been shown to control reproductive function via regulation of gonadotropin-releasing hormone (GnRH) secreting neurons [58], a key player in the hypothalamus-pituitary-gonadal axis. Leptin levels correlated with LH pulse frequency and amplitude in primiparous cows [59]. Intracerebroventricular administration of leptin induces hypersecretion of LH in fasted cows but not in well-fed cows[60].

Leptin receptor (Ob-R) is a member of class 1 cytokine receptor family [61] and express in six isoforms consisting of a full-length isoform (Ob-Rb), short isoforms (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Rf) and soluble isoform (Ob-Re) [62]. Expression of Ob-Rb, Ob-Ra and Ob-Rc were reported in peripheral tissues of cattle [63-65]. Expression of Ob-R transcript is the second most abundant in the uterus next to the liver among the peripheral tissues of prepubertal dairy heifers [64]. Among the various isoforms, the sum of Ob-Ra and Ob-Rb transcripts accounted for nearly the total amount of Ob-R, and Ob-Ra accounted for most of the Ob-R in the bovine uterus [64]. Estrogen suppresses Ob-R expression in prepubertal dairy heifers [64]. Ob-R levels in cyclic heifers were high during the luteal phase and the lowest on day 5 of the estrous cycle, with intermediate levels at estrus [66]. The lowest expression of Ob-R coincides with the first peak of endometrial EGF concentrations [13,44]. Together with the regulatory role of estrogen in endometrial EGF concentrations, Ob-R may be involved in the etiology of the abnormality in the endometrial EGF profile.

I had a particular interest in the role of the leptin system to determine the response of repeat breeder cows to the therapeutic treatment either with progesterone and estradiol [67] or seminal plasma (SP) [68]. Both treatments normalized the EGF profile in about 60% of repeat breeder cows [69] but factors segregating the consequence of treatment are unknown. Currently, this is a major obstacle to make the measures more effective. However, there is a preliminary observation that may be a clue to solve this problem. Repeat breeder beef cows with excessively high BCS and high leptin levels are resistant to treatment, and reduction of BCS by feed restriction and forced exercise reduced leptin levels and restored response to treatment [69]. It is likely that alteration of the leptin system may prevent repeat breeder cows from responding to treatment. Alternatively, I could find some difference in the leptin system in repeat breeder cows that were responding or not responding to treatment.

In chapter 2, I hypothesized that the leptin system may have a role in determining the response of therapeutic treatments to normalize the EGF profile in repeat breeder cows. The leptin system, the plasma leptin concentration and Ob-R expression in the endometrium, were examined in heifers, normal cows (fertile control), and repeat breeder cows together with EGF concentrations, before and after SP treatment to normalize the EGF profile. The data indicated that endometrial Ob-R, but not circulating leptin levels, may be related to the abnormality of the EGF profile in the endometrium.

Chapter 1

Recovery of the epidermal growth factor profile in the endometrium of dairy cows and some factors affecting the recovery

Introduction

Repeat breeding in cattle has been described in 1950's [7] when genetic improvement by AI is started. Repeat breeder cows are defined as the cows which failed to conceive after several (usually three or more) inseminations without anatomical and infectious abnormalities, and with an apparently normal estrous cycle [7–11]. As a definition, they are apparently normal and show regular estrus that is followed by ovulation. Therefore, they tend to be subjected AI repeatedly without producing a pregnancy until mid to the late stage of the lactation period. As a result, repeat breeder cows delay the time of conception and are culled before they make profits to the farm in their life cycle.

The previous studies found an abnormality in the cyclic change of EGF in the uterine endometrium and the abnormality has been linked to the reduced fertility in repeat breeding [13,43]. Endometrial EGF concentrations showed two peaks around days 3 and 14 (day 0 = estrus) of the estrous cycle. The loss of the two peaks characterizes the alteration of the endometrial EGF profile [22,43,44]. Two types of therapeutic treatments have been reported, i.e., hormonal treatment [67] and treatment with bovine SP or osteopontin (a potential active agent in the SP) [68,70] for this abnormality in the endometrium. These treatments normalized the endometrial EGF profile in about 70% [67] and 60% [68], respectively, and restored fertility in about 70% and 60% of the treated repeat breeder cows. However, for further improvement of the treatment and reduction of economic loss by repeat breeding in dairy cows, it is important to understand the etiology of this abnormality.

High milk production levels, heat stress, and obesity have been linked to this abnormality in a preliminary analysis with a small number of cows [69] The endometrial EGF profile recovered in approximately 75% of cyclic dairy cows by 60 dpp, while the recovery was delayed in cows producing large amount (50 kg per day or more) of milk. In these cows, approximately 40% of them showed an altered EGF profile [22]. From a physiological point of view, increased incidence of the abnormality in the endometrial EGF profile could be attributed, at least in part, to an increase in dry matter intake (DMI) to sustain high levels of milk production. Increased DMI increases hepatic blood flow [21] and, in turn, increases estradiol and progesterone clearance from the circulation [6]. These changes in ovarian steroid hormones may cause alteration of EGF in the

endometrium since E_2 and P_4 are the primary regulators of EGF in the endometrium [22]. In addition, some factors linking metabolic status and reproduction play a role in this abnormality may also have some roles in this problem. Drastic metabolic change associated with initiation of lactation, i.e., a negative energy balance (NEB) due to a lack of DMI for high levels of milk production may also be a potential factor delaying postpartum recovery of the EGF profile.

I hypothesized that the recovery of the cyclic change in the endometrial EGF concentration in lactating cows delays even after the regular estrous cycle resumed. This may prevent cows from getting conceived and the cows become repeat breeders. Therefore, the present study analyzed postpartum recovery of the EGF profile in cows showing the regular estrous cycle after 40 dpp and analyzed factors affecting the recovery of the EGF cycle in lactating dairy cows.

Materials and Methods

Animals

All animal experiments were performed in accordance with the Guidelines for Care and Use of the Experimental Animal Protocol of Hokkaido University, Japan (Experimental protocols # 16-0071 and 19-0030). The present study used Holstein cows housed on a commercial dairy farm in Hokkaido, Japan. They were multiparous lactating (> 10,000 kg, 305-days fat-corrected milk (FCM)) cows between two and five years of age and between 1 and 6 in parity. Cows experiencing postpartum diseases (e.g., ketosis, displaced abomasum, fatty liver, milk fever, metritis and endometritis), mastitis and lameness that are required more than three veterinary visits were excluded from the study. Cows showing irregular estrous cycles after 40 dpp were also excluded from the study.

Estrus detection

Estrus was diagnosed by using a telemetry activity monitor system using the algorithm provided by the manufacturer (estrus = day 0). Estrus was confirmed by palpation per rectum with the absence of corpus luteum and the presence of ovulatory follicle and uterine contraction by herdsmen. Cows were confirmed for ovulation within 36 h of the detection of the estrus by palpation per rectum every 12 h between 6:00 and 7:00 or between 18:00 and 19:00. Estrus with an inter-ovulatory interval of 18-24 days was considered normal.

Collection of endometrial tissue

Three pieces of endometrial tissue between 25 and 50 mg were obtained by biopsy using a biopsy instrument (3050100, Fujihira Industry, Tokyo, Japan) with caudal epidural anesthesia (3 ml of 2% lidocaine; 2% xylocaine, AstraZeneca, Osaka, Japan) as previously described [13,44]. Tissues were frozen in liquid nitrogen within 5 min of collection and stored at -30°C. A piece of the three tissues was used to measure the EGF concentration; the other two pieces were used elsewhere.

Measurement of endometrial EGF concentrations and judgment of the EGF profile

Concentrations of EGF in uterine tissues were determined by a double-antibody sandwich EIA using 96-well microtiter plates that has been validated for bovine EGF assay [43,68]. Anti-human EGF mouse monoclonal antibody (MAB636, R&D Systems, Inc., Minneapolis, MN, USA) and anti-human EGF rabbit antiserum (5022-100, Biogenesis Ltd., Poole, UK) were used as solid-phase and detection antibodies, respectively. Neither antibody showed significant cross-reactivity with other cytokines tested by the manufacturers. The assay system was verified for the measurement of bovine EGF using increasing concentrations of recombinant bovine EGF [68]. The intra- and inter-assay coefficients of variation (CV) were 5.5% and 7.8%, respectively. The sensitivity of the assay was 10 pg/well. The endometrial EGF profile was judged normal when the EGF concentration on day 3 was 4.70 ng/g tissue weight or greater [43,44].

Study design and data analysis

A total of 452 lactation postpartum dairy cows were used. Endometrial tissue samples were obtained by biopsy from all animals on day 3 in every estrous cycle until 90 dpp or until the cows showed the two consecutive estrous cycles with the normal EGF profile. Milk yield on each day of the estrus was calculated as mean of milk yield (kg) for 15 days with 7 days before and after the day of the estrus, respectively. All cows were recorded BCS [71] once a week by herdsmen and the BCS score immediately before the calving was used as BCS at calving. Cyclic change of the EGF concentration in the endometrium was judged as recovered when the cows showed two consecutive estrous cycles with the normal EGF profile. The day of recovery was defined as the estrus day of the first estrous cycle out of the two consecutive cycles.

For risk factor analysis, 57 cows in which a part of production and reproductive records are missing or culled before 90 dpp were excluded and data from a total of 395 cows were used for risk factors analysis. Categorical variable of parity (≤ 3 and > 3), BCS at calving (≤ 3.25 and

> 3.25), day of first ovulation (≤ 21, 22-30, and > 30 dpp), milk yield at peak level (< 50 and ≥ 50 kg), and time of peak period (< 45, 45-60, and > 60 dpp). The univariable logistics regression was used for comparison of the odds of cows with recovered and not recovered EGF for various risk factors. All statistical procedures were carried out by JMP 15 pro (SAS Institute Japan, Tokyo, Japan)

Results

The endometrial EGF concentration on day 3 (= EGF profile) of 452 cows were examined in every estrous cycle from calving to 90 dpp or until cows showed the normal EGF profile in 2 consecutive estrous cycles. Four hundred and one (88.7%) out of the 452 cows showed the first normal EGF profiles while 51(11.3%) cows repeated an altered EGF profile by 90 dpp (Fig 1-1). About 75% (338 out of 452) of all cows showed the first normal EGF profile within 60 dpp. In contrast, 106 (23.5%) and 192 (42.5%) cows recovered the cyclic change of the endometrial EGF concentration by 60 and 90 dpp, respectively (Fig 1-2). Among 192 cows that recovered the cyclic change of the EGF concentration by 90 dpp, one-third of cows (64 cows, 33.3%) showed the recovery at their first estrous cycle while the two-thirds of cows (128 cows, 66.7%) showed at least one estrous cycle with an altered EGF profile before the recovery (Table 1-1). Those cows showed the recovery at the first estrous cycle were 14.2% (64/452) of all cows used in the study.

Three hundred and fifty-eight (88.8%) cows were conceived, while 45 (11.2%) cows failed to conceive within 180 dpp (Table 1-2). Most of them 90% (147 out of 163) and 84% (123 out of 147) of the cows showed the first normal EGF profile within 40 dpp and 41-70 dpp were pregnant by 150 dpp, respectively (Table 1-2). In the cows showing the first normal EGF profile by 90 dpp, less than 10% of those remained open by 180 dpp. On the other hand, about 30% (13 out of 48) remained open by 180 dpp in the cows without recovery of the EGF profile.

The logistics regression analysis confirmed factors that include parity, BCS at calving, day of first ovulation, milk yield at peak level, and peak milk period are associated with delay or no recovery of the cyclic change of the endometrial EGF concentrations during the estrous cycle (Table 1-3). Specifically, cows with parity of 3 or less or BCS at calving greater than 3.25 seems to delay or less likely to recover EGF cycle by 90 dpp ($P < 0.01$). Cows showing the first ovulation later than 30 dpp reduced likelihood to recover the EGF cycle ($P < 0.05$). Further, cows with high peak yield (50 kg per day) or reaching to the peak yield later than 60 dpp were also at risk ($P < 0.05$).

Discussion

The present study examined the time of recovery of cyclic change in endometrial EGF in two forms. The first is time showing the first normal EGF profile and the second is the recovery of the cyclic change of the EGF profile. The latter was defined as the day of estrus of the first estrous cycle out of the two consecutive cycles with the normal EGF profile. During the voluntary waiting period (VWP) of the first 60 days of the postpartum period when AI is hold even with presentation of good estrus signs, about three fourth of high-yielding cows show at least one estrous cycle with the normal EGF profile while only about one-fourth of cows showed the recovery of the cyclic change of the endometrial EGF concentrations. During the time between 60 and 80 dpp when most cyclic cows subjected to AI for the first time, the cumulative recovery rate reached 30 to 40% and were similar to the conception rate by the first AI immediately after VWP in the herd used in the present study.

Accordingly, the cows showing at least one estrous cycle with the normal EGF profile by 90 dpp of the observation period, regardless of the timing before 90 dpp, showed a higher pregnancy rate at the end of the study period of 180 dpp than the cows not showing any estrous cycle with the normal EGF profile by 90 dpp (> 90 % vs. 72.9%, respectively, $P < 0.05$). The difference of fertility between the two groups is even bigger in the earlier postpartum period of 150 dpp (89.6% vs. 60.8%, respectively). This has significant impact on the economy of commercial farms since delayed pregnancy generally leads to a loss of 12,000-15,000 yen per day for each cow.

The present study used postpartum cows that repeated the regular estrous cycles without health and reproductive problems requiring more than three veterinary visits for each episode. In other words, the present study used cows with an ideal postpartum recovery. Nevertheless, resumption of the regular estrous cycle is not accompanied by the recovery of the cyclic change of endometrial EGF concentrations in many cows. Only 64 out of 452 cows (14.2%) recovered the cyclic change of the EGF concentrations at the first estrous cycle after calving. In most of cows, the EGF cycle required a few regular ovarian cycles. It has been reported that secretion of P_4 and E_2 of the first few estrous cycles after calving are low in high-yielding cows [72-74]. A slow luteinizing hormone (LH) pulses by postpartum NEB have been linked to the low ovarian steroid hormone secretion [75,76]. Alternatively, inflammation of the uterus may also be partly responsible for suppressed ovarian secretion during the early postpartum period [77] when dominant follicles from the first and second follicle wave reach to the ovulatory stage. The uterus of the postpartum cows is contaminated with bacteria and it takes at least three to four weeks

before clearance from the uterus [78,79]. *E. coli*, a gram-negative bacterium and a typical contaminant of the uterus, releases endotoxin and reduce ovarian steroid secretion via suppression of the hypothalamus-pituitary-gonad axis and by direct effects on follicles [80]. The present study excluded all cows presenting any signs of abnormality in the uterus. However, many cows show some degree of the inflammation of the uterus during the first three to four weeks is part of the normal recovery process of the postpartum period.

The present study examined the effect of risk factors on the recovery of EGF cycle in 90 dpp. Logistics regression analysis was used to determine the effects of risk factors. High BCS at calving, high milk yield (50 kg per day) at peak level, and the first ovulation after 30 dpp could be linked to metabolic challenge associated with initiation and continuous increase of milk production during the early postpartum period [74,81,82]. Most of the cows enter a period of NEB after initiating lactation [83]. However, the decline in body condition of the cows used in the present study was not as severe as that prevents the reproductive endocrine axis since all cows showed the regular estrous cycle. This may suggest that the drastic change in metabolic status during the postpartum period may prevent the recovery of the EGF cycle in some mode other than suppression on the hypothalamus-pituitary-gonadal axis. It has been implicated that the IGF system [84] and the leptin system [85] may be responsible for mediating detrimental effects of NEB on reproduction in dairy cows. Further study is necessary to determine the role of these systems in etiology of the abnormality of the EGF cycle. The present results also pointed out that delayed peak of milk yield later than 60 dpp was a risk factor for the recovery of the EGF cycle. The delay may be related to the response to stressors (e.g., nutritional or managerial stress) during the early lactation period and associated with an increase in the incidence of repeat breeding syndrome [86].

In conclusion, the results of the present study confirmed the association between the recovery of the normal EGF profile and fertility. Further, the results indicated that the cows which resumed the regular estrous cycle but failed to recover the normal EGF profile become the repeat breeder cows.

Tables and figures

Table 1-1. Patterns of the EGF profiles in cows recovered and not recovered the cyclic change of the endometrial EGF concentration and conception rate at 150 dpp

Recovery of the EGF cycle (%)	No.(%) of cows		Order of the estrous cycle relative to the last one before 90 dpp				No.(%) of cows conceived by 150 dpp	
			Cycle -3	Cycle -2	Cycle -1	Last cycle		
Recovered	62	(13.7)	-	-	Normal	Normal	56/ 61	(91.8)
	2	(0.4)	-	Normal	Normal	Normal	1/ 2	(50.0)
	2	(0.4)	Altered	Normal	Normal	Normal	2/ 2	(100.0)
	2	(0.4)	Normal	Altered	Normal	Normal	2/ 2	(100.0)
	16	(3.5)	Altered	Altered	Normal	Normal	13/ 16	(81.3)
	108	(23.9)	-	Altered	Normal	Normal	98/108	(90.7)
Subtotal	192	(42.5)					172/191	(90.0)[A]
Unrecovered	9	(2.0)	-	-	Altered	Normal	7/ 9	(77.8)
	2	(0.4)	-	Normal	Altered	Normal	0	
	54	(11.9)	Altered	Normal	Altered	Normal	46/ 51	(90.2)
	24	(5.3)	-	Altered	Altered	Normal	17/ 19	(89.5)
	3	(0.7)	Normal	Altered	Altered	Normal	0	
	22	(4.9)	Altered	Altered	Altered	Normal	14/ 21	(66.7)
	3	(0.7)	-	-	Normal	Altered	2/ 2	(100.0)
	49	(10.8)	-	Altered	Normal	Altered	21/ 24	(87.5)
	16	(3.5)	Altered	Altered	Normal	Altered	10/ 16	(62.5)
	4	(0.9)	-	Normal	Altered	Altered	1/ 1	(100.0)
	22	(4.9)	Altered	Normal	Altered	Altered	14/ 21	(66.7)
	1	(0.2)	Normal	Altered	Altered	Altered	0	
	20	(4.4)	Altered	Altered	Altered	Altered	11/ 20	(55.0)
	30	(6.6)	-	Altered	Altered	Altered	15/ 28	(53.6)
	1	(0.2)	-	-	Altered	Altered	0	
Subtotal	260	(57.5)					158/212	(74.5)[B]
Total	452	(100.0)					330/403	(81.9)

Cows were monitored for their EGF profile at every estrous cycle after calving either until showing two consecutive estrous cycles with the normal EGF profile (recovery of the cyclic change of EGF) or 90 dpp.

The normal EGF profile; the EGF concentration on day 3 $\geq$ 4.70 ng/g tissue weight.

[§] Four hundred and three cows with fertility results until 150 dpp were used.

[A, B] Values with different letters between groups differ ($P < 0.05$)

Table 1-2. Effects of the time of the first estrous cycle with the normal EGF profile on fertility

The first estrous cycle with the normal EGF profile (dpp)	No.of cow (n)	No.(%) of cows conceived by the time*				Subtotal	Not conceived by 180 dpp
		90	91 – 120	121 – 150	151 – 180		
≤ 40	163	55 (33.7)^{A}	54 (33.1)^{BC}	38 (23.3)	2 (1.2)^{C}	149 (91.4)^{A}	14 (8.6)^{A}
41 – 70	147	14 (9.5)^{B}	67 (45.6)^{A}	42 (28.6)	10 (6.8)^{B}	133 (90.5)^{A}	14 (9.5)^{A}
71 – 90	45	-	20 (44.4)^{AB}	14 (31.1)	7 (15.6)^{A}	41 (91.1)^{A}	4 (8.9)^{A}
Not recovered	48	-	11 (22.9)^{C}	15 (31.1)	9 (18.8)^{A}	35 (72.9)^{B}	13 (27.1)^{B}
All groups	403	69 (17.1)	152 (37.7)	109 (27.1)	28 (6.9)	358 (88.8)	45 (11.2)
Cumulative	-	69 (17.1)	221 (54.8)	330 (81.9)	358 (88.8)	-	-

Forty-nine out of 452 cows were excluded from the analysis due to incomplete breeding records.

* The day of AI that produce a pregnancy.

^{A,B,C} Values with different letters within the same column differ ($P < 0.05$).

Table 1-3. Risk factors for the recovery of EGF cycle by 90 days postpartum (dpp) period

Factor	Total	No.(%) of cows recovered by 90 dpp	No.(%) of cows not recovered by 90 dpp	Day of recovery* (dpp)	Odds ratio (95% confidence interval)	P-value
Parity						
> 3	157	87 (55.4)	70 (44.6)	45.8 ± 1.4	Reference	
≤ 3	238	105 (44.1)	133 (55.9)	45.8 ± 1.1	0.6 (0.423-0.953)	< 0.01
BCS at calving						
≤ 3.25	200	127 (63.5)	73 (36.5)	44.5 ± 1.2	Reference	
> 3.25	195	65 (33.2)	130 (66.7)	47.1 ± 1.3	0.3 (0.190-0.435)	< 0.01
Day of first ovulation (dpp)						
≤ 21	227	105 (46.3)	122 (53.7)	42.8 ± 1.0^{B}	Reference	
22 – 30	77	56 (72.7)	21 (27.3)	37.8 ± 1.7^{C}	3.1 (1.760-5.453)	< 0.01
> 30	91	31 (34.1)	60 (65.9)	62.8 ± 1.7^{A}	0.6 (0.362-0.996)	< 0.05
Milk yield at peak levels (kg)#						
< 50	292	161 (55.1)	131 (44.9)	42.4 ± 1.0^{A}	Reference	
≥ 50	103	31 (30.1)	72 (69.9)	56.5 ± 1.7^{B}	0.3 (0.217-0.566)	< 0.01
Time of peak period (dpp)§						
45 – 60	196	104 (55.4)	92 (44.6)	47.1 ± 1.2^{A}	Reference	
< 45	79	43 (39.4)	36 (60.6)	34.7 ± 1.8^{B}	1.1 (0.626-1.785)	0.84
> 60	120	45 (37.5)	75 (62.5)	51.1 ± 1.5^{A}	0.5 (0.333-0.844)	< 0.01

Fifty-seven cows out of 452 cows were excluded due to the absence of the data.

* Values are mean ± SDs; The estrus day of the first estrous cycle out of the two consecutive cycles of normal EGF profile.

Avarage of milk yield for the 15 days before and after 7 days of the day of estrus.

§ Day of the estrus with highest milk yield records.

^{A,B,C} Values with different letters within the same factor differ ($P < 0.05$).

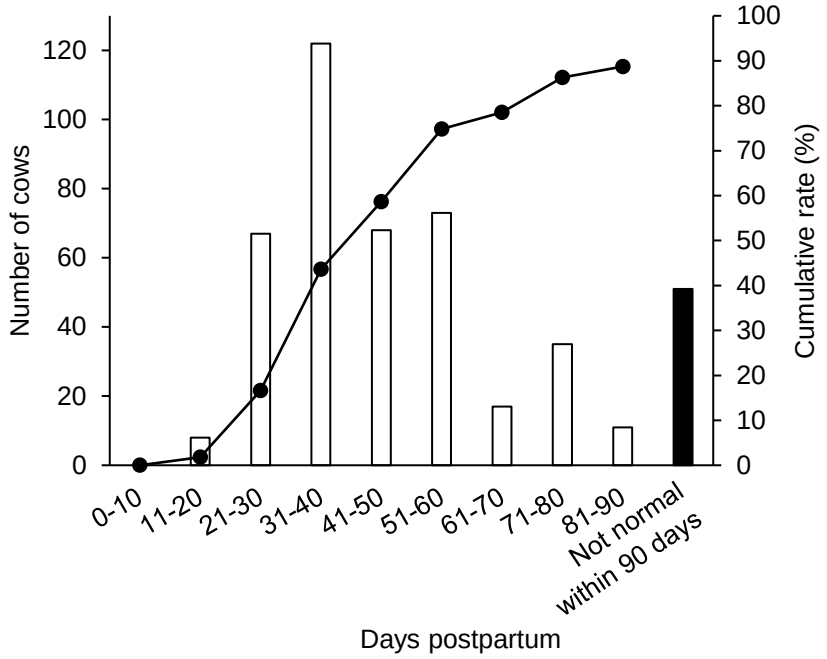

Fig 1-1. Days of estrus of the cycle in which the cows showed the EGF concentration on day 3 in the normal range (≥ 4.70 ng/g tissue weight) for the first time after calving (first normal EGF profile). In total 452 cows, 401 (88.7%) cows showed the first normal EGF profile between 14 and 89 dpp. About 75% (338 out of 452) of total cows showed the first normal EGF profile within 60 dpp. Until 90 dpp, 51 (11.3%) cows without the normal EGF profile were remaining. The clear bar indicates the numbers of cows showing the first normal EGF profile. The solid bar indicates the number of cows without showing a normal EGF profile. The closed circle (●—) indicates a cumulative rate of the first normal EGF profile during 90 dpp.

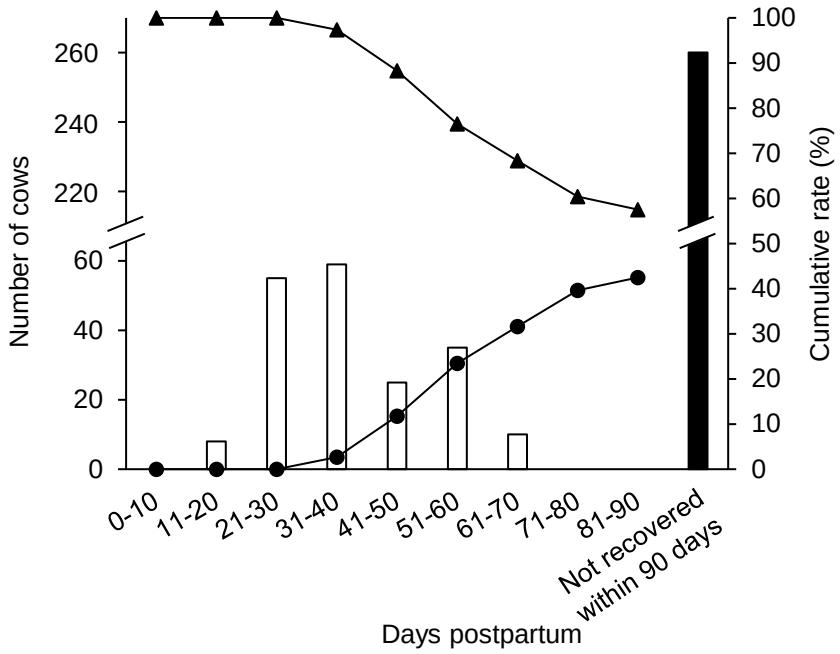

Fig 1-2. Days of the estrus of the first estrous cycle in the cows showed normal EGF profiles for 2 consecutive cycles (recovered EGF cycle). The recovery of the EGF cycle was found in 192 out of 452 (42.5%). Those cows showed the first normal EGF cycle between 14 and 65 dpp. By 60 dpp, 106 (23.5%) cows showed recovered EGF cycle. About 46% (209 out of 452) showed at least one normal EGF profiles and 11% (51 out of 452) did not show normal EGF profile in 90 dpp. The clear bar indicates the numbers of cows showing recovered EGF cycle. The solid bar indicates the number of cows that did not recover EGF profiles. The closed circle (●—)indicates a cumulative rate of recovered EGF profile. The closed triangle (▲—) indicates percentages of the remaining cows without the recovery of the EGF profile.

Summary

The present study aimed at examining changes in EGF cycle and the risk factors that affect the recovery of the EGF cycle. Four hundred fifty-two lactating cows were examined for the EGF profile in every estrous cycle from calving to 90 dpp or until the cows showed two consecutive estrous cycles with the normal EGF profile (recovery of the EGF cycle). Production and reproductive data were also recorded. About 75% of all cows showed the first normal EGF profile by 60 dpp, but only 42.5% recovered the EGF cycle by 90 dpp. The number of cows pregnant by 150 dpp was higher in a group showing the EGF normal profile. Parity, BCS at calving, day of first ovulation, milk yield at peak level, and peak milk period were associated with a delay of the EGF cycle. The results of the present study confirmed the association between the recovery of the normal EGF profile and fertility and the cows which failed to recover the normal EGF profile could be a source of repeat breeder cows

Chapter 2

Leptin receptor expression and its change in association with the normalization of EGF profile after seminal plasma treatment in repeat breeder dairy cows

Introduction

An alteration of the endometrial EGF profile during the estrous cycle causes reduced fertility in repeat breeder and high-yielding cows [22]. Endometrial EGF concentrations showed two peaks around days 3 and 14 of the estrous cycle. The loss of the two peaks characterizes the alteration of the endometrial EGF profile [13,44]. Recently, two types of treatment have been reported for this alteration in the endometrium [67,68,87]. The first is hormonal treatment combined with a high dose of estradiol benzoate and an intravaginal progesterone releasing device [67]. The second is the vaginal infusion of SP [68]. Hormonal and SP treatments normalized the endometrial EGF profile in approximately 70% [67] and 60% [68] of the repeat breeder cows, respectively. However, it is important to understand the factors that distinguish repeat breeder cows responding to treatment from those not responding to treatment to explore clues to improve treatment and determine predisposing factors for the alteration of endometrial EGF profile.

High milk production levels, heat stress, and obesity have been linked to this abnormality. The endometrial EGF profile recovered in approximately 75% of cyclic dairy cows by 60 days postpartum, while the recovery was delayed in cows producing 50 kg/day or more of milk. Moreover, approximately 40% of the cows showed an altered EGF profile [22,69]. The increased abnormality in the endometrial EGF profile could be attributed, at least in part, to an increase in dry matter intake (DMI) to sustain high levels of milk production. Increased DMI increases hepatic blood flow [21] and, in turn, increases E_2 and P_4 clearance from the circulation [6]. In addition, a negative energy balance due to a lack of DMI for high levels of
milk production may also be a potential factor delaying the EGF profile recovery time.

Leptin and its receptors (Ob-R) serve as mediators between metabolic status and reproductive function [61,88]. Leptin is primarily secreted by adipose tissue and plays a central role in regulating feeding behavior and energy homeostasis. Low leptin concentrations indicate inadequate nutritional stores and prevent unwanted pregnancy [61]. The suppression of reproductive activity by leptin is primarily mediated by the suppression of GnRH secretion from the hypothalamus [58,61]. However, the expression of Ob-R is found in peripheral tissues, including the ovary, uterus, oocyte, and early embryo [64,89]. The association between single nucleotide polymorphisms (SNPs) in leptin and fertility in heifers has been reported [90].

Together, the effect of leptin on fertility is directly in part and is not associated with postpartum fat mobilization by negative energy balance.

Expression of the Ob-R transcript is the most abundant in the uterus next to the liver in peripheral tissues [64]. However, studies on the role of leptin and Ob-R in the uterus have focused on only establishing pregnancy at implantation or placentation phases in rodents and humans [91-93]. Our understanding of the role of leptin and its receptors during the estrous cycle and preimplantation period is limited. Although estrogen treatment in intact and ovariectomized heifers did not change plasma leptin and leptin expression levels in adipose tissue, estrogen has been shown to modulate Ob-R expression in the uterus [64]. Expression of Ob-R transcript is the most abundant in the uterus next to the liver among the peripheral tissues of prepubertal dairy heifers. Among the various isoforms, the sum of Ob-Ra (a short isoform) and Ob-Rb (a full-length isoform) transcripts accounted for nearly the total amount of Ob-R, and Ob-Ra accounted for most of the Ob-R in the bovine uterus. Estrogen suppresses Ob-R expression in prepubertal dairy heifers [64]. Ob-R levels in cyclic heifers were high during the luteal phase and lowest on day 5 of the estrous cycle, with intermediate levels at estrus [66] The lowest expression of Ob-R coincides with the first peak of endometrial EGF concentrations [13,44]. Together with the regulatory role of estrogen in endometrial EGF concentrations, Ob-R may be linked to the endometrial EGF profile.

The objective of the present study was to examine whether the leptin system may have a role in determining the response of repeat breeder cows to treatment for EGF normalization by SP. Therefore, the present study compared the plasma leptin concentration and endometrial Ob-R expression in repeat breeder cows between those who responded and did not respond to the SP treatment to normalize the EGF profile. Ob-R expression was also determined after the treatment to examine whether the recovery of the EGF profile and fertility could be linked to changes in Ob-R expression. In addition, the expression of leptin and leptin receptors was examined in fertile and lactating cows to obtain reference values of blood leptin and endometrial leptin receptor levels in fertile cattle.

Materials and Methods

Animals

All animal experiments were performed in accordance with the Guidelines for Care and Use of the Experimental Animal Protocol of Hokkaido University, Japan (Experimental protocol #16-0071). The present study used Holstein heifers and cows housed on four commercial dairy farms in Hokkaido, Japan. The heifers were virgin and between 14 and 15 months of age on the

day of the first biopsy for EGF measurement. They showed at least three estruses with inter-estrus intervals between 18 and 20 before the biopsy. Repeat breeder cows were diagnosed by local practitioners using the criteria of failing to conceive after three artificial inseminations (AI) without a detectable abnormality in clinical signs, the estrous cycle, and genital organs. All repeat breeder cows were then confirmed to meet the definition of repeat breeders and performed additional examinations, including transrectal ultrasonography of the genital organs, uterine cytology, and oviductal patency by the veterinarian before enrollment in the study, as described previously [68]. Normal and repeat breeder cows were multiparous lactating (> 10,000 kg, 305-days FCM) cows between three and six years of age. The normal cows were between 75 and 90 days postpartum, while repeat breeder cows were between 170 and 230 days postpartum. None of the animals received any therapeutic treatment prior to recruitment for the study.

Collection of endometrial tissue and plasma samples

Three pieces of endometrial tissue between 25 and 110 mg were obtained by biopsy using a biopsy instrument (3050100, Fujihira Industry, Tokyo, Japan) with caudal epidural anesthesia (3 ml of 2% lidocaine; 2% xylocaine, Fujisawa Pharmaceutical, Osaka, Japan) as previously described [13,44]. Tissues were frozen in liquid nitrogen within 10 min of collection and stored at –80 °C. One of the three biopsy samples was used to measure EGF concentration; the other two pieces were used for Ob-R expression. Blood samples were collected from the jugular vein or tail vein.

Measurement of endometrial EGF concentrations and judgment of the EGF profile

Concentrations of EGF in uterine tissues were determined by a double-antibody sandwich EIA using 96-well microtiter plates that have been validated for bovine [43,68]. Anti-human EGF mouse monoclonal antibody (MAB636, R&D Systems, Inc., Minneapolis, MN, USA) and anti-human EGF rabbit antiserum (5022-100, Biogenesis Ltd., Poole, UK) were used as solid-phase and detection antibodies, respectively. Neither antibody showed significant cross-reactivity with other cytokines tested by the manufacturers. The assay system was verified for the measurement of bovine EGF using increasing concentrations of recombinant bovine EGF [68]. The intra- and inter-assay CVs Swere 5.8% and 6.3%, respectively. The sensitivity of the assay was 10 pg/well. The endometrial EGF profile was judged normal when the EGF concentration on day 3 was 4.70 ng/g tissue weight or greater [43,44].

Measurement of plasma leptin concentrations

Plasma leptin concentration was determined by enzyme immunoassay using a bovine leptin ELISA kit (55R-1959, Fitzgerald Industries International, North Acton, MA, USA) according to the manufacturer's instructions. Intra- and inter-assay CVs were reported to be < 15% by the manufacturer. The sensitivity of the assay is reported as < 1.56 ng/ml. All plasma samples were assayed in triplicate.

RNA isolation and quantitative real-time PCR for leptin receptor (Ob-R)

Total RNA was isolated and purified using affinity chromatography (79306, Qiazol and 74104, RNeasy Mini Kit, Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Quantitative real-time PCR assays were used to measure the Ob-R. Total RNA (2 μg) was reverse transcribed using Super Script II (18064022, Invitrogen, Carlsbad, CA, USA), and cDNA (150 ng) was amplified using 2x Universal Master Mix (4440038, Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers for Ob-R were designed to detect all Ob-R isoforms [63]: forward GTGCTGGCCATCAATTCAATT; reverse GGGTGACAGCATCCAGGAA; probe carboxyfluorescein-CAGCAAAGTAAATATCG-minor groove binding dye. Reactions were performed in triplicate on an ABI 7000 Thermocycler using standard thermocycling conditions (Applied Biosystems): 1 cycle at 50 ℃ for 2 min (Uracil N-glycosylase activation), 1 cycle at 95 ℃ for 10 min (DNA polymerase activation) followed by 40 cycles at 95 ℃ for 15 s (denaturation) and 60 ℃ for 1 min (annealing and extension). TaqMan Ribosomal RNA control reagents (4308329, Applied Biosystems) were used to detect 18s ribosomal RNA. Ob-R data were analyzed by the standard curve method prepared by serial dilution of the standard plasmid containing a homologous sequence for Ob-R.

Preparation of SP samples

SP samples were collected as previously described [68]. Semen was collected twice a week from nine Holstein bulls with known fertility using an artificial vagina at a commercial AI center (Genetics Hokkaido, Tokachi Shimizu, Hokkaido, Japan). SP was separated by centrifugation at 1,000 × g for 10 min, frozen at -20 ℃, and transported to Hokkaido University at -20 ℃. At the university laboratory, all frozen SP samples were thawed and centrifuged at 5,000 × g for 20 min at 4 ℃, and the resulting supernatants were used as SP samples. SP from nine bulls was pooled, kept in 0.5 ml aliquots, and stored at -80 ℃.

Infusion of SP into the vagina and the uterus

SP was infused into the vagina of repeat breeder cows, as described previously [68]. Briefly, at the time of infusion, an aliquot of SP (0.5 ml) was thawed on farms, diluted with 9.5 ml of PBS, and aspirated with a 10-cc syringe. A disposable plastic AI catheter was attached to the syringe containing the SP sample. The AI catheter was introduced into the vagina, and diluted SP was deposited in the vagina near the external orifice of the cervix. The AI catheter was gently withdrawn after the infusion. In heifers and fertile cows, 10 ml of PBS alone was infused.

Study design

The protocol for this study is outlined in Fig 2-1. Cows and heifers in four commercial dairy herds were observed for estrus (day 0) two or three times a day or were equipped with an activity monitor for estrus detection. Endometrial tissue samples were obtained by biopsy from all animals on day 3 in a natural estrous cycle, and endometrial EGF concentrations were determined. Twenty-six repeat breeder cows with altered EGF profiles were recruited for this study. A total of 24 apparently normal lactating cows and 18 heifers with a normal EGF profile were used as controls. Blood samples were collected from all animals on the day of the first biopsy for the plasma leptin assay.

On the day of the next estrus to the first endometrial biopsy for EGF assay, all 26 recruited repeat breeder cows were infused with SP into the vagina between 4 and 8 h after estrus detection, while all 18 heifers and 20 out of 24 control cows were infused with PBS. Four cows recruited as control cows that developed mastitis or severe lameness after the first EGF examination were excluded from the study. On day 3, endometrial tissues were biopsied again to determine the EGF concentrations. The expression of Ob-R was examined in all cows, in which the endometrial tissue samples remained after the EGF assay of both the first and second examinations: 18 out of 26 repeat breeder cows, 9 out of 18 heifers, and 9 out of 20 control cows. All cows examined for Ob-R expression were subjected to AI by professional AI technicians up to two times immediately after the study period. Pregnancy was diagnosed by rectal palpation between 55 and 60 days after AI.

Statistical analysis

Plasma leptin concentrations in the animal groups were compared using one-way ANOVA. The correlation between plasma leptin and endometrial EGF concentrations, leptin and Ob-R, and Ob-R before and after SP treatment were analyzed using Pearson correlation.

Endometrial EGF concentrations and Ob-R levels before and after infusion were analyzed by two-way ANOVA, followed by Tukey's test and Student's paired t-test. Endometrial EGF concentrations and Ob-R levels were compared between heifers, control cows, and repeat breeder cows. Then, a comparison between heifers, control cows, and repeat breeder cows with normal and unnormalized EGF profiles after SP infusion. Data were analyzed using JMP Pro 15 software (SAS Institute Japan, Tokyo, Japan).

Results

All heifers and control cows showed a normal endometrial EGF profile on day 3 (i.e., the normal EGF profile) in both the first and second examinations and all were conceived by the second AI (Table 1). SP treatment normalized the endometrial EGF profile in 14 out of 26 (53.8%) repeat breeder cows and 8 out of 18 (44.4%) repeat breeder cows conceived after SP treatment.

Plasma leptin concentrations were similar in all groups and between repeat breeder cows showing normal and unnormalized EGF profiles after SP treatment (Table 2-1). Plasma leptin levels were not correlated with EGF concentrations (Fig 2-2) or Ob-R levels (Fig 2-3) in the endometrium. In contrast, a potential relationship between EGF concentration and Ob-R levels in the endometrium was observed. Endometrial EGF concentrations and Ob-R levels before treatment showed a negative correlation ($r = 0.653$, $P < 0.01$ for all cows; $r = 0.541$, $P < 0.05$ for heifers and control cows, Fig 2-4). Heifers showed higher EGF concentrations and lower Ob-R levels than control cows ($P < 0.01$) (Table 2-1). Ob-R levels in repeat breeder cows before SP treatment were similar to those in control cows. Normalization of the endometrial EGF concentrations on day 3 (i.e., the EGF profile) in repeat breeder cows coincided with a decrease in Ob-R levels in the endometrium. Ob-R in repeat breeder cows showing a normal EGF profile after SP treatment were at intermediate levels between heifers and control cows. Ob-R levels in those showing an unnormalized EGF profile after SP treatment remained at similar levels before treatment. Ob-R levels before and after PBS infusion in fertile animals (heifers and control cows) were consistent and showed a positive correlation ($r = 0.965$, $P < 0.01$, Fig 2-5). As a result, Ob-R levels in repeat breeder cows with a normal EGF profile after SP treatment were off the regression line of the fertile animals, while those showing an unnormalized EGF profile after SP treatment appeared along the regression line.

Discussion

The objective of the present study was to examine whether the leptin system (plasma leptin and endometrial Ob-R) could be a determining factor of the response of the EGF profile to

SP treatment, which has been demonstrated to recover the normal EGF profile and fertility in repeat breeder cows. The leptin system was also examined in heifers and lactating cows to obtain reference values for the system in fertile cattle. To our knowledge, this is the first report comparing leptin and its receptor at the time of breeding in heifers and postpartum cows between 75 and 90 days postpartum. The results indicated an association between endometrial EGF concentration and Ob-R levels, but not leptin concentrations, in relation to fertility.

Ob-R levels differed between heifers and control cows, although all heifers and control cows conceived immediately after the study. Heifers with supposedly high fertility showed lower Ob-R values than cows. Ob-R levels were consistent between the two consecutive estrous cycles in both heifers and control cows ($r = 0965$, $P < 0.01$). However, the normalization of the EGF profile after SP treatment, associated with restoration of fertility [68,87], coincided with a decrease in Ob-R levels in repeat breeder cows. Together, these results may indicate that Ob-R levels could have a wide range in fertile animals and that relatively low levels or tending to decrease even within the normal range are advantageous for conception.

I expected a difference in the Ob-R levels between heifers and cows. In the present study, EGF and Ob-R were examined on day 3 of the estrous cycle when EGF concentrations peaked [13,44] and Ob-R expression was low. In cyclic heifers, Ob-R in the metestrus (day5) was at the lowest level and showed an almost two-fold increase in diestrus (day 12) [66]. Estrogen treatment suppressed endometrial Ob-R levels in cyclic and ovariectomized heifers, whereas estrogen increases EGF levels in the endometrium. Plasma estradiol levels show a slower increase and lower peak in high-yielding cows due to an increase in dry matter intake associated with high levels of milk production [6,21]. Similar changes in plasma estradiol levels have been reported in repeat breeder cows [22]. Suppressed estradiol activity in high-yielding cows and repeat breeder cows may result in an increase in endometrial Ob-R levels compared to heifers.

The concentration of plasma leptin increases during pregnancy, starts to decline 1 to 2 weeks before parturition, and reaches a nadir during early lactation [94-96]. The plasma leptin concentration remained depressed during early lactation. Corresponding changes occurred in the abundance of leptin mRNA in the subcutaneous adipose tissues. The postpartum reduction in plasma leptin was due to a decrease in adipose tissue caused by the negative energy balance because plasma leptin remained high in cows not milked after parturition [94,95]. In the present study, however, plasma leptin levels in control cows were similar to those in heifers. The absence of a decrease in plasma leptin in control cows could be attributed to the advanced days of postpartum and selection criteria at recruitment in the present study. A previous study [95] examined the plasma leptin concentration up to 56 days postpartum during the period when milk

yield increased to the maximal levels, while the present study used cows at a later stage of lactation between 75 and 90 days postpartum when nutritional status may have improved in average cows. In addition, cows that had experienced postpartum diseases and a decrease in body condition of more than 1.25 were excluded from serving as fertile controls in the present study.

The present results also contradict those of a previous report showing that repeat breeder cows have lower leptin concentrations than fertile cows [97]; this can also be explained by the different lactation stages of the cows used in the two studies. A previous study [97] used fertile cows in the late stage of lactation as they used fertile cows in the matched postpartum days to repeat breeder cows that failed to conceive after up to ten times the AI. It is probable that the body condition or amount of adipose tissue may have recovered to the levels at which leptin concentration is normal. However, repeat breeder cows could still be under the influence of malnutrition associated with parturition and initiation of lactation.

The functional relationship between EGF and Ob-R in fertile cattle and repeat breeder cows is beyond the scope of the present study. However, finding that the normalization of the EGF profile and a decrease in Ob-R occurred simultaneously after SP treatment that restored fertility appears important to elucidate the role of Ob-R in fertility and take measures against repeat breeding caused by an alteration of the endometrial EGF profile in dairy cows.

Table 2-1. Effect of seminal plasma (SP) treatment on endometrial epidermal growth factor (EGF) concentration and expression of leptin receptor (Ob-R) in endometrium of repeat breeder cattle

Cattle group	n	EGF concentration (ng/g tissue weight)		Leptin (ng/ml)	Ob-R expression (arbitrary unit)		Conception (%)
		Before infusion	After infusion		Before infusion	After infusion	
Heifers	18	9.29 ± 2.01^{ax}	9.64 ± 1.99^{ax}	4.36 ± 1.10	10.90 ± 0.55^{ax} (9)	11.06 ± 1.99^{ax} (9)	9 (100.0)
Control	20	6.38 ± 0.93^{byA}	7.36 ± 1.53^{byB}	4.32 ± 1.19	20.13 ± 4.24^{by} (9)	20.76 ± 4.01^{byz} (9)	9 (100.0)
Repeat breeder	26	2.10 ± 0.87^{cA}	4.66 ± 2.57^{cB}	4.15 ± 0.91	23.78 ± 6.88^{b} (18)	20.28 ± 7.57^{b} (18)	8 (44.4)
Normalized	14	2.26 ± 1.01^{zA}	6.86 ± 1.07^{yB}	4.41 ± 0.84	24.23 ± 7.27^{yA} (9)	15.57 ± 2.71^{xyB} (9)	6 (66.7)
Unnormalized	12	1.90 ± 0.66^{z}	2.11 ± 0.64^{z}	3.85 ± 0.94	23.33 ± 6.88^{y} (9)	24.99 ± 8.03^{z} (9)	2 (22.2)

Fertile cattle (heifers and control) were infused with PBS into the vagina, while repeat breeder cows were infused with SP.

Values are means ± SDs.

The numbers in parentheses are the number of cattle examined for Ob-R expression.

^{a,b,c} Means of EGF concentrations and Ob-R levels with different letters differ between the groups of cattle ($P < 0.01$).

^{x,y,z} Means of EGF concentration and Ob-R in repeat breeder cows with normal and unnormalized EGF profiles were compared with fertile heifers and control. cows. Means with different letters differ between groups ($P < 0.01$).

^{A,B} Means of EGF concentration and Ob-R with different letters differ before and after infusion ($P < 0.05$).

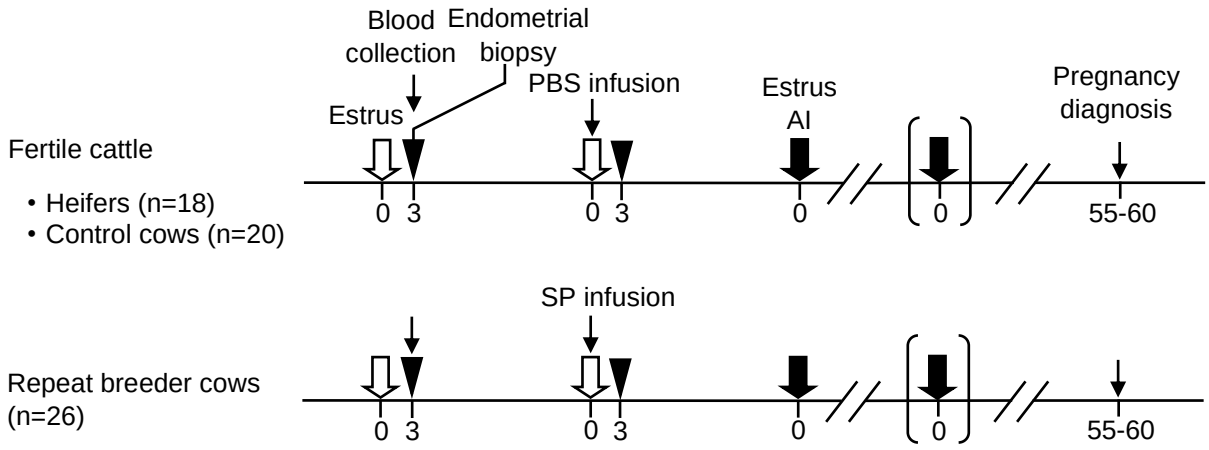

Fig 2-1. A schematic diagram of SP treatment for study. In all cattle recruited for the present study, endometrial tissues were obtained for the EGF assay by biopsy on day 3 of the estrous cycle (day 0 = estrus). Twenty-six repeat breeder cows with an altered EGF profile, 18 heifers, and 20 control cows with the normal EGF profile were used as a fertile control groups. Blood samples were collected on the day of the first biopsy and used for plasma leptin assay. On the day of estrus in the next cycle, SP was infused into the vagina of repeat breeder cows while PBS was infused in heifers and control cows, then endometrial tissues were obtained on day 3 of the estrous cycle for the second time for the EGF assay. Endometrial Ob-R expression was examined in all cattle with remaining tissue samples on both days (n = 9 for all groups). All animals, in which Ob-R expression was examined, were subjected to AI up to two times and pregnancy was diagnosed between 55–60 days after the last AIs.

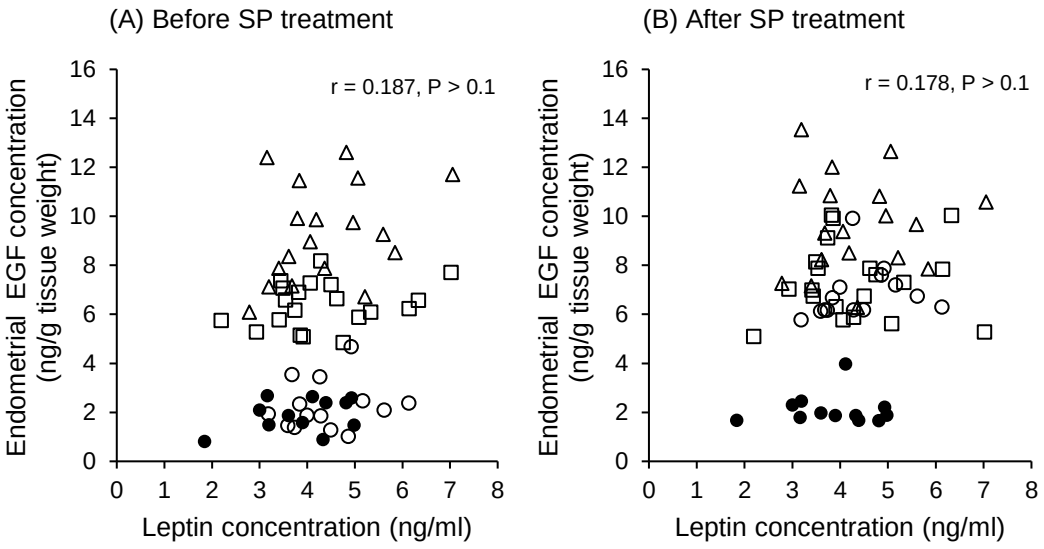

Fig 2-2. Correlation between plasma leptin and EGF concentration before (A) and after (B) PBS or SP infusion. Endometrial tissues were biopsied on day 3 of estrous cycle from all cows twice, before and after intra-vaginal infusion with PBS or SP in fertile animals (heifers and control cows) and repeat breeder, respectively. Plasma leptin concentrations were examined at before infusion in all animals. Different symbols indicate different group of animals: Δ heifers; □ control cows; ○ repeat breeder cows with the normal EGF profile after SP infusion; ● repeat breeder cows with unnormalized EGF profile after SP infusion

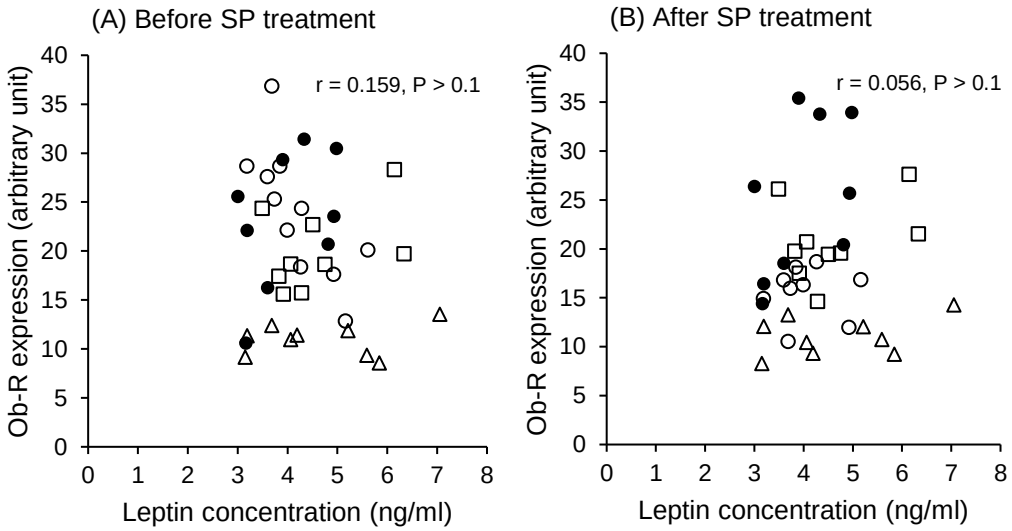

Fig 2-3. Correlation between plasma leptin and endometrial Ob-R before (A) and after (B) PBS or SP infusion. Heifers and control cows were infused with PBS into the vagina on the day of estrus in the second estrous cycle while repeat breeder cows were given SP. Remaining endometrial tissues from EGF assay both before and after infusion of 9 heifers ,9 control cows, and 18 repeat breeder cows were examined for Ob-R expression levels. Different symbols indicate different group of animals: Δ heifers; □ control cows; ○ repeat breeder cows with the normal EGF profile after SP infusion; ● repeat breeder cows with unnormalized EGF profile after SP infu

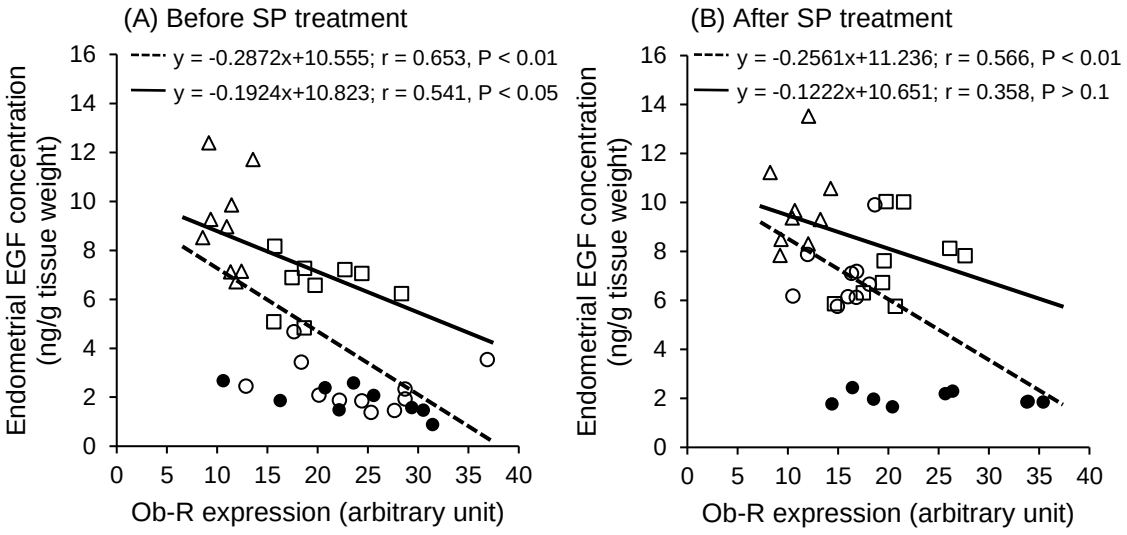

Fig 2-4. Correlation between Ob-R expression and EGF concentration in the endometrial tissue before (A) and after (B) PBS or SP infusion. Endometrial EGF concentrations were examined in all cows twice on the day 3 after estrus, and some cows were selected for examination of Ob-R expression levels using remaining endometrial tissue obtained for EGF assay. Different symbols indicate different group of animals: Δ heifers; □ control cows; ○ repeat breeder cows with the normal EGF profile after SP infusion; ● repeat breeder cows with unnormalized EGF profile after SP infusion. Solid line: linear regression line of fertile animals, broken lines: linear regression line of all animals.

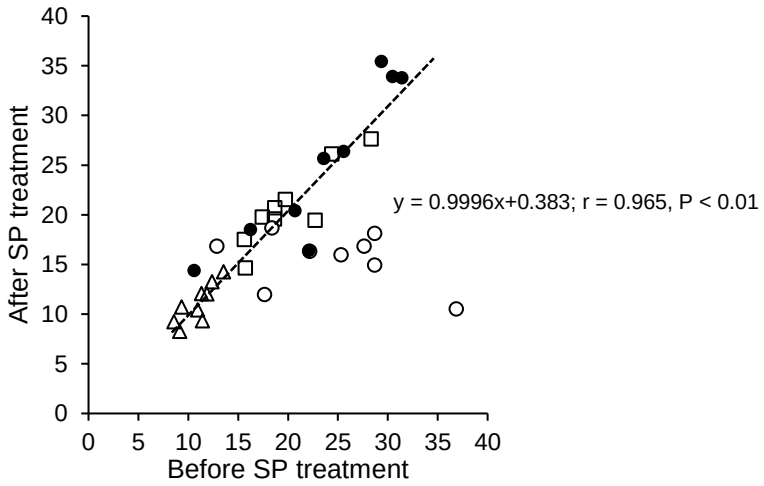

Fig 2- 5. Correlation between endometrial Ob-R before and after SP or PBS infusion. Remaining endometrial tissues obtained for EGF assay were used to examine Ob-R expression of 9 heifers, 9 control cows, and 18 repeat breeder cows. Regression line indicates linear relation between endometrial Ob-R of fertile animals before and after an infusion. Different symbols indicate different group of animals: Δ heifers; □ control cows; ○ repeat breeder cows with the normal EGF profile after SP infusion; ● repeat breeder cows with unnormalized EGF profile after SP infusion

Summary

Factors associated with high milk production levels have been linked to alterations in the endometrial EGF profile, a cause of reduced fertility in dairy cows. Therefore, I examined the leptin system that connects nutritional status and reproduction in dairy cattle related to reduced fertility in repeat breeder cows. Plasma leptin concentrations were measured in 18 heifers, 20 high-yielding control cows, and 26 repeat breeder cows, showing an altered EGF profile. Then, all repeat breeder cows were infused with SP into the vagina at the next estrus to normalize the EGF profile, while heifers and control cows were infused with vehicle alone. All animals were examined for EGF profiles. Eighteen repeat breeder cows, nine heifers, and nine control cows were also determined for leptin receptor (Ob-R) expression levels in the estrous cycle before and after the infusion. SP normalized the EGF profile in 53.8% of the repeat breeder cows. Leptin concentrations were similar in all groups, regardless of the treatment results for the EGF profile. In contrast, Ob-R levels in repeat breeder and control cows were similar and higher than those in heifers before SP treatment. Ob-R in repeat breeders showing a normal EGF profile after treatment decreased to an intermediate level between heifers and control cows and may provide a clue to take measures against repeat breeding in dairy cows.

Summary and conclusions

An abnormality in the EGF profile could be attributed to reduced fertility in high-yielding cows and repeat breeders in commercial dairy herds. The etiology of this abnormality was not well understood, although some metabolic and endocrine changes associated with initiation of lactation have been linked to this abnormality. The present study was conducted to describe recovery process of the cyclic change of the endometrial EGF profile and the factors affecting the recovery in lactating dairy cows during the postpartum period. The study is in two parts: the first part (chapter 1) examined the process of the recovery of the EGF cycle and the factors that affected the recovery of the EGF cycle. In the second part (chapter 2), role of the leptin system in determining response to SP treatment in repeat breeder cows and fertile cattle (heifers and control cows) was investigated.

In chapter 1, the recovery process of the EGF cycle in postpartum dairy cows showing the regular estrous cycle were examined. Firstly, EGF concentrations on day 3 (day 0=estrus) were determined in all cows at every estrous cycle from calving to 90 days postpartum (dpp) or until the cows showed the normal EGF profile in consecutive two estrous cycles (recovery EGF cycle). Then, effects of recovery process of the EGF profile on fertility was determined. I found that the first normal EGF cycle was found in about 75% (338 out of 452 cows) of cows within 60 dpp and about 90% (401 out of 452 cows) by 90 dpp. In contrast, about 40% (192 out of 452 cows) showed the recovery of the EGF cycle. More than 90% of cows showing the first normal EGF profile within 70 dpp conceived by 150 dpp and only less than 10% of them remained open by 180 dpp, while about 30% of cows not showing the normal EGF profile remained open. These results confirmed the association between recovery of the normal EGF profile and fertility in previous studies and indicated that in apparently normal postpartum cows without recovery of the normal EGF profile could be a source of repeat breeder cows in commercial dairy herds.

Then, the factors associated with recovery of EGF cyclicity including parity, BCS at calving, day of the first ovulation, milk yield at peak level, and peak period were investigated. The results indicated that cows with parity lower than 3, BCS at calving over 3.25, and a delay of the first ovulation (> 30 dpp) were less likely to recover the EGF cyclicity. Milk yield at peak greater than 50 kg/day or delaying the day of peak period to later than 60 dpp were also the risk factors for the recovery of EGF cyclicity. These results indicate that physiological changes associated with initiation of lactation may play a role in preventing the endometrial EGF cycle from recovery. The findings provided supportive information to enhance the importance of

periparturient (i.e., feeding to control BCS at calving in optimal range) and postpartum management in improving fertility of dairy herds.

In chapter 2, involvement of the leptin system, a potential linkage of metabolic status to reproduction, was studied. Particularly, potential role of the leptin system in determining the response of repeat breeder cows to treatment aiming at normalizing the EGF profile was examined. The leptin system and the endometrial EGF profile were examined in repeat breeder cows before and after SP treatment. Heifers and fertile cows were given PBS and used as controls. Plasma leptin concentrations were similar in all groups of cattle. The correlation between plasma leptin and endometrial EGF concentrations was not found both before and after SP treatment. The endometrial EGF concentrations and expression of Ob-R were examined in all cattle groups. The expression of Ob-R was lower in heifers than in the cows (both fertile and repeat breeder cows). The Ob-R level in repeat breeder cows showing the normal EGF concentration after SP treatment were at intermediate levels between heifers and repeat breeder cows with unnormalized EGF profile. Altogether, the results indicated that endometrial Ob-R could be at a wide range in fertile animals, and low levels of Ob-R may be advantageous for conception. Further, high levels of Ob-R may prevent repeat breeder cows from normalizing the endometrial EGF profile. Although the role of Ob-R in the regulation of endometrial EGF remained to be studied, the involvement of the leptin system in the local regulatory mechanism of the uterus has been shown.

In this study, I have confirmed association of the recovery of EGF cycle with restoration of fertility and determined risk factors for delaying recovery of the EGF cycle in lactating cows. This analysis revealed potential role of metabolic factors in etiology of abnormality in the EGF profile. Then, the role of leptin system, a linkage of metabolic status and reproduction, in the etiology and, thus, mechanism of normalization of the EGF profile in repeat breeder cows were studied. After treatment of repeat breeder cows, decrease in the endometrial Ob-R expression coincided with normalization of the EGF profile. This may give a clue to improving measures against the abnormality of the EGF profile in repeat breeder cows in dairy herds.

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