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Original Article

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

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Abstract. Factors associated with high milk production levels have been linked to alterations in the endometrial epidermal growth factor (EGF) profile, a cause of reduced fertility in dairy cows. Therefore, we 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 seminal plasma (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.
Key words: Epidermal growth factor (EGF), Leptin, Ob-R, Repeat breeder, Seminal plasma

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An alteration of the endometrial epidermal growth factor (EGF) profile during the estrous cycle causes reduced fertility in repeat breeder and high-yielding cows [1]. 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 [2, 3]. Recently, we reported two types of treatments for this alteration in the endometrium [4–6]. The first is hormonal treatment combined with a high dose of estradiol benzoate and an intravaginal progesterone releasing device [4]. The second is the vaginal infusion of seminal plasma (SP) [5]. Hormonal and SP treatments normalized the endometrial EGF profile in approximately 70% [4] and 60% [5] 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 [1, 7]. 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 [8] and, in turn, increases estradiol and progesterone clearance from the circulation [9]. 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 [10, 11]. 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 [11]. The suppression of reproductive activity by leptin is primarily mediated by the suppression of gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus [11, 12]. However, the expression of *Ob-R* is found in peripheral tissues, including the ovary, uterus, oocyte, and early embryo [13, 14]. The association between single nucleotide polymorphisms (SNPs) in leptin and fertility in heifers has been reported [15]. 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 [13]. However, studies on the role of leptin and *Ob-R* in the uterus have focused on establishing

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pregnancy at implantation or placentation phases in rodents and humans [16–18]. 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 [13]. 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 [13]. *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 [19]. The lowest expression of *Ob-R* coincides with the first peak of endometrial EGF concentrations [2, 3]. 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 one of the authors before enrollment in the study, as described previously [5]. Normal and repeat breeder cows were multiparous lactating (> 10,000 kg, 305-day 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 [2, 3]. 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 [5, 20]. 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 [5]. The intra- and inter-assay coefficients of variation (CV) were 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 [3, 20].

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 [21]: 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°C for 2 min (Uracil N-glycosylase activation), 1 cycle at 95°C for 10 min (DNA polymerase activation) followed by 40 cycles at 95°C for 15 sec (denaturation) and 60°C 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 [5]. 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°C, and transported to Hokkaido University at –20°C. At the university laboratory, all frozen SP samples were thawed and centrifuged at 5,000 × *g* for 20 min at 4°C, 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°C.

Infusion of SP into the vagina and the uterus

SP was infused into the vagina of repeat breeder cows, as described previously [5]. 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. 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 1). Plasma leptin levels were not correlated with EGF concentrations (Fig. 2) or *Ob-R* levels (Fig. 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. 4). Heifers showed higher EGF concentrations and lower *Ob-R* levels than control cows ($P < 0.01$) (Table 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

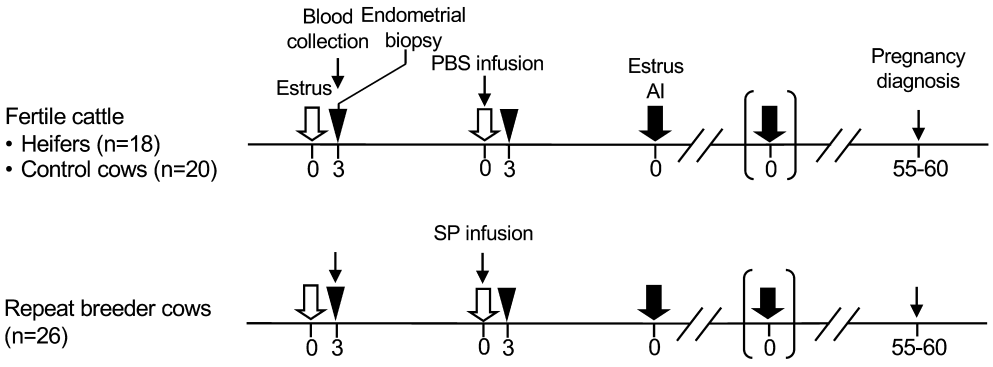

Fig. 1. A schematic diagram of seminal plasma (SP) treatment for study. In all cattle recruited for the present study, endometrial tissues were obtained for the EGF assay by biopsy on day 3 of the estrous cycle (day 0 = estrus). Twenty-six repeat breeder cows with an altered EGF profile, 18 heifers, 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 artificial insemination (AI) up to two times and pregnancy was diagnosed between 55–60 days after the last AIs.

Table 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 ($P < 0.05$).

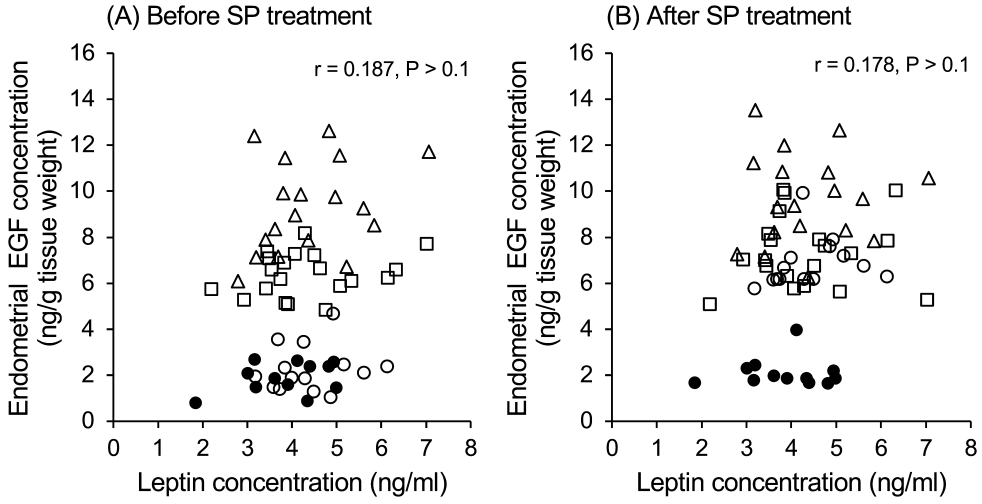

Fig. 2. Correlation between plasma leptin and EGF concentration before (A) and after (B) PBS or SP infusion. Endometrial tissues were biopsied on day 3 of estrous cycle from all cows twice, before and after intra-vaginal infusion with PBS or SP in fertile animals (heifers and control cows) and repeat 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.

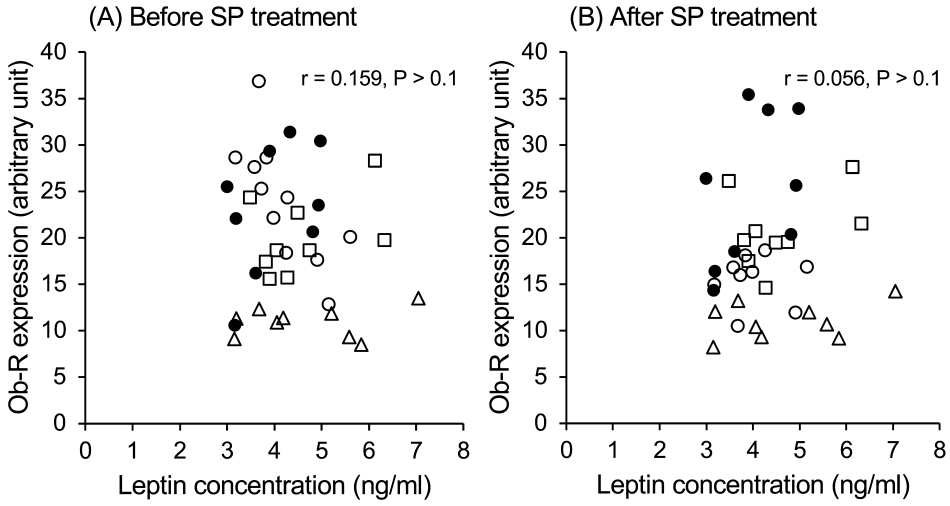

Fig. 3. Correlation between plasma leptin and endometrial *Ob-R* before (A) and after (B) PBS or SP infusion. Heifers and control cows were infused with PBS into the vagina on the day of estrus in the second estrous cycle while repeat breeder cows were given SP. Remaining endometrial tissues from EGF 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 infusion.

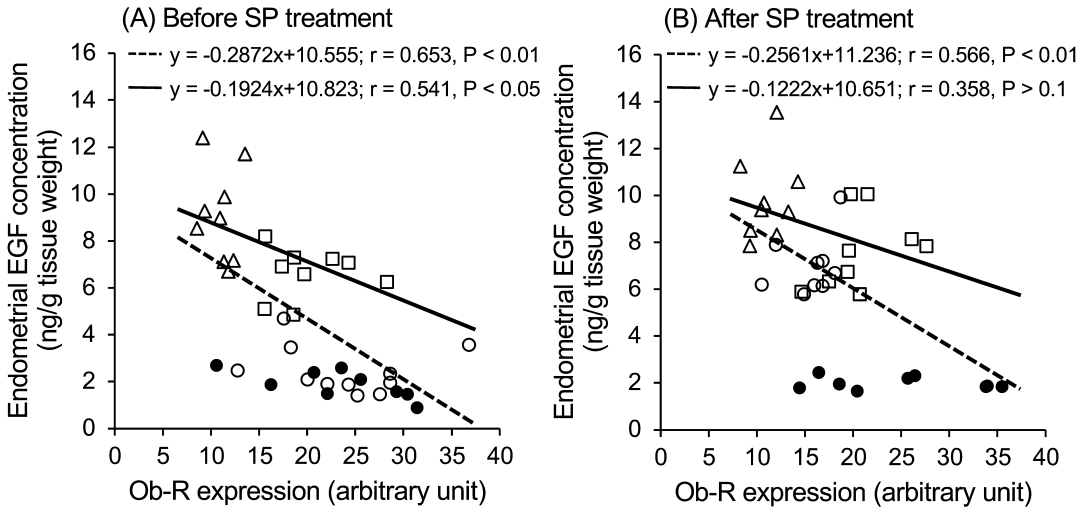

Fig. 4. Correlation between *Ob-R* expression and EGF concentration in the endometrial tissue before (A) and after (B) PBS or SP infusion. Endometrial EGF concentrations were examined in all cows twice on the day 3 after estrus, and some cows were selected for examination of *Ob-R* expression levels 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.

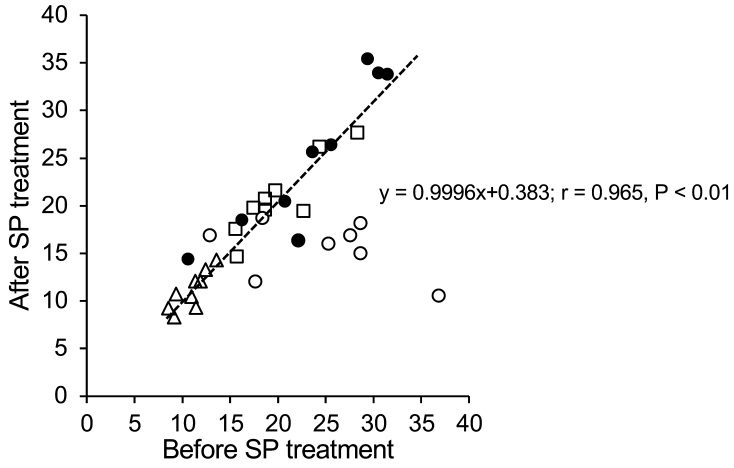

Fig. 5. Correlation between endometrial *Ob-R* before and after SP or PBS infusion. Remaining endometrial tissues obtained for EGF assay were used to examine *Ob-R* expression of 9 heifers, 9 control cows, and 18 repeat breeder cows. Regression line indicates linear relation between endometrial *Ob-R* 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.

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. 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 ($r = 0.965$, $P < 0.01$). However, the normalization of the EGF profile after SP treatment, associated with restoration of fertility [5, 6], 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.

We 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 [2, 3], and *Ob-R* expression was low. In cyclic heifers, *Ob-R* in the metestrus (day 5) was at the lowest level and showed an almost two-fold increase in diestrus (day 12) [19]. 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 [8, 9]. Similar changes in plasma estradiol levels have been reported in repeat breeder cows [1]. 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 [22–24]. 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 [22, 23]. 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 [23] 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 [25]; this can also be explained by the different lactation stages of the cows used in the two studies. A previous study [25] 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.

Conflict of interests: The authors declare that there are no conflicts of interest related to this study.

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