Journal of Reproduction and Development, Vol. 54, No. 1, 2008

—Research Note—

Effect of Suckling on the Reproductive Performance and Metabolic Status of Obese Japanese Black Cattle during the Early Postpartum Period

Chiho KAWASHIMA[1)], Katsuya KIDA[1)], Miki MATSUHASHI[2)], Motozumi MATSUI[3)], Takashi SHIMIZU[2)], Nobuyoshi MATSUNAGA[5)], Mitsuo ISHII[4)], Yoh-Ichi MIYAKE[3)] and Akio MIYAMOTO[2)]

[1)]Field Center of Animal Science and Agriculture, [2)]Graduate School of Animal and Food Hygiene, [3)]Department of Clinical Veterinary Science, [4)]Research Center for Animal Hygiene and Food Safety and [5)]Department of Agricultural and Life Science, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-8555, Japan

Abstract. The aim of our study was to investigate the effects of suckling on reproductive performance and metabolic status of obese (mean body condition score of more than 4.0 on a scale of 1–5) maternal Japanese Black cows during early postpartum period. We used 7 postpartum Japanese Black cattle. Four cows were suckled *ad libitum* (suckled) until completion of their first artificial insemination (AI), while 3 cows were not suckled at all because they were separated from their calves immediately after parturition (non-suckled). Body weight and plasma concentrations of metabolites and hormones were measured from wk 1 to 9 postpartum. Ovarian activity was detected using plasma progesterone concentration, and all cows received their first AI after application of the Ovsynch protocol at approximately 4 months postpartum. Although body weights of non-suckled cows increased during experimental period ($P<0.05$), those of suckled cows remained unchanged. Plasma concentrations of glucose of non-suckled cows were higher at wk 2 postpartum ($P<0.05$) and their levels of non-esterified fatty acid tended to be lower at wk 1 and 2 postpartum compared with suckled cows ($P<0.1$); however, these differences between groups were not observed with progression of postpartum period. In addition, plasma insulin concentrations of non-suckled cows were higher than those of suckled cows during experimental period ($P<0.05$). During sampling period (wk 0 to 9 postpartum), onset of normal ovarian cycle was observed in all non-suckled and 2 of 4 suckled cows, and it was delayed in other 2 suckled cows compared with non-suckled cows; however, 3 suckled cows conceived at the first AI after application of the Ovsynch protocol; none of non-suckled cows conceived at this time. Overall, we suggest that suckling seems to reduce increase of body weight after parturition, although it does not improve obesity, and influences conception despite delay in resumption of normal ovarian cyclicity in obese Japanese Black cows.

Key words: Japanese Black cattle, Metabolic status, Obesity, Reproductive performance, Suckling

(**J. Reprod. Dev. 54:** 46–51, 2008)

Suckling reduces release of gonadotropin-releasing hormone (GnRH) [1, 2], secretion of luteinizing hormone (LH) [3, 4] and LH pulses [5], and therefore suckled cows have an extended period of anovulation and anestrous after parturition [6, 7]. In addition, their energy status also influences reproductive performance. Restricted energy intake, which is induced by pregnancy, parturition, onset of lactation and the stress of suckling, during the peripartum period induces reduced reproductive performance [8]. On the other hand, obesity in beef cows is associated with insulin resistance [9]. Insulin resistance may induce anestrus because it is an important factor in ovarian activity [10–12]. In beef cows, reduced reproductive performance is directly linked to decreased economical efficiency of the herd. Based on energy status and reproductive performance, we hypothesize that suckling may help obese beef cows to recover from their obese condition after parturition. The effects of suckling on the reproductive performance and energy status of obese maternal Japanese Black cows are unclear because this situation would not normally occur in maternal beef cows under standard farming conditions. Therefore, the aim of the present study was to investigate whether suckling influences the reproductive performance and metabolic status of obese maternal Japanese Black cows during the early postpartum period.

Accepted for publication: October 3, 2007
Published online: November 13, 2007
Correspondence: K. Kida (e-mail: kidak@obihiro.ac.jp)

Materials and Methods

The experimental procedures complied with the Guide for Care and Use of Agricultural Animals of Obihiro University.

Animals

This experiment was carried out at the Field Center of Animal Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine. We used 7 postpartum Japanese Black cattle (5 primiparous and 2 multiparous cows, parity: 1.4 ± 0.7) between June and October 2006. All cows calved toward the end of June. Body weight, body height and body condition score at wk 1 postpartum were 560.6 ± 97.3 kg, 132.4 ± 4.6 cm and 4.3 ± 0.3, respectively. The cows were classified into two groups (suckled

cows, n=4; non-suckled cows, n=3). They were suckled *ad libitum* from parturition to completion of their first artificial insemination (AI). The non-suckled cows were immediately separated from their calves after calving. We collected samples from wk 1 to 9 postpartum, with the period of 0–6 days after parturition regarded as wk 1 postpartum. In October, we confirmed the presence of corpora lutea in the ovaries of all the cows, and they received their first AI after application of the Ovsynch protocol (d 0, GnRH; d 7, $PGF_{2\alpha}$; d 9, GnRH; d 10, AI) at approximately 4 months postpartum. The cows and calves were housed in a paddock, offered timothy hay (54.1% DM for TDN and 12.5% DM for CP) and grazed from 0900 to 1600 h during the experimental period.

Sampling

Body weight was measured once a week from wk 1 to 9 postpartum using a scale. Blood samples were obtained by caudal venipuncture after measurement of body weight using heparinized 5 ml tubes (Venoject II, VP-H050K; Terumo, Tokyo, Japan) for biochemical analysis once a week and using sterile 10 ml tubes containing 200 μl of stabilizer solution (0.3 M EDTA and 1% acetyl salicylic acid, pH 7.4) for hormonal analysis twice weekly during the same period. These tubes were centrifuged at 3,000 rpm for 20 min at 4 C, and the plasma samples were kept at –30 C until biochemical and hormonal analyses.

Definition of ovarian activity

When a cow's plasma progesterone (P_4) concentration increased to more than 1 ng/ml, the cow was confirmed as having returned to luteal activity [13]. Luteal phases were considered normal if the plasma P_4 concentration for at least 3 time points (more than 7 days) was more than 1 ng/ml and at least 2 of these time points were more than 2 ng/ml [14]. An ovarian cycle was considered normal if its luteal phase was normal [14], and the day the plasma P_4 concentration increased to more than 1 ng/ml was considered the day of initiation of a normal ovarian cycle.

Measurement of P_4, growth hormone (GH), insulin-like growth factor-I (IGF-1) and insulin

Plasma P_4 concentrations were determined by enzyme immunoassay (EIA) after extraction using diethyl ether as described previously [15]; the extraction efficiency was 93%. The standard curve ranged from 0.05 to 50 ng/ml, and the ED_{50} of the assay was 3.2 ng/ml. The mean intra- and interassay coefficients of variation (CVs) were 6.7 and 7.2%, respectively.

The plasma GH, IGF-1 and insulin concentrations were determined by EIA using the biotin-streptavidin amplification technique. The GH concentration was measured by EIA as described previously [16]. The standard curve ranged from 0.78 to 100 ng/ml, and the ED_{50} of this assay system was 6.2 ng/ml. The intra- and interassay CVs were 8.1 and 8.5%, respectively. The total plasma IGF-1 concentration was determined by EIA [12] after protein extraction using acid-ethanol (87.5% ethanol and 12.5% 2 N hydrochloric acid) to obtain IGF-1 free from binding proteins [17]. The IGF-1 standard curve ranged from 0.39 to 50 ng/ml. The intra- and interassay CVs were 5.7 and 6.6%, respectively, and the ED_{50} of this assay system was 2.5 ng/ml. Insulin concentrations were determined by EIA [12]. The standard curve ranged from 39 to 5,000 pg/ml. The intra- and interassay CVs were 9.7 and 14.5%, respectively, and the ED_{50} of this assay system was 800 pg/ml.

Biochemical analyses

In each sample, the concentrations of glucose, total cholesterol (T-cho), non-esterified fatty acid (NEFA), 3-hydroxybutyric acid (3HB), aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (γ-GTP) were measured using a clinical chemistry automated analyzer (TBA120FR, Toshiba Medical Systems, Otawara, Japan).

Statistical analysis

All biochemical, hormonal, and body weight data were analyzed by repeated measures ANOVA. There was interaction between group and time for body weight, glucose, NEFA and IGF-1 ($P<0.05$); therefore, mean values of these data were calculated for each group and each sampling period, and significant differences were analyzed by Student's *t*-test. For other data, no significant interaction between group and time was observed. For the interaction of normal ovarian cycles, we were unable to identify any significant differences because less than three suckled cows resumed normal ovarian cyclicity during the experimental period. The results are expressed as the mean ± standard deviation (SD). Differences of $P<0.05$ were considered significant.

Results

Body weight and metabolic status of the suckled and non-suckled cows during the sampling period

Fig. 1 shows the actual body weights, changes of body weight when the body weight during wk 1 postpartum was regarded as 100% and body condition scores of the suckled and non-suckled cows throughout the sampling period. The actual body weights and body condition scores did not differ between the two groups because the body size of each cow varied. However, the body weights of the non-suckled cows when body weight during wk 1 postpartum was regarded as 100% were greater than those of the suckled cows throughout the experimental period ($P<0.05$). Although the body weights of the non-suckled cows when body weight during wk 1 postpartum was regarded as 100% increased with progression of the postpartum period ($P<0.05$), those of the suckled cows were unchanged during the sampling period. The increase in body weight from wk 1 postpartum to the first AI after application of the Ovsynch protocol for the suckled and non-suckled cows were 5.0 ± 3.9 and 29.0 ± 11.4 kg, respectively ($P<0.05$, Table 1).

The concentrations of metabolites and metabolic hormones are shown in Figs. 2 and 3, respectively. The plasma concentrations of glucose of the non-suckled cows were higher at wk 2 postpartum than those of the suckled cows ($P<0.05$), and the plasma NEFA concentrations of the suckled cows tended to be higher than those of the non-suckled cows at wk 1 and 2 postpartum ($P<0.1$); however, no differences were observed between the suckled and non-suckled cows with progression of the postpartum period. The levels of other metabolites were the same for the suckled and non-

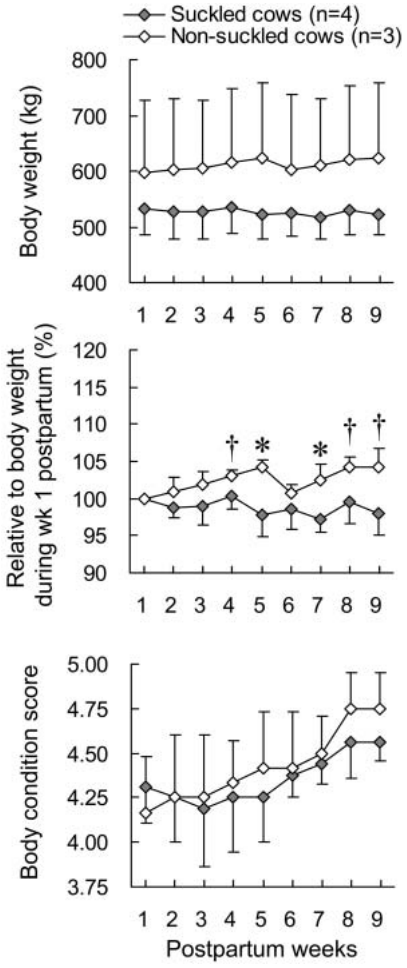

Fig. 1. Change of actual body weight, body weight when body weight at wk 1 postpartum was regarded as 100 % and body condition score in suckled (n=4) and non-suckled (n=3) cows during the sampling period (mean ± SD). There was interaction between group and time ($P<0.05$) for the change of body weight when body weight at wk 1 postpartum was regarded as 100%. The body weights of the non-suckled cows when body weight at wk 1 postpartum was regarded as 100% increased with progression of the postpartum period ($P<0.05$), and that of the suckled cows was unchanged during the experimental period. The symbols * and † indicate differences of $P<0.05$ and $P<0.1$ between the suckled and non-suckled cows, respectively.

suckled cows throughout the sampling period (Fig. 2). The plasma concentrations of IGF-1 of the non-suckled cows at wk 2 postpartum were higher than those of the suckled cows, although the plasma IGF-1 concentrations at the end of the sampling period were higher in the suckled cows than in the non-suckled cows (Fig. 3). In addition, the plasma insulin concentrations were higher in the non-suckled cows than in the suckled cows throughout the sampling period ($P<0.05$, Fig. 3). The plasma concentrations of GH were similar between the suckled and non-suckled cows (Fig. 3).

Resumption of ovarian cycles and conception at the first AI after application of the Ovsynch protocol in the suckled and non-suckled cows

Two suckled cows and 3 non-suckled cows resumed normal ovarian cycles during the sampling period [wk 0 to 9 postpartum (Fig. 4)]. The weeks of initiation of normal ovarian cycles for the 2 suckled cows and 3 non-suckled cows were 7.0 ± 0.5 and 3.3 ± 0.2 weeks postpartum, respectively (Table 1). All cows received their first AI after application of the Ovsynch protocol, and the mean number of days to first service was 117.3 ± 3.4 days postpartum. Consequently, conception was confirmed for 3 suckled, but no non-suckled, cows at their first AI after application of the Ovsynch protocol (Table 1).

Discussion

This study investigated the effects of suckling on the reproductive performance and metabolic status of obese (mean body condition score of more than 4.0) maternal Japanese Black cows during the early postpartum period. Our data showed that the energy status, indicated by the plasma concentrations of glucose and NEFA, of the suckled cows was lower than that of the non-suckled cows at the several time points during the early postpartum period, although this difference disappeared with progression of the postpartum period. Under such an energy status, our data also showed that the body weights of the non-suckled cows increased gradually after calving, whereas they did not change in the suckled cows.

In Japanese Black cows, secretion of GH and insulin remains essentially unchanged between the lactation and non-lactation periods because Japanese Black cows produce only one-tenth the total milk production of Holstein cows [18]. On the other hand, a previous study showed that the plasma NEFA concentrations of suckled cows fed diets supplying 100% of their energy requirements did not change after calving and that suckled cows fed diets supplying 70% of their energy requirements had a low energy status (higher NEFA, and lower glucose and insulin) compared with cows fed sufficient energy [19]. Although we did not measure the actual energy intake of individual cows in this study, the intake of suckled cows may be restricted by suckling; therefore, we surmised that the energy status during the early postpartum period was low in the suckled cows compared with the non-suckled cows.

We observed that the plasma IGF-1 concentrations of the non-suckled cows were higher at wk 2 postpartum and lower at the end of the experimental period compared with the suckled cows. The plasma concentration of IGF-1 is changed by acute feed intake [20–22] and the estrous cycle [14, 23]. The energy status of the non-suckled cows at wk 2 postpartum was better than that of the suckled cows in this study. Moreover, onset of luteal activity in the non-suckled cows was confirmed at wk 3.3 postpartum. Therefore, we surmised that the higher energy status and timing of ovulation influenced the higher plasma IGF-1 concentration at wk 2 postpartum in the non-suckled cows, although there were no clear differences in the plasma concentrations of IGF-1 at the end of the experimental period between the two groups.

A previous study reported that LH pulse activity decreases in

Table 1. Initiation of normal ovarian cycles during the experimental period, conception and increase of body weight from wk 1 postpartum to the first AI after application of the Ovsynch protocol in suckled and non-suckled cows

	Suckled cows (n=4)	Non-suckled cows (n=3)
Number of cows resuming normal ovarian cycles during the experimental period	2/4	3/3
Initiation of normal ovarian cycles (week)	7.0 ± 0.5†	3.3 ± 0.2
Number of cows with confirmed the conception at the first AI after application of the Ovsynch protocol	3/4	0/3
Increase of body weight from wk 1 postpartum to the first AI after application of the Ovsynch protocol (kg)	5.0 ± 3.9[a]	29.0 ± 11.1[b]

Values are means ± SD. a, b: Differences of P<0.05 between the suckled and non-suckled cows. †: Average data for 2 cows resuming normal ovarian cycles during the exprimental period.

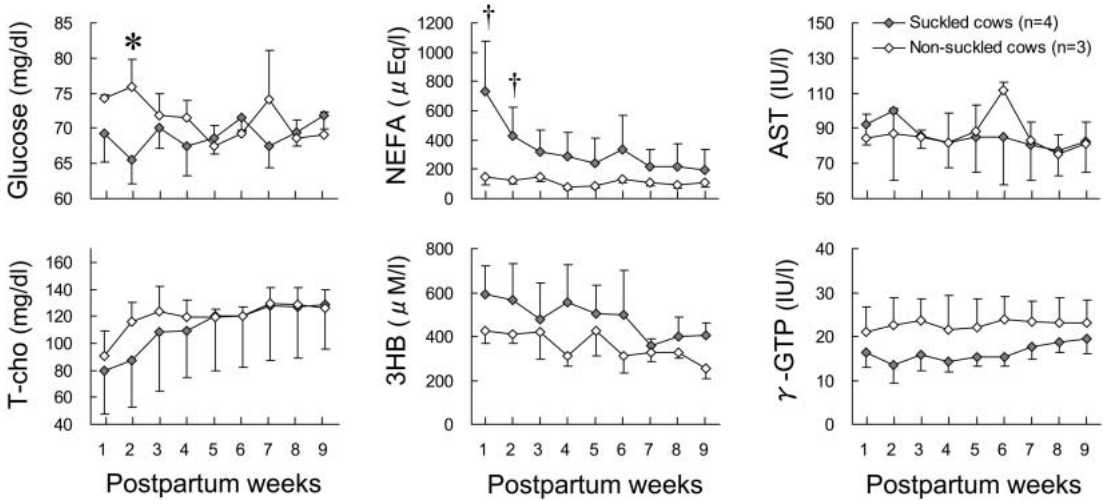

Fig. 2. Metabolites of the suckled (n=4) and non-suckled (n=3) cows during the sampling period (mean ± SD). A significant interaction between group and time was observed for glucose and NEFA (P<0.05). The symbols * and † indicate differences of P<0.05 and P<0.1 between the suckled and non-suckled cows, respectively. There were no differences in the other metabolites between the suckled and non-suckled cows.

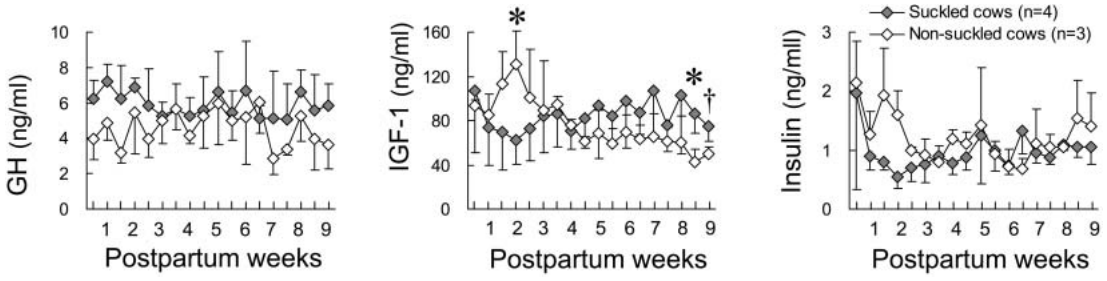

Fig. 3. Metabolic hormones of the suckled (n=4) and non-suckled (n=3) cows during the sampling period (mean ± SD). A significant interaction between group and time was observed for IGF-1 (P<0.05). The symbols * and † indicate differences of P<0.05 and P<0.1 between the suckled and non-suckled cows, respectively. The plasma insulin concentrations of the non-suckled cows were higher than those of the suckled cows during the sampling period (P<0.05). The plasma GH concentration did not differ between the suckled and non-suckled cows.

suckled rather than non-suckled postpartum cows [6] because suckling suppresses pulsatile LH release by inhibiting GnRH discharge from the hypothalamus [1, 2]. Therefore, resumption of ovarian activity in suckled cows is delayed compared with non-suckled cows [6, 7]. In the present study, onset of a normal ovarian cycle was observed in all non-suckled and 2 of 4 suckled cows, and initiation of a normal ovarian cycle was delayed in the other 2 suckled cows compared with the non-suckled cows. This data supports the results of previous studies [6, 7], although we did not measure the LH pulse in this study. However, conception was confirmed in 3 suckled, but no non-suckled, cows at the first AI after application of the Ovsynch protocol, although we confirmed the presence of a corpus luteum and carried out the first AI after application of the Ovsynch protocol. In the present study, the body weights of the

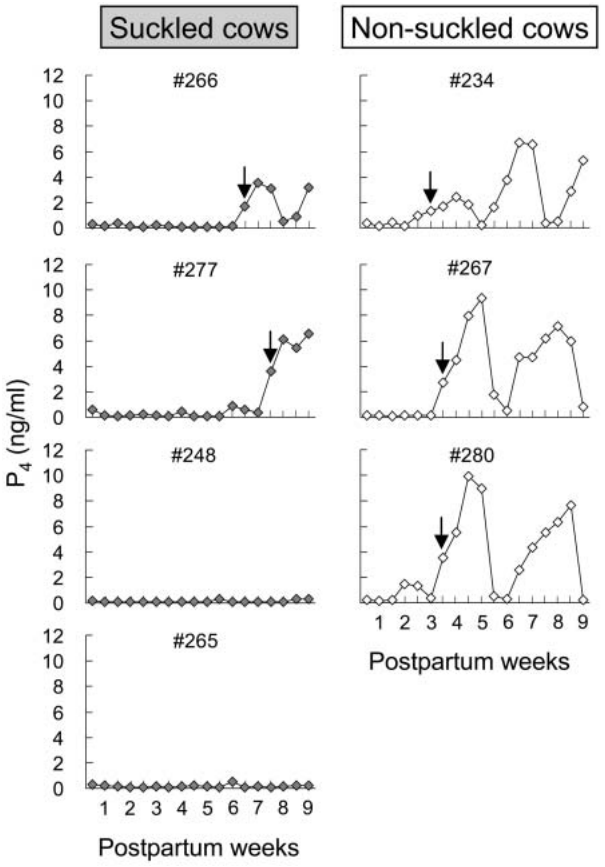

Fig. 4. Change in the P_4 concentrations of the suckled (n=4) and non-suckled (n=3) cows during the sampling period (solid, suckled; open, non-suckled). Arrows indicate the date of resumption of a normal ovarian cycle ($P_4 \geq 1$ ng/ml). Onset of a normal ovarian cycle was observed for all non-suckled and 2 of 4 suckled cows; however, the other 2 suckled cows did not show onset of a normal ovarian cycle during the sampling period.

non-suckled cows increased after parturition, and their body weights at the first AI after application of the Ovsynch protocol increased by 29.0 ± 11.4 kg compared with those at wk 1 postpartum. On the other hand, the body weights of the suckled cows increased by 5.0 ± 3.9 kg. In addition, the plasma insulin concentrations were higher in the non-suckled cows than in the suckled cows during the experimental period. The plasma insulin concentration is high in obese cows, and obesity is associated with insulin resistance [9]. Abnormal insulin secretion from the pancreas may reduce ovarian function because insulin is a stimulators of secretion of estradiol-17β in granulosa cells [10, 11] and of proliferation of follicular cells [24, 25]. Therefore, we hypothesized that conception was prevented in the non-suckled cows by their greater obesity and higher insulin secretion compared with the suckled cows. In addition, uterine involution proceeds more rapidly in suckled cows than in non-suckled cows [7]. Thus, a difference in uterine involution after parturition between the suckled and non-suckled cows may have affected the conception in this study. Further studies are necessary to investigate the effects of obesity on metabolic status, such as insulin resistance, the mechanisms of infertility and the effects of suckling on uterine involution in obese Japanese Black cows.

In conclusion, we suggest that suckling seems to reduce increase of body weight after parturition, although it does not improve obesity, and influence conception despite a delay in resumption of normal ovarian cyclicity in obese Japanese Black cows.

Acknowledgments

The authors thank Dr. K. Okuda (Okayama University, Japan) for P_4 antiserum; Dr. D. Schams (TU-Munich Weihenstephan, Germany) for insulin antiserum; and Dr. Parlow (NIDDK) for GH for use as the standard and tracer and IGF-1 antiserum. This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, the 21st Century COE Program (A-1) of the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Secure and Healthy Livestock Farming Project of the Ministry of Agriculture, Forestry and Fisheries, and the Japan Livestock Technology Association. C. Kawashima was supported by the COE Program.

References

1. **Zalesky DD, Forrest DW, McArthur NH, Wilson JM, Morris DL, Harms PG**. Suckling inhibits release of luteinizing hormone-releasing hormone from the bovine median eminence following ovariectomy. *J Anim Sci* 1990; 68: 444–448.
2. **Williams GL, Gazal OS, Guzman Vega GA, Stanko RL**. Mechanisms regulating suckling-mediated anovulation in the cow. *Anim Reprod Sci* 1996; 42: 289–297.
3. **Lamming GE, Wathes DC, Peters AR**. Endocrine patterns of the post-partum cow. *J Reprod Fertil Suppl* 1981; 30: 155–170.
4. **Edwards S**. The effects of short term calf removal on pulsatile LH secretion in the postpartum beef cow. *Theriogenology* 1985; 23: 777–785.
5. **Garcia-Winder M, Imakawa K, Day ML, Zalesky DD, Kittok RJ, Kinder JE**. Effect of suckling and ovariectomy on the control of luteinizing hormone secretion during the postpartum period in beef cows. *Biol Reprod* 1984; 31: 771–778.
6. **Williams GL**. Suckling as a regulator of postpartum rebreeding in cattle: a review. *J Anim Sci* 1990; 68: 831–852.
7. **Yavas Y, Walton JS**. Postpartum acyclicity in suckled beef cows: a review. *Theriogenology* 2000; 54: 25–55.
8. **Ciccioli NH, Wettemann RP, Spicer LJ, Lents CA, White FJ, Keisler DH**. Influence of body condition at calving and postpartum nutrition on endocrine function and reproductive performance of primiparous beef cows. *J Anim Sci* 2003; 81: 3107–3120.
9. **McCann JP, Reimers TJ**. Effects of obesity on insulin and glucose metabolism in cyclic heifers. *J Anim Sci* 1986; 62: 772–782.
10. **Armstrong DG, Gong JG, Webb R**. Interactions between nutrition and ovarian activity in cattle: physiological, cellular and molecular mechanisms. *Reprod Suppl* 2003; 61: 403–414.
11. **Butler ST, Pelton SH, Butler WR**. Insulin increases 17beta-estradiol production by the dominant follicle of the first postpartum follicle wave in dairy cows. *Reproduction* 2004; 127: 537–545.
12. **Kawashima C, Fukihara S, Maeda M, Kaneko E, Amaya Montoya C, Matsui M, Shimizu T, Matsunaga N, Kida K, Miyake Y-I, Schams D, Miyamoto A**. Relationship between metabolic hormones and ovulation of dominant follicle during the first follicular wave postpartum in high-producing dairy cows. *Reproduction* 2007; 133: 155–163.
13. **Stevenson JS, Britt JH**. Relationships among luteinizing hormone, estradiol, progesterone, glucocorticoids, milk yield, body weight and postpartum ovarian activity in Holstein cows. *J Anim Sci* 1979; 48: 570–577.
14. **Kawashima C, Kida K, Hayashi KG, Amaya Montoya C, Kaneko E, Matsunaga N, Shimizu T, Matsui M, Miyake Y-I, Schams D, Miyamoto A**. Changes in plasma metabolic hormone concentrations during the ovarian cycles of Japanese Black and Holstein cattle. *J Reprod Dev* 2007; 53: 247–254.
15. **Miyamoto A, Okuda K, Schweigert FJ, Schams D**. Effects of basic fibroblast growth factor, transforming growth factor-beta and nerve growth factor on the secretory func-

tion of the bovine corpus luteum *in vitro*. *J Endocrinol* 1992; 135: 103–114.

16. **Kawashima C, Sakaguchi M, Suzuki T, Sasamoto Y, Takahashi Y, Matsui M, Miyamoto A**. Metabolic profiles in ovulatory and anovulatory primiparous dairy cows during the first follicular wave postpartum. *J Reprod Dev* 2007; 53: 113–120.
17. **Daughaday WH, Mariz IK, Blethen SL**. Inhibition of access of bound somatomedin to membrane receptor and immunobinding sites: a comparison of radioreceptor and radioimmunoassay of somatomedin in native and acid-ethanol-extracted serum. *J Clin Endocrinol Metab* 1980; 51: 781–788.
18. **Shingu H, Hodate K, Kushibiki S, Ueda Y, Watanabe A, Shinoda M, Matsumoto M**. Breed differences in growth hormone and insulin secretion between lactating Japanese Black cows (beef type) and Holstein cows (dairy type). *Comp Biochem Physiol C Toxicol Pharmacol* 2002; 132: 493–504.
19. **Grimard B, Humblot P, Ponter AA, Mialot JP, Sauvant D, Thibier M**. Influence of postpartum energy restriction on energy status, plasma LH and oestradiol secretion and follicular development in suckled beef cows. *J Reprod Fertil* 1995; 104: 173–179.
20. **Enright WJ, Spicer LJ, Prendiville DJ, Murphy MG, Campbell RM**. Interaction between dietary intake and ovariectomy on concentrations of insulin-like growth factor-I, GH and LH in plasma of heifers. *Theriogenology* 1994; 41: 1231–1240.
21. **Armstrong DG, McEvoy TG, Baxter G, Robinson JJ, Hogg CO, Woad KJ, Webb R, Sinclair KD**. Effect of dietary energy and protein on bovine follicular dynamics and embryo production *in vitro*: associations with the ovarian insulin-like growth factor system. *Biol Reprod* 2001; 64: 1624–1632.
22. **Taylor VJ, Cheng Z, Pushpakumara PG, Beever DE, Wathes DC**. Relationships between the plasma concentrations of insulin-like growth factor-I in dairy cows and their fertility and milk yield. *Vet Rec* 2004; 155: 583–588.
23. **Darwash AO, Lamming GE, Woolliams JA**. Estimation of genetic variation in the interval from calving to postpartum ovulation of dairy cows. *J Dairy Sci* 1997; 80: 1227–1234.
24. **Spicer LJ, Alpizar E, Echternkamp SE**. Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production *in vitro*. *J Anim Sci* 1993; 71: 1232–1241.
25. **Spicer LJ, Stewart RE**. Interactions among basic fibroblast growth factor, epidermal growth factor, insulin, and insulin-like growth factor-I (IGF-I) on cell numbers and steroidogenesis of bovine thecal cells: role of IGF-I receptors. *Biol Reprod* 1996; 54: 255–263.