Relationship between metabolic hormones and ovulation of dominant follicle during the first follicular wave *post-partum* in high-producing dairy cows

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Abstract

Recent studies suggest that IGF-I is a crucial regulatory factor in follicular growth during early *post-partum* period. The aim of the present study was to determine in detail the changing profiles of metabolic and reproductive hormones in relation to ovulation of the dominant follicle (DF) of the first follicular wave *post-partum* in high-producing dairy cows. Plasma concentrations of related hormones in 22 multiparous Holstein cows were measured from 4 weeks pre-partum to 3 weeks *post-partum*, and the development of DF was observed with colour Doppler ultrasound. Thirteen cows showed ovulation by 15.2 days *post-partum*. Anovulatory cows showed higher GH and lower IGF-I levels than those in ovulatory cows during the peri-partum period. Each DF developed similarly, and a clear blood flow in the follicle wall was observed despite ovulation or anovulation. In addition, detailed endocrine profiles were analyzed in 9 out of the 22 cows. Five cows showed an increase in plasma oestradiol-17 β (E2) with follicular growth followed by E2 peak, LH surge and ovulation. In these cows, plasma IGF-I concentrations remained high until 10 days *post-partum* followed by a gradual decrease. Subsequently, the insulin level increased together with the E2 peak towards ovulation. These profiles were not observed in anovulatory cows. In conclusion, our data strongly support the concept that IGF-I and insulin represent 'metabolic signals' of the resumption of ovarian function *post-partum* in high-producing dairy cows. Moreover, we provide the first visual evidence that both ovulatory and anovulatory DFs of the first follicular wave *post-partum* are similarly supplied with active blood flow.

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Introduction

The negative energy balance early *post-partum* that is characterized by the loss of body weight and mobilization of body fat stores does not affect the first follicular wave *post-partum* (Beam & Butler 1999). In fact, the medium-sized follicles appear 5 days after calving and grow into large follicles at 10 days *post-partum* in most dairy cows (Savio *et al.* 1990a, 1990b). However, almost a half of these follicles are not able to ovulate and become atretic or cystic. Most ovulations are observed within 3 weeks postpartum (Lamming & Bulman 1976, Lucy *et al.* 1992b, Darwash *et al.* 1997). Our recent study showed that the recovery of ovarian function and subsequent reproductive performance in ovulatory cows within 3 weeks *post-partum* was superior to that of

anovulatory cows (Kawashima *et al.* 2006). It is generally accepted that the cow with early resumption of the ovarian function has higher fertility (Staples *et al.* 1990, Senatore *et al.* 1996, Darwash *et al.* 1997). However, the factors that control the first ovulation *post-partum* have not been fully elucidated.

Insulin and insulin-like growth factor-I (IGF-I), metabolic hormones that are changed by feed intake, stimulate oestradiol-17 β (E2) production in the granulosa cells (Gutierrez *et al.* 1997, Glister *et al.* 2001, Armstrong *et al.* 2003, Butler *et al.* 2004) and proliferation of follicular cells (Spicer *et al.* 1993, Spicer & Stewart 1996). The growth of ovulatory follicles is regulated by metabolic hormones such as IGF-I or insulin and is susceptible to the endocrine and metabolic status during early lactation (Spicer *et al.* 1990, Lucy

et al. 1992b). The liver-derived IGF-I is a factor that regulates the final maturation of the dominant follicle (DF) during the first follicular wave *post-partum* (Beam & Butler 1998). The circulating IGF-I in ovulatory cows at the first follicular wave *post-partum* was higher than that in anovulatory cows (Beam & Butler 1998).

Most of the above studies investigated a few metabolic factors during the first follicular wave *post-partum*. Therefore, the aim of the present study was to investigate the time-dependent relationship in detail among metabolic hormones, including IGF-I, growth hormone (GH) and insulin, and metabolites such as glucose, non-esterified fatty acids (NEFA), total cholesterol (T-cho) and aspartate aminotransferase (AST), as well as the body condition score (BCS) and ovulation during the first follicular wave *post-partum* in a homogeneous herd at early lactation period in high-producing dairy cows. In addition, we used colour Doppler ultrasonography to monitor local blood flow during the growth of individual follicles.

Materials and Methods

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

Animals

The experiment was carried out in the Field Centre of Animal Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine. We used 22 multiparous Holstein cows that calved between October 2004 and April 2005. We collected samples from 4 weeks pre-partum to 3 weeks *post-partum*, with the period of 0–6 days after parturition regarded as a parturient week (week 0). Additionally, 9 out of the 22 cows that calved between February 2005 and April 2005 were used to observe the competence of the DF in detail; we collected blood samples (4 times/day) from the day of the appearance of a DF (8.5 mm in diameter) to 7 days after the first ovulation (ovulatory cows) or 20 days after calving (anovulatory cows). The cows were housed in a free-stall barn throughout the experimental period and offered a total mixed ration consisting of grass, corn silage and concentrate. Milking was performed twice daily (0600 and 1800 h). The average 305-day milk yield was approximately 9700 kg.

Sampling

BCS was assessed once a week during the experimental period by the same operator on a 0–5 scale with 0.25 intervals, where 0 = thin and 5 = very fat (Ferguson *et al.* 1994).

Blood samples (serum) for biochemical analysis were obtained once a week by caudal venipuncture after measurement of BCS using unheparinized, silicone-coated 9 ml tubes (Venoject, Autosep, Gel + Clot. Act., VP-AS109K; Terumo Corporation, Tokyo, Japan) and the tubes were coagulated for 30 min at 38 °C in an incubator. Blood samples (plasma) for hormonal analysis were obtained by caudal venipuncture twice weekly during the same period using sterile 10 ml tubes containing 200 µl stabilizer solution (0.3 M EDTA (pH 7.4), 1% acetyl salicylic acid). In addition, in 9 out of the 22 cows, we obtained blood samples 4 times/day at 6-h intervals for detection (presence or absence) of luteinizing hormone (LH) surge. To minimize the stress of blood sampling, blood was drawn 2 times/day for detection of E2 peak and 1 time/day for profile of P4 and metabolic hormones. These tubes were centrifuged at 3000 r.p.m. for 20 min at 4 °C, and the serum or plasma samples were kept at –30 °C until biochemical and hormonal analyses.

Ovaries of all cows were scanned twice weekly from 7 days to 30 days after calving by transrectal ultrasonography using a colour Doppler ultrasound scanner (SSD-5500; Aloka Co., Tokyo, Japan) equipped with a 7.5 MHz convex transducer (UST-995-7.5; Aloka Co.). Follicles ≥ 5 mm were measured for maximum diameter and follicles ≥ 8.5 mm were regarded as DFs (Ginther *et al.* 2003). After morphological evaluation, the flow mode was activated for blood flow mapping and the presence or absence of blood flow was assessed for each follicle. Colour signals were used to generate images in which blood flow with a velocity higher than 2 mm/s could be located as areas of colour within the follicle wall.

Identification of ovulation during the first follicular wave *post-partum*

When the P4 concentration in plasma was increased to more than 1 ng/ml, the cow was confirmed as having returned to luteal activity (Stevenson & Britt 1979). Furthermore, the first ovulation and luteal formation were identified using an ultrasound scanner equipped with a 7.5 MHz convex transducer.

Measurement of P4 and E2

The P4 determination in plasma was performed by enzyme immunoassay (EIA) after extraction by diethyl ether, as described previously (Miyamoto *et al.* 1992), and the extraction efficiency was 93%. The standard curve ranged from 0.05 to 50 ng/ml, and the 50% of effective dose (ED50) of assay was 3.2 ng/ml. The intra- and interassay coefficient of variation (CV) values averaged 6.7 and 7.2% respectively. The E2 determination in plasma was performed by EIA after extraction by diethyl ether as described previously (Acosta *et al.* 2000) and the extraction efficiency was 85%. The standard

curve ranged from 1.95 to 2000 pg/ml, and the ED50 of assay was 5.2 pg/ml. The intra- and interassay CV values averaged 6.7 and 8.7% respectively.

Measurement of FSH, LH, GH, IGF-I and insulin

The determination of plasma follicle-stimulating hormone (FSH), LH, GH, IGF-I and insulin was performed by EIA using the biotin–streptavidin amplification technique.

The FSH assay was performed as described previously (Watanabe *et al.* 1997). The standard curve for FSH ranged from 0.18 to 12 ng/ml, and the ED50 of the assay was 1.7 ng/ml. The intra- and interassay CV values averaged 8.3 and 14.6% respectively.

The LH assay was described previously in detail (Mutayoba *et al.* 1990). The standard curve for LH ranged from 0.09 to 50 ng/ml, and the ED50 of the assay was 3.1 ng/ml. The intra- and interassay CV values averaged 8.3 and 9.4% respectively.

The GH concentration was measured by the EIA as described by Roh *et al.* (1997), but with slight modification. A diluted rabbit antibody added to bovine GH (100 µl, $\times 150\,000$, D Schams, TU-Munich, Weihenstephan, Germany) was distributed in all wells of a microplate coated with anti-rabbit γ -globulin antiserum, then incubated for 24 h at room temperature and the plate was decanted. After chicken serum diluted in assay buffer (100 µl, 1%) was added to each well, 15 µl GH standard (0.78–100 ng/ml, NIDDK-bGH, AFP-9984C) dissolved in assay buffer or plasma were incubated in wells for 24 h. After decanting the plate, biotin-labelled GH was distributed in all wells and then incubated for 3 h. Finally, colorimetric treatments were carried out. Intra- and interassay CV values were 8.1 and 8.5% respectively, and the ED50 in this assay system was 6.2 ng/ml.

The total IGF-I determination in plasma was performed by the EIA after protein extraction by an acid–ethanol mixture (87.5% ethanol and 12.5% 2 N hydrochloric acid; Daughaday *et al.* 1980) to obtain free IGF-I from binding protein. Thirty microlitres of human IGF-I standard (Roche; 0.39–50 ng/ml) dissolved in assay buffer or sample were added to each well coated with anti-rabbit γ -globulin antiserum. In addition, 100 µl biotin-labelled hIGF-I ($\times 10\,000$) and rabbit anti-hIGF-I ($\times 40\,000$, NIDDK, AFP18111298) diluted in assay buffer were distributed in all wells and then incubated for 72 h at 4 °C. Finally, colorimetric treatments were carried out. Intra- and interassay CV values were 5.7 and 6.6% respectively, and the ED50 in this assay system was 2.5 ng/ml.

The insulin determination was carried out by EIA. Insulin standard (Sigma) was diluted with charcoal-treated serum (insulin-free) during preparation. Thirty microlitres of insulin standard (39–5000 pg/ml) or plasma were added to each well coated with anti-guinea

pig goat γ -globulin antiserum. In addition, 100 µl anti-bovine guinea pig insulin ($\times 150\,000$, D Schams) dissolved in assay buffer were distributed in all wells and then incubated for 24 h at 4 °C. After decanting the plates, 100 µl biotin-labelled bovine insulin ($\times 50\,000$) were distributed in all wells and then incubated for 2 h at 4 °C. Finally, colorimetric treatments were carried out. Intra- and interassay CV values were 9.7 and 14.5% respectively, and the ED50 in this assay system was 800 pg/ml.

Biochemical analyses

In each sample, the concentrations of glucose, NEFA, T-cho and AST were measured using a clinical chemistry automated analyser (TBA120FR; Toshiba Medical Systems Co., Tochigi, Japan).

Statistical analyses

All data from 22 cows was analyzed by repeated ANOVA. There was a tendency for a group $\times$ time interaction on plasma concentrations of E2 ($P=0.09$) and plasma E2 concentrations changed over time in the ovulatory cows (time effect; $P<0.05$). Mean concentrations of E2 were calculated for each group and each sampling period, and significant differences were detected by Fisher's protected least significance difference test.

For other data, no significant interaction between group and time was observed. For all metabolites, metabolic hormones and BCS in 22 cows during the peri-partum period, mean concentrations for each cow were calculated for the pre- or post-partum periods. For the diameter of DFs and metabolic hormones in 22 cows at 1, 1.5 and 2 weeks post-partum, the mean diameter and mean concentrations during the sampling period were calculated and analyzed by repeated measures ANOVA. The significant differences between ovulatory and anovulatory cows were analyzed by Student's *t*-test.

In 5 cows which ovulated, we observed in detail their endocrine profiles. In 9 out of the 22 cows, we analyzed metabolic hormones before and after E2 peaks. We divided this into two phases: before E2 peak (–10 to –1 days relative to E2 peak) and after E2 peak (0–10 days relative to E2 peak), and the significant differences between the two phases were analyzed by paired *t*-test.

Results were expressed as means $\pm$ S.E.M.; differences with $P<0.05$ were considered significant.

Results

Metabolites, BCS and metabolic hormones during the peri-partum period related to the ovulation of the DF at the first follicular wave post-partum

The numbers of cows showing ovulation and anovulation of a DF during the first post-partum follicular wave were

13 (59.1%) and 9 (40.9%) respectively. In ovulatory cows, the interval between calving and ovulation was 15.2 ± 0.8 days, and after the first ovulation, cows had an ovarian cycle followed by more than 7 days of a luteal phase. The first month milk yield was 45.5 ± 0.4 kg/day in ovulatory cows and 45.1 ± 0.5 kg/day in anovulatory cows.

Figure 1 shows the change of metabolites, BCS and metabolic hormones during the peri-partum period. The serum concentration of glucose was not significantly different between the ovulatory and anovulatory cows during the pre-partum period. Thereafter, the serum glucose concentrations of the anovulatory cows were lower than those of the ovulatory cows during the *post-partum* period (ovulatory, 59.2 ± 1.3 vs anovulatory, 52.7 ± 1.3 ; $P < 0.001$). For BCS, there was no significant difference between ovulatory and anovulatory cows during the pre-partum period; however, BCS in ovulatory

Figure 1 Metabolites, metabolic hormones and BCS from 4 weeks pre-partum to 3 weeks *post-partum* for cows that developed either ovulatory ($n=13$) or anovulatory ($n=9$) dominant follicle during the first follicular wave *post-partum* (mean $\pm$ S.E.M.; solid, ovulation; open, anovulation). The first sampling week after parturition was regarded as week 0. The serum glucose concentrations in the anovulatory cows were lower than those in the ovulatory cows during the *post-partum* period ($P < 0.001$). BCS in ovulatory cows tended to be higher than that in anovulatory cows during the *post-partum* period ($P = 0.08$). The plasma concentration of GH in anovulatory cows was higher than that in ovulatory cows during the peri-partum period (pre-partum, $P < 0.01$; *post-partum*, $P < 0.0001$). On the other hand, the plasma concentration of IGF-I in the ovulatory cows was higher than that in the anovulatory cows during the peri-partum periods (pre-partum, $P < 0.05$; *post-partum*, $P < 0.01$). The concentrations of NEFA, T-cho, AST and insulin were similar between the ovulatory and the anovulatory cows during the period of study.

cows tended to be higher than that in anovulatory cows during the *post-partum* period ($P = 0.08$; Fig. 1). The serum concentrations of NEFA, T-cho and AST were similar between the ovulatory and the anovulatory cows during the period of study (Fig. 1).

The plasma concentration of insulin was not significantly different between the ovulatory and anovulatory cows during the pre- and *post-partum* periods. The plasma concentration of GH in anovulatory cows was higher than that of ovulatory cows during both pre- and *post-partum* periods (pre-partum, $P < 0.01$; *post-partum*, $P < 0.0001$; Fig. 1). On the other hand, the plasma concentration of IGF-I in ovulatory cows was higher than that of anovulatory cows during both pre- and *post-partum* periods (pre-partum, $P < 0.05$; *post-partum*, $P < 0.01$; Fig. 1).

The function and morphology of DF during the first follicular wave *post-partum*, and metabolic status either ovulatory or anovulatory cows

The DF of the first follicular wave appeared on day 7 *post-partum*, and the diameter of the DF was approximately 10 mm and blood flow in each follicle was observed by 14 days *post-partum* despite ovulation or anovulation (Fig. 2). In the ovulatory cows, a corpus luteum with blood flow was observed by 17 days *post-partum* (Fig. 2). On the other hand, the DF of anovulatory cows continued to grow maintaining identifiable blood flow (Fig. 2).

The plasma concentration of FSH did not differ between the ovulatory and anovulatory cows until 14 days *post-partum* (ovulation, 2.18 ± 0.09 ng/ml vs anovulation, 2.05 ± 0.09 ng/ml). The ratio of the existence of the DF at the first scanning was 61.5% (8/13) in the ovulatory cows and 44.4% (4/9) in the anovulatory cows, and the diameter of the follicle at that time was not significantly different between the ovulatory and anovulatory cows (ovulation, 9.9 ± 0.7 mm vs anovulation, 9.9 ± 1.0 mm).

The diameter of the DF had similar growth in both groups (time effect; $P < 0.01$, Fig. 3) and did not differ between the ovulatory and the anovulatory cows during the period of study. The plasma concentrations of E2 in ovulatory cows was higher at 1.5 weeks *post-partum* than those at 1 and 2 weeks *post-partum* ($P < 0.05$; Fig. 3), and higher than those in anovulatory cows at 1.5 weeks *post-partum* ($P < 0.01$; Fig. 3). However, there was no significant difference between the ovulatory and anovulatory cows at 2 weeks *post-partum*, because of the decrease of preovulatory E2 concentrations. In the ovulatory cows, plasma GH concentrations were lower ($P < 0.01$) and IGF-I concentrations were higher ($P < 0.05$) when compared with anovulatory cows, while the plasma insulin concentrations did not differ between the ovulatory and anovulatory cows during this period (Fig. 3).

Ovulatory cow

Anovulatory cow

7 days pp

14 days pp

17 days pp

21 days pp

5mm

Figure 2 Representative images of dominant follicles during the first follicular wave *post-partum* of ovulatory and anovulatory cows. Red colour represents the blood flow towards the transducer, and blue indicates blood flow away from the transducer. The colour gain of the flow mode was set to detect the movement of at least 2 mm/s. Scale bar represents 5 mm. The white dotted lines that delineate circles indicate the frame of the follicle and the green dotted lines that delineate circles indicate the frame of the corpus luteum.

The metabolic endocrine factors related to ovulation of the DF during the first follicular wave post-partum

To observe the relationship between the growth of the DF and the endocrine and metabolic factors (E2, insulin, IGF-I and GH) in detail, 9 out of the 22 cows showing ovulation or anovulation of DFs during the first follicular wave *post-partum* were studied. The number of ovulatory cows was 5 (55.6%), E2 peak could be confirmed on 16.0 ± 1.1 days *post-partum*, the interval between E2 peak and LH surge being approximately 21 ± 7 h, and the interval between calving and ovulation being 17.0 ± 1.5 days. On the other hand, E2 peak and LH surge could not be confirmed in the anovulatory cows.

In the ovulated cows, E2 peak could be confirmed on 16.0 ± 1.1 days *post-partum*, and the interval between E2 peak and LH surge was approximately 21 ± 7 h (Fig. 4). On the other hand, E2 peak and LH surge could not be confirmed in the anovulated cows (Fig. 4). In ovulated cows, insulin concentrations during and after the E2 peak period showed a strong tendency to be higher than those before the E2 peak ($P=0.06$; Table 1). The GH concentrations from before until after the E2 peak did not change. However, plasma IGF-I remained high until 7–10 days *post-partum*, followed by a gradual decrease (Fig. 4). Therefore, IGF-I concentration during the after E2 peak was lower than that during the before E2 peak ($P<0.05$, Table 1).

Discussion

The present study focused on the changes in metabolic hormones, such as IGF-I, GH and insulin that occurs during the peri-partum period in dairy cows and their relation to the occurrence of ovulation. The results suggest that the plasma concentration of IGF-I relates to the early growth of the ovulatory DF and insulin relates to the maturation and ovulation of the ovulatory DF at the first follicular wave *post-partum*.

Our results showed that higher plasma GH concentrations and lower glucose and IGF-I concentrations

were the clear features in anovulatory cows during the pre-partum period compared with those of the ovulatory cows. Previous studies have shown that the expression of GH receptor 1A and IGF-I mRNA in the liver declines at parturition, and GH concentrations are elevated during the *post-partum* period, because IGF-I concentrations decrease to low levels, reducing the negative feedback on GH (Kobayashi *et al.* 1999, Pushpakumara *et al.* 2003, Radcliff *et al.* 2003). The plasma IGF-I levels in the ovulatory cows at the first follicular wave *post-partum* were higher than those in the anovulatory cows (Beam & Butler 1998). The high GH concentrations in the anovulatory cows in the present study with lower IGF-I concentrations during the peri-partum period may have caused GH insensitivity in the liver. These findings suggest that energy status, which reflects on GH, IGF-I and glucose levels, and ovulation are closely related to each other during the early *post-partum* period. However, in the present study, the metabolites such as NEFA, AST and T-cho did not differ between ovulatory and anovulatory cows during the peri-partum period, indicating that the lipid metabolism between these two groups was at similar levels despite the different GH levels between two groups. In the anovulatory cows, body fat stores might have already been reduced during the peri-partum period, resulting in lower BCS than that in ovulatory cows. Therefore, ovulatory cows may be able to handle better the negative energy balance after parturition when compared with anovulatory cows. The present study could not examine the exact feed intake and nutritional status, because all cows were provided with feed and water available *ad libitum*. Thus, further studies are necessary to investigate the effect of feed intake on metabolic hormones and on the maturation and ovulation of the first *post-partum* DF.

It was observed that the rate of follicular growth did not differ between the ovulatory and anovulatory cows during the *post-partum* period. The local blood flow was detected in DF of all cows despite ovulation or anovulation. Angiogenesis, the formation of new network of blood vessels, is essential for follicular

development and ovulation (Yamada *et al.* 1994, Jiang *et al.* 2003). In addition, a recent report suggested that the angioprotein-tie system that acts on angiogenesis in concert with vascular endothelial growth factor controls the blood vessels to maintain active angiogenesis in developing bovine follicles (Hayashi *et al.* 2003). The maintenance of follicle vasculature and appropriate blood supply to the large follicles is essential for follicle dominance (Acosta *et al.* 2005). However, our results

showed that an insufficient blood flow supply is not the cause of anovulation. The plasma E2 concentrations during this period increased only in the ovulatory cows, while those in the anovulatory cows did not, which are well consistent with previous reports (Beam & Butler 1997, 1998). Thus, it is likely that an insufficient ability of the granulosa cells to secrete E2 is a determinant for anovulation rather than insufficient angiogenesis.

The plasma concentration of FSH did not differ between the ovulatory and anovulatory cows until 14 days *post-partum*. Our data confirmed the previous studies that FSH appears to be insensitive to metabolic status (Lamming *et al.* 1981, Beam & Butler 1998). On the other hand, serum concentrations of IGF-I during the first follicular wave *post-partum* were reported to be at low levels in anovulatory dairy cows (Beam & Butler 1998). In the present study, the plasma IGF-I remained elevated until 7–10 days *post-partum*, followed by a gradual decrease, whereas IGF-I in the anovulatory cows was kept at a low level throughout the *post-partum* period. The IGF-I stimulates steroidogenesis and the proliferation in follicular cells (Spicer *et al.* 1993, Spicer & Stewart 1996), and the ratio of oestrogen to progesterone in follicular fluid was correlated positively with plasma IGF-I in lactating cows (Lucy *et al.* 1992a). In addition, IGF-I works with FSH synergically in promoting follicular development and E2 synthesis (Fortune *et al.* 2004). Thus, the data suggests that IGF-I is an important factor for the development of the DF to the maturation stage by amplifying the endocrine signal of FSH to upregulate E2 secretion from the future DF *post-partum*.

The present data suggested that plasma insulin level in the ovulatory cows increased together with E2 peak. In dairy cows, the serum insulin levels increase together with E2 during the development of the DF (Armstrong *et al.* 2003). Insulin has been shown to stimulate E2 production in the granulosa cells (Gutierrez *et al.* 1997, Glistler *et al.* 2001). In addition, E2 secreted from a follicle also enhances insulin secretion from the

Figure 3 The function and morphology of the dominant follicle during the first follicular wave *post-partum* and metabolic status, which were data extracted from Fig. 1, either ovulatory ($n=13$) or anovulatory ($n=9$) cows at 1, 1.5 and 2 weeks *post-partum* (mean $\pm$ S.E.M.; solid, ovulation; open, anovulation). * Indicates differences of $P<0.01$ between the ovulatory and anovulatory cows at 1.5 weeks *post-partum*. a and b indicate differences of $P<0.05$ within ovulatory cows. The diameter of the dominant follicle was similar in both groups (time effect; $P<0.01$) and did not differ between the ovulatory and the anovulatory cows during the period of study. The plasma concentrations of E2 in ovulatory cows were higher than those in anovulatory cows at 1.5 weeks *post-partum* ($P<0.05$); however, there were no significant differences between ovulatory and anovulatory cows at 2 weeks *post-partum* because of decrease of preovulatory E2 concentrations. In ovulatory cows, plasma GH concentrations were lower ($P<0.01$) and IGF-I concentrations were higher ($P<0.05$) when compared with anovulatory cows; however, the plasma insulin concentrations did not differ between the ovulatory and anovulatory cows during this period.

Figure 4 Daily representations of plasma concentrations of insulin, GH, IGF-I and P4 in 9 out of the 22 cows. We obtained blood samples 4 times/day for detection (presence or absence) of LH surge, 2 times/day for detection of E2 peak and 1 time/day for profile of P4 and metabolic hormones. E2 concentrations are shown at 6-h intervals from 10 days before to 10 days after E2 peak in ovulated ($n=5$) and from 10 days to 20 days *post-partum* in anovulated cows ($n=4$; mean $\pm$ S.E.M.). Insulin, GH, IGF-I and P4 concentrations are indicated by squares and E2 concentrations are indicated by circles; solid in ovulated cows (■, ●) and open in anovulated cows (□, ○). Dotted lines in both graphs indicate time of E2 peak in ovulated cows, and the orange vertical bar of the graph for ovulated cows indicates the range of LH surge (21 ± 7 h after E2 peak).

Table 1 The metabolic-endocrine factors between before and after E2 peak period in ovulatory cows.

	Relative to E2 peak		Significance of differences
	Before	After	
Insulin (ng/ml)	0.29 ± 0.03	0.47 ± 0.08	$P=0.06$
GH (ng/ml)	11.7 ± 1.5	9.6 ± 0.8	NS
IGF-I (ng/ml)	98.2 ± 21.5	61.1 ± 13.7	$P<0.05$

NS, not significant. Values are means $\pm$ S.E.M. Data were divided into two phases which were before E2 peak (-10 to -1 days relative to E2 peak) and after E2 peak (0 – 10 days relative to E2 peak).

pancreas (Morimoto *et al.* 2001). These findings clearly indicate that plasma insulin and E2 from follicles positively interact with each other during the development of the DF. The increase in circulating insulin level by the feeding modification results in advanced first ovulation *post-partum* in the cow (Gong *et al.* 2002) and the increase in plasma LH concentration in heifer (McCann & Hansel 1986). The LH pulse frequency was reported to be greater in ovulatory cows than in anovulatory cows (Lamming *et al.* 1981, Canfield & Butler 1990, Jolly *et al.* 1995). Therefore, plasma insulin levels closely relate to the follicle growth and ovulation by regulating LH secretion. On the other hand, LH was not controlled by insulin infusion in early lactation cows (Butler *et al.* 2004). The energy status resulting in high insulin levels, but not directly insulin infusion, may induce the change of pulsatile LH release, follicle growth and ovulation. Consequently, the nutritional status which leads to the increase of insulin level together with E2 peak, is one of the key features of ovulatory cows. Undoubtedly, further studies are needed to characterize the factors responsible for the suboptimal GnRH and LH release after parturition in dairy cows.

As shown in Fig. 5, our results showed that the changes in the concentration of two metabolic hormones (IGF-I and insulin) may regulate the development of ovulatory follicle in the first follicular wave *post-partum*. In ovulatory cows, high IGF-I levels were maintained during the growth of DF, and then insulin levels increased together with the increase in E2 secretion from DF. These findings indicate that IGF-I is an essential factor for the growth of the DF, and insulin may stimulate the DF to mature and reach ovulation. Collectively, it is

Figure 5 The summary of the results as a schematic representation of the growth and maturation of the dominant follicle at the first follicular wave *post-partum*. High IGF-I levels may stimulate the follicular cell function such as the steroidogenesis and the proliferation to appear healthy dominant follicle, and after that the levels decrease because of negative energy balance. Subsequently, E2 was secreted more. In contrast, the increase in insulin level together with E2 peak may enhance the maturation of the dominant follicle. Consequently, E2 enhanced by IGF-I induces LH surge and the dominant follicle reaches ovulation.

suggested that the DF which is stimulated by high IGF-I levels during the first follicular wave *post-partum*, become ovulatory with the increase in E2 secretion, resulting in the occurrence of LH surge and ovulation.

In conclusion, the present study provides convincing evidence that IGF-I is an important factor for the development the DF during the first follicular development *post-partum*. After that, the increase in insulin level together with E2 peak may ensure the maturation and ovulation. Our findings strongly support the concept that IGF-I and insulin represent 'metabolic signals' for the resumption of ovarian function *post-partum* in high-producing dairy cows. Moreover, we provide the first visual evidence that both ovulatory and anovulatory DFs of the first follicular wave *post-partum* are similarly supplied with the active blood flow, suggesting that an insufficient angiogenesis is not a determinant for anovulation.

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