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

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Short title: The first follicular wave postpartum in dairy cows

Key words: Cow, Dominant follicle, First follicular wave, Metabolic hormone, Postpartum

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Abstract

Recent studies suggest that insulin-like growth factor-1 (IGF-1) is a crucial regulatory factor in follicular growth during early postpartum 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 postpartum in high-producing dairy cows. Plasma concentrations of related hormones in 22 multiparous Holstein cows were measured from - 4 to 3 wk postpartum, and the development of DF was observed with colour Doppler ultrasound. Thirteen cows showed ovulation by 15.2 d postpartum. Anovulatory cows showed higher growth hormone and lower IGF-1 levels than in ovulatory cows during the peripartum 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 analysed in 9 out of 22 cows. Five cows showed an increase in plasma oestradiol-17β (E2) increase with follicular growth followed by E2 peak, LH surge and ovulation. In these cows, plasma IGF-1 concentrations remained high until 10 days postpartum followed by a gradual decrease. Subsequently, the insulin level increased together with the E2 peak toward ovulation. These profiles were not observed in anovulatory cows. In conclusion, our data strongly support the concept that IGF-1 and insulin represent 'metabolic signals' of the resumption of ovarian function postpartum in high-producing dairy cows. Moreover, we provide the first visual evidence that both ovulatory and anovulatory dominant follicle of the first follicular wave postpartum are similarly supplied with active blood flow.

Introduction

The negative energy balance early postpartum that is characterised by the loss of body weight and mobilization of body fat stores does not affect the first follicular wave postpartum (Beam & Butler 1999). In fact, the medium-sized follicles appear 5 days after calving, and reach into the large follicles at 10 days postpartum in most dairy cows (Savio *et al.* 1990a, b). However, almost a half of these follicles are not able to ovulate, but becomes 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 observed that the recovery of ovarian function and subsequent reproductive performance in ovulatory cows within 3 weeks postpartum 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 postpartum have not been fully elucidated.

Insulin and insulin-like growth factor 1 (IGF-1), metabolic hormones that are changed by feed intake, stimulate oestradiol-17β (E2) production in the granulosa cells (Gutierrez *et al.* 1997, Glister *et al.* 2001, Armstrong *et al.* 2003, Butler *et al.* 2004) and the proliferation of follicular cells (Spicer *et al.* 1993, Spicer & Stewart 1996). The growth of ovulatory follicles is regulated by the metabolic hormones such as IGF-1 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-1 is a factor that regulates the final maturation of the dominant follicle during the first follicular wave postpartum (Beam & Butler 1998). The circulating IGF-1 in ovulatory cows at the first follicular wave postpartum 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 postpartum. Therefore, the aim of the present study was to investigate the time-dependent relationship in detail among metabolic hormones including IGF-1, 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 postpartum

in a homogeneous herd at early lactation period in high-producing dairy cows. In addition, we used colour Doppler ultrasonography to monitor a 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 prepartum to 3 weeks postpartum when the period of 0-6 days after parturition was regarded as a parturient week (0 week). Additionally, 9 out of 22 cows that calved between February 2005 and April 2005 were used to observe the competence of the dominant follicle in detail, we collected blood samples (4times/day) from the appearance of a dominant follicle (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. The milking was performed twice daily (6:00 and 18:00). The average 305-day milk yield was approximately 9,700 kg.

Sampling

Body condition score (BCS) was assessed once a week during the experimental period by the same operator using a 0 to 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, Japan) and the tubes were coagulated for 30 minutes 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 ul of stabilizer solution (0.3 M EDTA, 1 % acetyl salicylic acid, pH 7.4). In addition, in 9 out of 22 cows, we obtained blood samples 4 times/day at 6 hours intervals for detection (presence or absence) of LH surge to minimized the stress of blood sampling 2, time/day for detection of E2 peak and 1 time/day for profile of P4 and metabolic hormones. These tubes were centrifuged at 3000 rpm for 20 minutes at 4 C and the serum or plasma samples were kept at -30 C until analysis for 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 ≥ 5mm were measured for maximum diameter and follicles ≥ 8.5mm were regarded as dominant follicles (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/sec could be located as areas of colour within the follicle wall.

Identification of ovulation during the first follicular wave postpartum

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 enzymeimmunoassay (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 ED50 of assay was 3.2 ng/ml. The intra- and interassay coefficients of variation (CVs) 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 CVs averaged 6.7 % and 8.7 %, respectively.

Measurement of FSH, LH, GH, IGF-1 and insulin

The determination for plasma FSH, LH, GH, IGF-1 and insulin was performed by EIA using the biotin-streptavidin amplification technique.

The FSH assay was performed as previously described (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 CVs averaged 8.3 % and 14.6 %, respectively.

The LH assay was previously described 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 CVs averaged 8.3 % and 9.4 %, respectively.

The GH concentration was measured by the EIA described by Roh *et al.* (1997) with slight modification. A diluted rabbit antibody to bovine GH (100µl, × 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 (100µl, 1%) was added to each well, 15µl of GH standard (0.78 to 100 ng/ml, NIDDK-bGH, AFP-9984C) dissolved in assay buffer or plasma was 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 CVs were 8.1 % and 8.5 %, respectively, and the ED50 in this assay system was 6.2 ng/ml.

The total IGF-1 determination in plasma was performed by the EIA after protein extraction by an acid-ethanol (87.5% ethanol and 12.5% 2N hydrochloric acid; Daughaday *et al.* 1980) to obtain IGF-1 free from binding protein. Thirty µl of human IGF-1 standard (Roche, Indianapolis, USA, 0.39 to 50 ng/ml) dissolved in assay buffer or sample was added to each well coated with anti-rabbit γ-globulin antiserum. In addition, 100µl of biotin-labelled hIGF-1 (× 10,000) and rabbit anti-hIGF-1 (× 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 CVs 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, Missouri, USA) was diluted with charcoal-treated serum (insulin-free) at preparation. Thirty μl of insulin standard (39 to 5000 pg/ml) or plasma were added to each well coated with anti-guinea pig goat γ-globulin antiserum. In addition, 100μl of anti-bovine guinea pig insulin (× 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, 100μl of biotin-labelled bovine insulin (× 50,000) was distributed in all wells, and then incubated for 2 h at 4 C. Finally, colorimetric treatments were carried out. Intra- and interassay CVs were 9.7 % and 14.5 %, respectively, and the ED50 in this assay system was 800 pg/ml.

Biochemical analyses

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

Statistical analyses

All the data from 22 cows were analyzed by repeated ANOVA. There was a tendency for a group x 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 peripartum period, mean concentrations for each cow were calculated for the prepartum or postpartum period. For the diameter of dominant follicles and metabolic hormones in 22 cows at 1, 1.5 and 2 weeks postpartum, the mean diameter and mean concentrations during the sampling period were calculated and analysed repeated measures ANOVA. The significant differences between ovulatory and anovulatory were analysed by Student’s t test.

In 5 cows which ovulated when we observed in detail endocrine profiles in 9 out of 22 cows, we analysed metabolic hormones at the before and after E2 peak. We divided into two phases that were before

E2 peak (-10 to -1 days relative to E2 peak) and after E2 peak (0 to 10 days relative to E2 peak), and the significant differences between two phases were analysed by paired t test.

Results were expressed as mean ± standard error of mean; differences with $P<0.05$ were considered significant.

Results

Metabolites, BCS and metabolic hormones during the peripartum period related to the ovulation of the dominant follicle at the first follicular wave postpartum

The numbers of cows showing ovulation and anovulation of a dominant follicle during the first postpartum 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 peripartum period. The serum concentration of glucose was not significantly different between the ovulatory and anovulatory cows during the prepartum period. Thereafter, the serum glucose concentrations of the anovulatory cows was lower than that of the ovulatory cows during the postpartum 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 prepartum period, however, BCS in ovulatory cows tended to be higher than in anovulatory cows during the postpartum 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 postpartum period. The plasma concentration of GH in anovulatory cows was higher than that of ovulatory cows during both prepartum and postpartum periods (prepartum; $P<0.01$, postpartum; $P<0.0001$, Fig. 1). On the other hand, the plasma concentration of IGF-1 in ovulatory

cows was higher than that of anovulatory cows during both prepartum and postpartum periods (prepartum; $P<0.05$, postpartum; $P<0.01$, Fig. 1).

The function and morphology of the dominant follicle during the first follicular wave postpartum, and metabolic status either ovulatory or anovulatory cows

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

The plasma concentration of FSH did not differ between the ovulatory and anovulatory cows until 14 days postpartum (ovulation: 2.18±0.09ng/ml vs. anovulation: 2.05±0.09ng/ml). The ratio of the existence of dominant follicle 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±0.7mm vs. anovulation, 9.9±1.0mm).

The diameter of the dominant follicle 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 were higher at 1.5 weeks postpartum than in 1 and 2 weeks postpartum ($P<0.05$, Fig. 3), and higher than that in anovulatory cows at 1.5 weeks postpartum ($P<0.01$, Fig. 3). However there was no significant difference between ovulatory and anovulatory cows at 2 weeks postpartum, because of decrease of preovulatory E2 concentrations. In ovulatory cows, plasma GH concentrations were lower ($P<0.01$) and IGF-1 concentrations were higher ($P<0.05$) compared to anovulatory cows, while the plasma insulin concentrations did not differ between the ovulatory and anovulatory cows during this period (Fig. 3).

The metabolic-endocrine factors related to ovulation of the dominant follicle during the first follicular wave postpartum

To observe the relationship between growth of the dominant follicle, and the endocrine and metabolic factors (E2, insulin, IGF-1 and GH) in detail, 9 out of 22 cows showing ovulation or anovulation of dominant follicles during the first follicular wave postpartum were studied. The number of ovulatory cows was 5 (55.6%), E2 peak could be confirmed on 16.0±1.1 days postpartum, the interval between E2 peak and LH surge was approximately 21±7 hours, and interval between calving and the ovulation was 17.0±1.5 days. On the other hand, E2 peak and LH surge could not be confirmed in anovulatory cows.

In ovulated cows, E2 peak could be confirmed on 16.0±1.1 days postpartum, and the interval between E2 peak and LH surge was approximately 21±7 hours (Fig. 4). On the other hand, E2 peak and LH surge could not be confirmed in 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-1 remained high until 7 to 10 days postpartum followed by a gradual decrease, (Fig. 4). Therefore, IGF-1 concentration during the after E2 peak was lower than that during the before E2 peak of E2 ($P<0.05$, Table 1).

Discussion

The present study focused on the changes in metabolic hormones, such as IGF-1, GH and insulin, occurring during the peripartum period in dairy cows and their relation to the occurrence of ovulation. The results suggest that the plasma concentration of IGF-1 relates to the early growth of ovulatory dominant follicle and insulin relates to the maturation and ovulation of the ovulatory dominant follicle at the first follicular wave postpartum.

Our results showed that higher plasma GH concentrations and lower glucose and IGF-1 concentrations were the clear feature in anovulatory cows during the prepartum period compared to those of the ovulatory cows. Previous studies have shown that the expression of the GH receptor 1A and the IGF-1 mRNA in the liver declines at parturition, and GH concentrations are elevated during the postpartum period, because IGF-1 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-1 levels in ovulatory cows at the first follicular wave postpartum were higher than that in anovulatory cows (Beam and Butler, 1998). The high GH concentrations in anovulatory cows in the present study with lower IGF-1 concentrations during the peripartum period may have caused GH insensitivity in a liver. These findings above suggest that energy status, which reflects on GH, IGF-1 and glucose levels, and ovulation are closely related to each other during the early postpartum 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 peripartum period, indicating that the lipid metabolism between these two groups was similar level despite the different GH levels between two groups. In anovulatory cows, body fat stores might have already been reduced during the peripartum period, resulting in their lower BCS than ovulatory cows. Therefore, ovulatory cows may be able to handle better the negative energy balance after parturition compared to anovulatory cows. The present study could not examine the exact feed intake and nutritional status, because all cows were provided with feed and water *ad libitum*. Thus, future studies are necessary to investigate the effect of feed intake on metabolic hormones and on the maturation and ovulation of the first postpartum dominant follicle.

It was observed that the rate of follicular growth did not differ between ovulatory and anovulatory cows during the postpartum period. The local blood flow was detected in dominant follicle 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 angiopoietin-tie system that acts on angiogenesis in concert with vascular endothelial growth factor (VEGF) 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, Beam & Butler 1998). Thus, it is likely that an insufficient ability of 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 postpartum. Our data confirmed the previous studies that FSH appears to be insensitive to metabolic status (Beam & Butler 1998, Lamming *et al.* 1981). On the other hand, serum concentration of IGF-1 during the first follicular wave postpartum were reported to be at low levels in anovulatory dairy cows (Beam & Butler 1998). In the present study, the plasma IGF-1 remained elevated until 7 to 10 days postpartum followed by a gradual decrease, whereas IGF-1 in the anovulatory cows was kept at a low level throughout the postpartum period. The IGF-1 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-1 in lactating cows (Lucy *et al.* 1992a). Also, IGF-1 works with FSH synergically in promoting follicular development and E2 synthesis (Fortune *et al.* 2004). Thus, the data suggest that IGF-1 is an important factor to develop the dominant follicle to the maturation stage by amplifying the endocrine signal of FSH to upregulate E2 secretion from the future dominant follicle postpartum.

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 dominant follicle (Armstrong *et al.* 2003). Insulin has been shown to stimulate E2 production in the granulosa cells (Gutierrez *et al.* 1997, Glister *et al.* 2001). In addition, E2 secreted from a follicle also enhances insulin secretion from the 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 dominant follicle. The increase in circulating insulin level by feeding modification results in advanced first ovulation postpartum 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 relates 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). Namely, 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 characterise the factors responsible for the suboptimal GnRH and LH release after parturition in dairy cows.

As shown in Fig 5, our results demonstrated that the changes in the concentration of two metabolic hormones (IGF-1 and insulin) may regulate the development of ovulatory follicle in the first follicular wave postpartum. In ovulatory cows, high IGF-1 levels were maintained during growth of dominant follicle, and then insulin levels increased together with the increase in E2 secretion from dominant follicle. These findings indicate that IGF -1 is an essential factor for the growth of the dominant follicle, and insulin may stimulate the dominant follicle to mature and reach ovulation. Collectively, it is suggested that the dominant follicle, which stimulated by high IGF-1 levels during the first follicular wave postpartum, 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-1 is an important factor to develop the dominant follicle during the first follicular development postpartum. After that, the increase of insulin level together with E2 peak may ensure the maturation and ovulation. Our findings strongly support the concept that IGF-1 and insulin represent 'metabolic signals' of the resumption of ovarian function postpartum in high-producing dairy cows. Moreover, we provide the first visual evidence that both ovulatory and anovulatory dominant follicle of the first follicular wave postpartum are similarly supplied with the active blood flow, suggesting that an insufficient angiogenesis is not a determinant for anovulation.

Acknowledgments

The authors thank Dr. K. Okuda, Okayama University, Japan, for P_4 antiserum, and Dr. Parlow, NIDDK, for the FSH, LH and GH standard, and IGF-1 antiserum. This study was supported by the Grant-in-Aid for Scientific Research of the Japan Society for the Promotion of Science (JSPS), the 21st Century COE Program (A-1) of the Ministry of Education, Culture, Sports, Science, and Technology, Japan, Secure and Healthy Livestock Farming Project of the Ministry of Agriculture, Forestry and Fisheries, and the Japan Livestock Technology Association. C. Kawashima and E. Kaneko are supported by the COE program.

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1

2

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-1 (ng/ml)	98.2±21.5	61.1±13.7	P<0.05

Values are mean±sem
NS; not significant.
Data were divided into two phases which were before E2 peak (-10 to -1 days relative to E2 peak) and after E2 peak (0 to 10 days relative to E2 peak)

Figure Legends

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

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

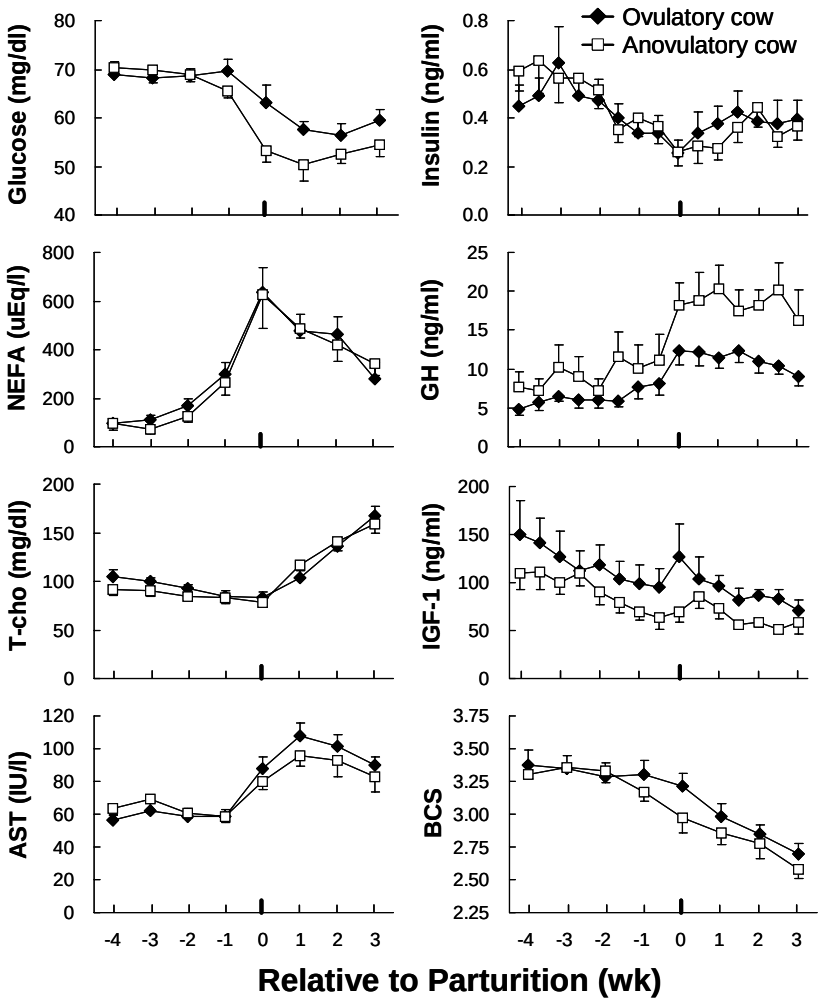
Figure 3 The function and morphology of the dominant follicle during the first follicular wave postpartum and metabolic status, which was extracted data from Fig. 1, either ovulatory (n=13) or anovulatory (n=9) cows at 1, 1.5 and 2 weeks postpartum (mean±sem, solid; ovulation, open; anovulation). ** indicates differences of $P<0.01$ between ovulatory and anovulatory cows at 1.5 weeks postpartum. a and b indicates differences of $P<0.05$ within ovulatory cows. The diameter of the dominant follicle was similar growth 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 that in

anovulatory cows at 1.5 weeks postpartum ($P<0.05$), however there was no significant differences between ovulatory and anovulatory at 2 weeks postpartum because of decrease of preovulatory E2 concentrations. In ovulatory cows, plasma GH concentrations were lower ($P<0.01$) and IGF-1 concentrations were higher ($P<0.05$) compared to anovulatory cows, however the plasma insulin concentrations did not differ between the ovulatory and anovulatory cows during this period.

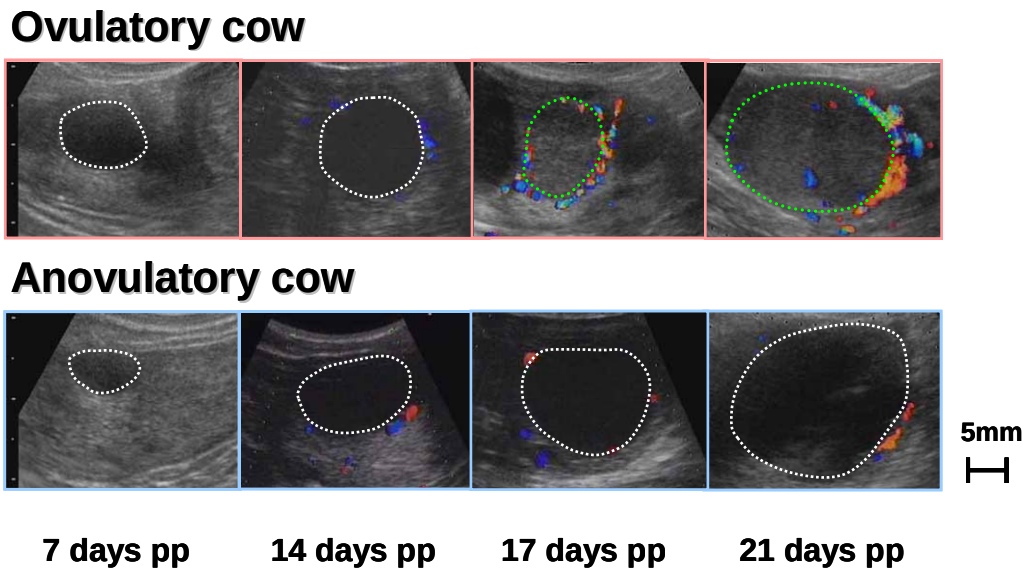
Figure 4 Daily representations of plasma concentrations of insulin, GH, IGF-1 and P4 in 9 out of 22 cows. We obtained blood samples 4 times/day for detection (presence or absence) of LH surge, 2 time/day for detection of E2 peak and 1 time/day for profile of P4 and metabolic hormones. E2 concentrations are shown at 6 h intervals from 10 d before to 10 d after E2 peak in ovulated (n=5) and from 10 d to 20 d postpartum in anovulated cows (n=4) (mean±sem). Insulin, GH, IGF-1 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 of 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).

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 postpartum. High IGF-1 levels may stimulate the follicular cell function such as the steroidogenesis and the proliferation to appear healthy dominant follicle, and after that, the levels decreases because of negative energy balance. Subsequently, E2 was more secreted. Contrastively, the increase in insulin level together with E2 peak may enhance the maturation of the dominant follicle. Consequently, E2 enhanced by IGF-1 induce LH surge and the dominant follicle reaches ovulation.

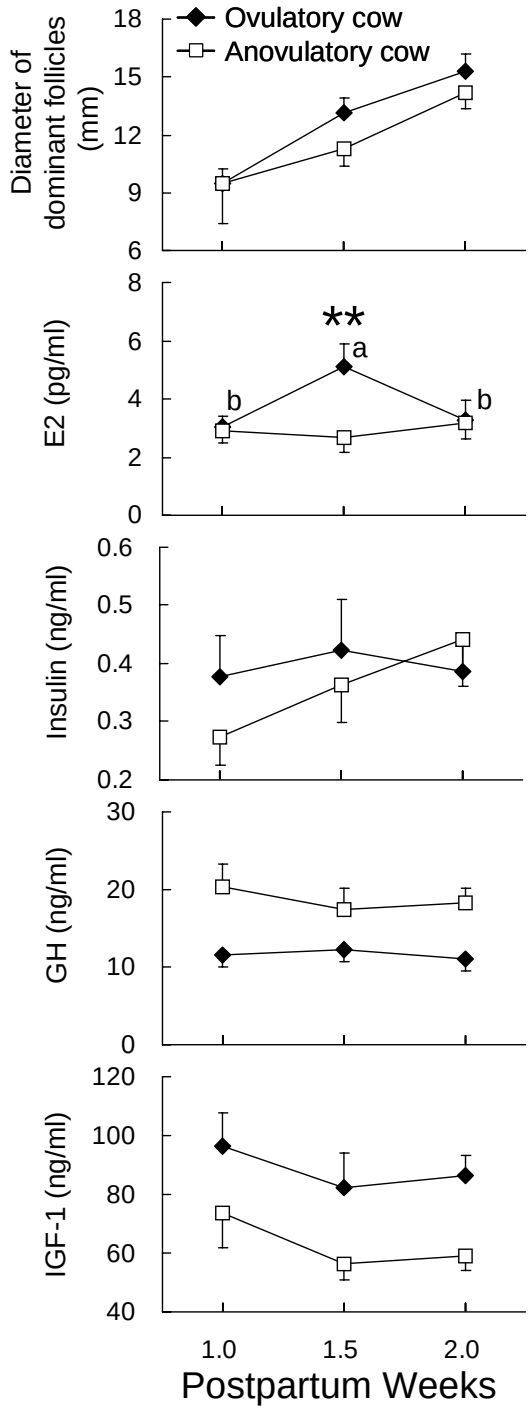
1 Figure 1


Ovulatory cow
Anovulatory cow
Glucose (mg/dl)
Insulin (ng/ml)
NEFA (uEq/l)
GH (ng/ml)
T-cho (mg/dl)
IGF-1 (ng/ml)
AST (IU/l)
BCS
Relative to Parturition (wk)

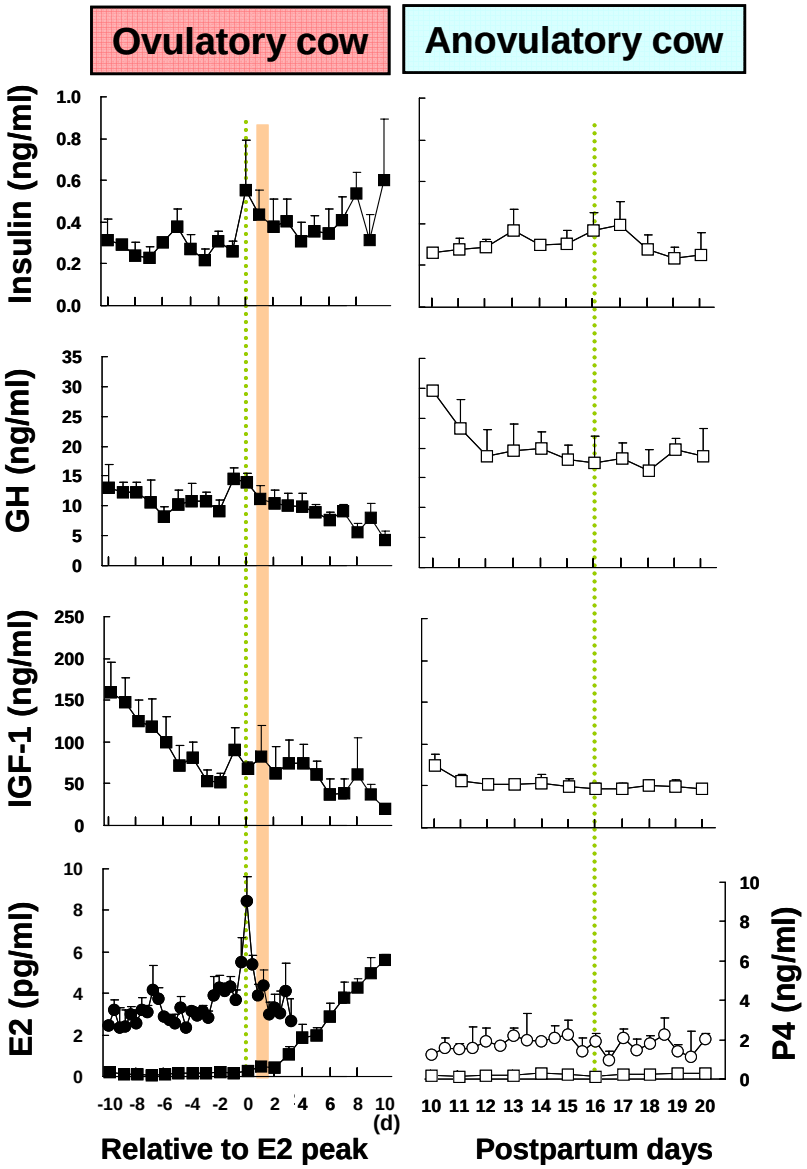
1 Figure 2


Ovulatory cow
Anovulatory cow
5mm
7 days pp
14 days pp
17 days pp
21 days pp

1 Figure 3


Ovulatory cow
Anovulatory cow
Diameter of dominant follicles (mm)
18
15
12
9
6
E2 (pg/ml)
8
6
4
2
0
**
a
b
b
Insulin (ng/ml)
0.6
0.5
0.4
0.3
0.2
GH (ng/ml)
30
20
10
0
IGF-1 (ng/ml)
120
100
80
60
40
1.0
1.5
2.0
Postpartum Weeks

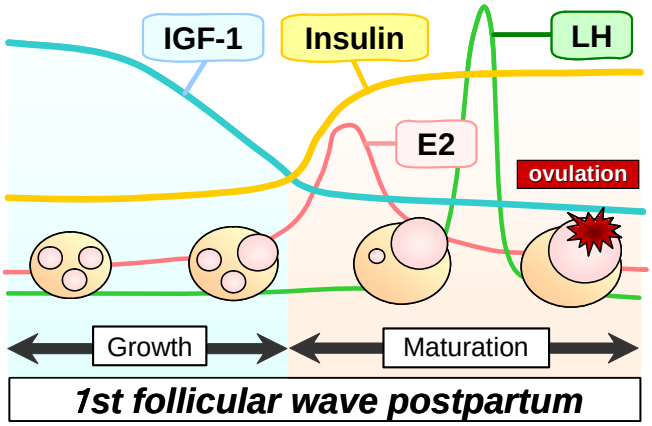
1 Figure 4


Ovulatory cow
Anovulatory cow
Insulin (ng/ml)
GH (ng/ml)
IGF-1 (ng/ml)
E2 (pg/ml)
P4 (ng/ml)
(d)
Relative to E2 peak
Postpartum days

1 Figure 5

2

3


IGF-1
Insulin
LH
E2
ovulation
Growth
Maturation
1st follicular wave postpartum