

—Original Article—

Influence of hepatic load from far-off dry period to early postpartum period on the first postpartum ovulation and accompanying subsequent fertility in dairy cows

Chiho KAWASHIMA¹⁾, Nozomi ITO¹⁾, Shuntarou NAGASHIMA²⁾, Motozumi MATSUI³⁾, Kumiko SAWADA⁴⁾#, Florian J. SCHWEIGERT⁵⁾, Akio MIYAMOTO²⁾ and Katsuya KIDA¹⁾

¹⁾Field Center of Animal Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-8555, Japan

²⁾Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-8555, Japan

³⁾Department of Applied Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-8555, Japan

⁴⁾DSM Nutrition Japan K.K., Minato, Tokyo 105-0014, Japan

⁵⁾Department of Physiology and Pathophysiology, Institute of Nutritional Science, University of Potsdam, Potsdam-Rehbrücke, Germany

#Present: DuPont Industrial Biosciences, Danisco Japan Ltd., Tokyo 100-6111, Japan

Abstract. The aim of the present study was to investigate nutritional and metabolic parameters during the dry and early postpartum periods of ovulatory and anovulatory cows, as well as their postpartum reproductive performance. Blood samples from 20 multiparous Holstein cows were collected once a week from the far-off dry period to 3 weeks postpartum. Early postpartum (0–3 weeks) ovulation was confirmed using plasma progesterone concentration profiles, and cows were considered ovulatory if they had resumed luteal activity by this point ($n = 9$), whereas cows that had not were considered anovulatory ($n = 11$). Data from the ovulatory and anovulatory cows were analyzed separately for the far-off dry period (7–4 weeks prepartum), the close-up dry period (3–1 weeks prepartum), and the early postpartum period (0–3 weeks). Serum gamma-glutamyl transpeptidase activity (far-off, $P = 0.065$; close-up, $P = 0.051$; and early postpartum, $P = 0.030$) and aspartate aminotransferase (close-up, $P = 0.050$ and early postpartum, $P = 0.087$) activities were higher in anovulatory than in ovulatory cows. The days open period was longer ($P = 0.019$) in anovulatory than in ovulatory cows, and the number of artificial inseminations per conception ($P = 0.025$) was greater. In conclusion, we found that continuously high gamma-glutamyl transpeptidase activities in serum, which may be induced by liver disorders, prevent subsequent ovulation and affect subsequent fertility, even if cows obtain sufficient ovulation-related energy and β -carotene.

Key words: Dairy cow, First ovulation, Hepatic enzyme, Liver function, Reproductive performance

(J. Reprod. Dev. 62: 289–295, 2016)

The resumption of ovarian activity plays a crucial role in subsequent fertility [1, 2], and in dairy cows, the time until first postpartum ovulation is positively correlated to the resumption of normal ovarian function, first service, and conception rate [2, 3]. Our previous study showed that during the first postpartum follicular wave, ovulatory cows resume ovarian function more quickly and, subsequently, exhibit reproductive performances that are superior to those of anovulatory cows [4].

Dairy cows experience a negative energy balance after parturition, which is caused by insufficient feed intake that is required to provide

the energy necessary for milk production. [5]. In cows falling into negative energy balance, a typical metabolic profile includes elevated concentrations of growth hormone (GH) and non-esterified fatty acid (NEFA), as well as depressed concentrations of glucose, insulin, and insulin-like growth factor-I (IGF-1), all of which are associated with ovarian activity [6–8]. In addition, previous studies have reported that circulating IGF-1 and glucose concentrations are higher, and GH concentrations lower, in ovulatory cows than in anovulatory cows during the first postpartum follicular wave [9, 10]. Moreover, cows that exhibit increased lipolysis are at a higher risk of developing fatty liver or hepatic lipidosis [11], and cows with severe fatty liver exhibit lower fertility [12].

β -Carotene plays a role in the reproductive performance of dairy cows, and a positive relationship between supplemental β -carotene and reproductive performance has been demonstrated in many studies [13]. In addition, although β -carotene concentrations generally decline throughout the dry period, due to colostrum formation and decreased feed intake [14–16], lower concentrations are found in

Received: October 20, 2015

Accepted: February 17, 2016

Published online in J-STAGE: March 3, 2016

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Correspondence: C Kawashima (e-mail: kawashima@obihiro.ac.jp)

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anovulatory cows than in ovulatory cows [17]. Our recent study showed that β -carotene supplementation during the close-up dry period increases the number of ovulatory cows by 3 weeks postpartum [18]. Therefore, metabolic status during the early postpartum period and β -carotene levels during the close-up dry period are associated with reproductive performance in dairy cows.

In addition, de Feu *et al.* [19] found that eliminating the dry period advanced first postpartum ovulation, but a high-energy diet after parturition had no effect on the onset of the ovarian cycle. Moreover, Cavestany *et al.* [20, 21] found that a highly supplemented prepartum diet also advanced first ovulation, and another one of our previous studies [22] showed that postpartum ovarian function is crucially influenced by energy status during the dry period. The results of these studies suggest that the energy supply during the dry period critically affects nutrient partitioning, metabolism, and the reproductive axis. However, to our knowledge, no studies exist that have conducted long-term monitoring of the effects of prepartum nutritional and metabolic status on postpartum fertility.

Therefore, to define how nutrition and metabolism from the far-off dry period to the early postpartum period are associated with the resumption of ovarian activity in dairy cows, we investigated nutritional and metabolic parameters in ovulatory and anovulatory cows. In addition, reproductive performance after parturition was evaluated for both ovulatory and anovulatory cows.

Materials and Methods

Animals

Our experimental procedures complied with the Guide for the Care and Use of Agricultural Animals of Obihiro University. The experiment was conducted at the Field Center of Animal Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. We used 20 multiparous Holstein cows that had calved between January and June of 2008 and January and June of 2009. The body weight and body condition score (BCS) of cows at the beginning of the experiment were 681.2 ± 84.2 kg and 2.84 ± 0.39 , respectively. The BCS was assessed on a scale of 0 to 5, with 0.25 intervals (0 = thin and 5 = very fat) [23].

The cows were fed a total mixed ration that consisted of grass, corn silage, and concentrate during the dry period (far-off dry period, dry matter [DM] basis: 109 g crude protein [CP]/kg and 6.2 MJ net energy for lactation [NE_L]/kg; close-up dry period, DM basis: 127 g CP/kg and 6.6 MJ NE_L /kg). After parturition, the cows were housed in a free-stall barn and fed the total mixed ration (DM basis: 155 g CP/kg and 6.2 MJ NE_L /kg). Milking was performed twice daily at 0500 and 1700 h, and the average 305-day milk yield of the herd was approximately 9,800 kg. We recorded the daily milk yield, total milk yield during the previous lactation, length of dry period, calving difficulty, and any diagnoses of peripartum disease for all experimental cows.

Blood sampling

Blood samples were obtained 2–3 h before feeding by caudal venipuncture once a week from the far-off dry period to 3 weeks postpartum; the parturient week (week 0) was the period 0 to 6 days postpartum. For β -carotene and biochemical analyses, we used

unheparinized, silicone-coated 9-ml tubes (Venoject[®], Autosep[®], Gel + Clot. Act., VP-AS109K; Terumo, Tokyo, Japan). For progesterone (P_4), GH, and IGF-1 analyses, we used sterile 10-ml tubes that contained 200 μ l stabilizer solution (0.3 M EDTA and 1% acetyl salicylic acid, pH 7.4). Blood samples collected for serum analyses were coagulated for 15 min at 38°C in an incubator. For hematocrit (Ht), anticoagulated blood was analyzed shortly after collection using an automatic cell counter (MEK-6358; Nihon Kohden, Tokyo, Japan). All tubes were centrifuged at $2000 \times g$ for 20 min at 4°C, and the serum and plasma samples were kept at -30°C until analysis. In addition, BCS was also assessed whenever blood samples were taken. The same experimenter performed both tasks.

Determination of ovulation

A plasma P_4 concentration over 1 ng/ml was set as the benchmark for luteal activity in the experimental cows [24]. Cows that resumed luteal activity by 3 weeks postpartum were considered ovulatory, whereas those that had not were considered anovulatory.

Artificial Insemination

Cows were artificially inseminated at observed estrus after 50 days postpartum. Artificial inseminations (AIs) were carried out from spring to autumn in both 2008 and 2009 and were performed either once or twice per estrus, using one of 6 frozen bull semen samples. For each AI, a 0.5 ml aliquot of frozen-thawed semen was used. Cows with clear vaginal mucous discharge and internal signs of estrus (detected by rectal palpation) were considered in estrus. Cows in estrus were artificially inseminated within 6 hours after estrus detection, and ovulation was confirmed next day. If there were apparent reproductive disorders (cystic follicles or anovulation), hormonal treatment with 25 mg prostaglandin $F_{2\alpha}$ (Dinoprost tromethamine, Veterinary Pronalgon F injection; Zoetis Japan, Tokyo, Japan), and 100 μ g gonadotropin-releasing hormone (Fertirelin acetate, Conceral; Intervet, Osaka, Japan) was applied after 120 days postpartum. Conception was confirmed using ultrasonography (HS-1500V; Honda Electronics, Aichi, Japan) at 60 days after each AI.

Measurement of β -carotene, hormones, and metabolites

Serum β -carotene concentrations were measured as described previously [25], after one-step denaturation and extraction into an organic solvent, using the iEx[™] assay system and a carotene photometer (iCheck[™]; BioAnalyt GmbH, Teltow, Germany). The extraction efficiency was $> 95\%$ and the intra- and inter-assay coefficients of variation (CVs) averaged 7.4% and 8.3%, respectively.

Plasma P_4 concentrations were determined using enzyme immunoassay (EIA) after extraction with diethyl ether, as described previously [26]; the extraction efficiency was 90%. The standard curve ranged from 0.05 to 50 ng/ml, the mean intra- and inter-assay CVs were 6.0% and 9.2%, respectively, and the 50% effective dose (ED_{50}) of the assay was 0.78 ng/ml.

Plasma GH concentrations were also determined using EIA and biotin-streptavidin amplification [10]. The standard curve ranged from 0.78 to 100 ng/ml, the mean intra- and inter-assay CVs were 8.2% and 8.9%, respectively, and the ED_{50} was 3.1 ng/ml.

The total plasma IGF-1 concentration was determined using the same method as that for plasma GH [10], after protein extraction with

Table 1. Parity, dry period, calving difficulty, milk yield, and disease diagnosis in ovulatory and anovulatory cows

	Ovulatory cows (n = 9)	Anovulatory cows (n = 11)	P-value
Parity	1.7 ± 0.9	2.0 ± 1.1	0.537
Dry period (days)	76.9 ± 15.8	68.5 ± 11.8	0.189
Calving difficulty *	1.1 ± 0.3	1.1 ± 0.3	0.942
Daily milk yield during previous lactation (kg)	30.3 ± 3.9	32.9 ± 5.5	0.230
Total milk yield during previous lactation (kg)	9,087.0 ± 1,465.9	10,241 ± 2,296.1	0.209
Daily milk yield by 30 days postpartum during present lactation (kg)	42.6 ± 3.6	41.3 ± 6.9	0.632
Total milk yield by 30 days postpartum during present lactation (kg)	1,022.2 ± 86.4	992.4 ± 165.8	0.632
Number of cows with disease during previous lactation	5 (55.6%)	9 (81.8%)	0.336
Number of cows with disease during 3 weeks postpartum	3 (33.3%)	7 (63.6%)	0.370

Values are presented as means ± SDs. Significance was set at $P < 0.05$. * 1, unassisted birth (natural, without human assistance); 2, easy calving with human assistance; 3, difficult calving with a few humans; 4, dystocia (requiring considerably more force than normal); and 5, surgical treatment or death of cow.

an acid-ethanol solution (87.5% ethanol and 12.5% 2N hydrochloric acid) to obtain IGF-1 free from binding proteins [27]. The IGF-1 standard curve ranged from 0.39 to 50 ng/ml, the mean intra- and inter-assay CVs were 5.9% and 6.1%, respectively, and the ED₅₀ was 4.8 ng/ml.

Serum concentrations of glucose, NEFA, b-hydroxybutyric acid (BHBA), total protein, albumin, total cholesterol (T-cho), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (GGT) were measured using a clinical chemistry automated analyzer (TBA120FR; Toshiba Medical Systems, Tochigi, Japan). In addition, for serum samples, other than those taken during the far-off period of one cow in each group, low-density lipoprotein cholesterol (LDL-cho), high-density lipoprotein cholesterol (HDL-cho) and triglyceride (TG) concentrations were measured using a clinical chemistry automated analyzer (TBA120FR; Toshiba Medical Systems), in order to evaluate lipid metabolism and liver function. Globulin levels were calculated as the difference between total protein and albumin, after which the albumin/globulin ratio (A/G) was calculated.

Statistical analysis

The actual length of the dry period ranged from 56 to 106 days, so we used data from 7 weeks to 1 week prepartum as the dry period for analysis, whereas data from 7 to 4 weeks prepartum, 3 weeks to 1 week prepartum, and 0 to 3 weeks postpartum were used as the far-off dry period, the close-up dry period, and the early postpartum period, respectively. The data were analyzed separately for the far-off dry period, close-up dry period, and early postpartum period, using StatView (StatView 5.0 software, Abacus Concepts; Berkeley, CA, USA) and the repeated-measures ANOVA procedure, which included time (week in each period), group (ovulatory or anovulatory), and individual cows as the repeated subjects. Moreover, the significance of time (week) within each group was analyzed using the Tukey-Kramer test. The Kolmogorov-Smirnov test (SAS Enterprise Guide version 4.3; SAS Institute, Cary, NC, USA) was used to verify that the data had normal distributions.

Differences in the disease diagnosis of ovulatory and anovulatory cows during the previous lactation period and 3 weeks postpartum, as well as the conception rate of the first AI, were analyzed using chi-square tests. Depending on whether the normality assumption

was met, between-group differences in the means of all other data were analyzed using the Student's *t*-test, Welch's *t*-test, or Wilcoxon's signed rank test (SAS Enterprise Guide version 4.3; SAS Institute). The results are expressed as percentages or means ± standard deviations (SDs), and probabilities of $P < 0.05$ were considered significant.

Results

By 3 weeks postpartum, 9 (45.0%) of the cows were ovulatory, and 11 (55.0%) were anovulatory. No difference was found between the parity, length of dry period, calving difficulty, or daily or total milk yield during the previous lactation and 30 days postpartum during the present lactation period of ovulatory and anovulatory cows (Table 1). One ovulatory and one anovulatory cow easily calved with human assistance, whereas all other cows had unassisted calving. Five ovulatory cows were diseased during the previous lactation (n = 1, mastitis; n = 2, mastitis and lameness; n = 1, lameness; n = 1, ketosis) and 3 were diseased 3 weeks postpartum (n = 1, hypocalcemia; n = 2, mastitis), whereas in anovulatory cows, 9 were diseased during the previous lactation (n = 3, mastitis; n = 3, mastitis and lameness; n = 2, mastitis and ketosis; n = 1, lameness) and 7 were diseased 3 weeks postpartum (n = 1, hypocalcemia; n = 6, mastitis). No significant differences were observed between the two groups (Table 1).

During the far-off dry period, serum GGT activities ($P = 0.065$) and TG concentrations ($P = 0.065$) tended to be higher and serum β-carotene concentrations tended to be lower ($P = 0.092$) in anovulatory cows than in ovulatory cows (Fig. 1). Furthermore, a significant treatment and time effect was observed for T-cho concentrations ($P = 0.022$) and GGT activities ($P = 0.047$) during the far-off dry period, and the decreases of serum T-cho concentrations and GGT activities during the far-off dry period were greater in anovulatory cows than in ovulatory cows. During the close-up dry period, the serum AST ($P = 0.050$) and GGT ($P = 0.051$) activities tended to be higher in anovulatory than in ovulatory cows, and a significant treatment and time effect was observed for serum TG concentrations ($P = 0.033$). In addition, the serum TG concentrations increased gradually as the calving day approached in ovulatory cows, whereas its concentration did not change in anovulatory cows. After calving, serum AST ($P =$

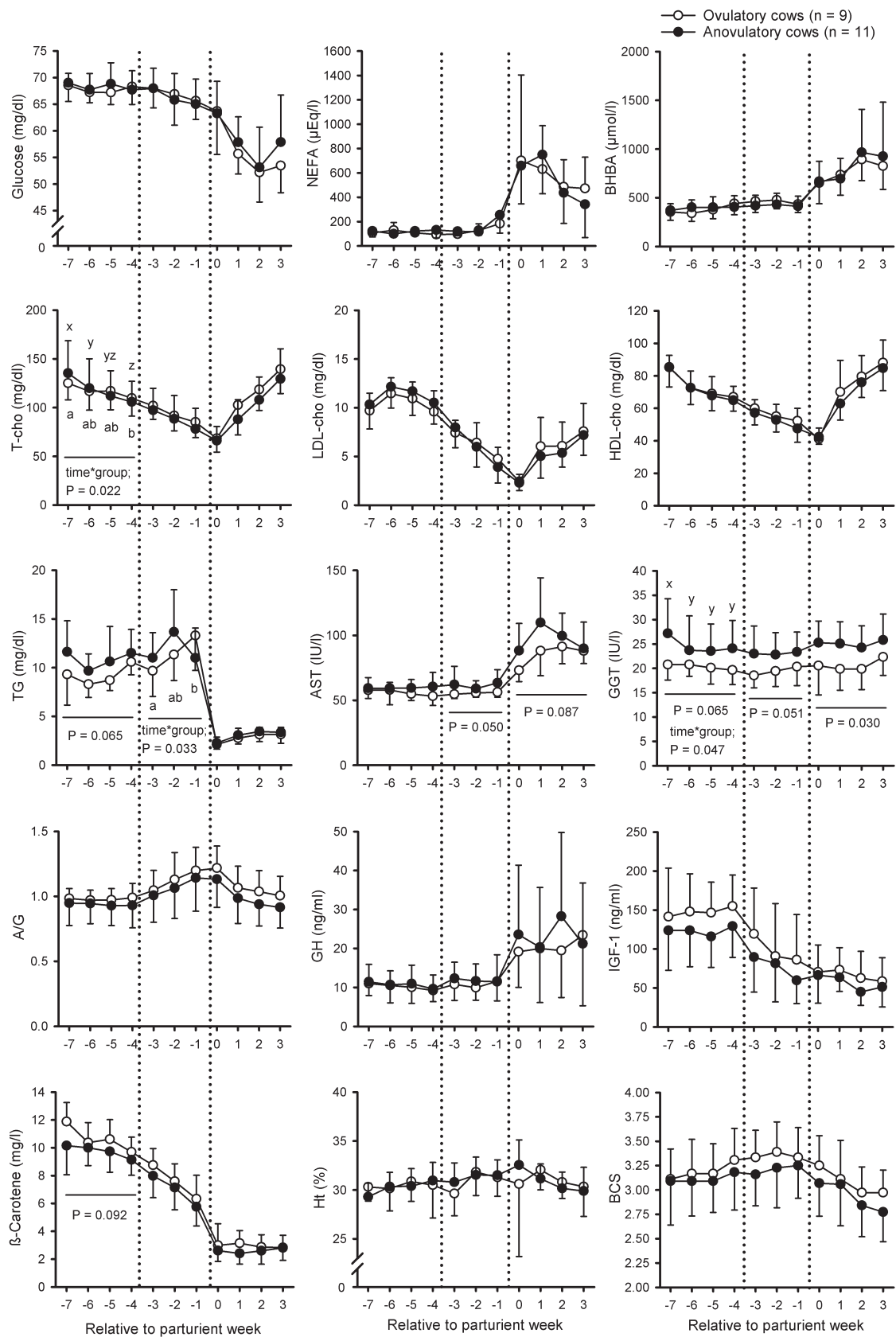


Fig. 1.

Table 2. Comparison of variables related to conception from artificial insemination (AI) in ovulatory and anovulatory cows

	Ovulatory cows	Anovulatory cows	P-value
Number of cows for analysis	8	10	
Days to first AI	79.4 ± 20.3	79.0 ± 9.4	0.963
Conception rate of first AI	75% (6/8)	20% (2/10)	0.054
Number of cows for analysis	6	7	
Days open	77.0 ± 15.7	145.4 ± 56.9	0.019
Number of AI per conception	1.0 ± 0.0	2.6 ± 1.4	0.025

Values are presented as means ± SDs. Two cows did not receive AI because of mastitis (ovulatory cows, n = 1; anovulatory cows, n = 1), so the data from these cows were excluded from the fertility analysis. Significance was set at $P < 0.05$.

0.087) and GGT ($P = 0.030$) activities were higher in anovulatory than in ovulatory cows. However, no significant differences were observed between ovulatory and anovulatory cows with regard to the other variables.

Two cows did not receive AI because of mastitis (ovulatory cows, n = 1; anovulatory cows, n = 1), so the data from these cows were excluded from the fertility analysis. In addition, five cows did not conceive during the lactation period (ovulatory cows, n = 2; anovulatory cows, n = 3), so data from these cows were excluded from the analyses of AI number per conception and days open. The conception rate after the first AI tended to be higher in ovulatory cows than in anovulatory cows ($P = 0.054$), although the number of days to the first AI did not differ between the two groups (Table 2). Anovulatory cows had a longer days open period than ovulatory cows ($P = 0.019$), and the number of AIs per conception was greater in anovulatory cows than in ovulatory cows ($P = 0.025$; Table 2).

Discussion

Anovulatory cows exhibited higher serum GGT activity than ovulatory cows from the far-off dry period to the early postpartum period. On the other hand, the energy status (including glucose, GH, and IGF-1 levels), which has been associated with the onset of ovarian activity after calving, did not differ between two groups. The peripartum blood level of IGF-1 is positively and closely related with the first postpartum ovulation [9, 10, 22, 28], and the results of our previous study [17] showed that ovulatory cows exhibit higher plasma β -carotene concentrations than anovulatory cows during the close-up dry period. However, in the present study, the plasma IGF-1 and serum β -carotene concentrations did not differ significantly during the close-up dry period or early postpartum period of either ovulatory or anovulatory cows. Generally, plasma IGF-1 concentrations are positively correlated with the level of

feed intake [7], and decreases in plasma β -carotene during the dry period [15–17] reflect a decrease in feed intake, since pasture and high quality forage are rich in β -carotene [29]. Therefore, our results suggest that the energy status of ovulatory and anovulatory cows were similar throughout the dry and early postpartum periods.

This outcome implies that both groups obtained sufficient β -carotene from their feed, although the serum β -carotene concentration in the far-off dry period was slightly higher for ovulatory cows than anovulatory cows. Thus, the present study also shows that the energy status of ovulatory and anovulatory cows was better than in previous studies [14, 16, 17, 30].

Apolipoproteins, which include very low-density lipoprotein (VLDL), LDL, and HDL, are mainly synthesized in the liver, whereas the major lipids of VLDL are TG [31]. Therefore, T-cho, LDL-cho, HDL-cho, and TG concentrations in the serum are reflective of liver function. In the present study, we found that liver function did not differ between ovulatory and anovulatory cows, since the concentrations of the above markers were similar in both groups. In addition, we assumed that mobilization from adipose tissue to the liver and lipid metabolism in the liver were similar between the two groups in serum NEFA and BHBA concentrations.

In addition, milk yield did not differ between ovulatory and anovulatory cows, again indicating that ovulatory and anovulatory cows shared the same energy status. However, although previous studies revealed the relationship between energy status during the peripartum period and the onset of the ovarian activity after parturition [9, 10, 17, 22, 28], the present study did not reveal the same relationship. This could be because previous studies did not measure blood GGT activity [10, 19–21] or because the differences of GGT activity in ovulatory and anovulatory cows were not confirmed [22]. Furthermore, cows with hepatic loads, which are induced by the mobilization of body fat and the accumulation of triglycerides in the liver, have been shown to exhibit delayed first ovulation after calving [32], as well as hampered oocyte maturation and developmental competence [33]. Therefore, cows without liver disorders exhibited high GGT activity, and the energy status could be the main factor that induces earlier resumption of ovarian activity after calving. On the other hand, cows with long-term elevated GGT activity, which can be induced by liver disorders, could delay first postpartum ovulation, even if cows are provided with ideal feed.

In the present study, we were not able to determine the cause of liver disorders, such as high GGT activities. However, previous studies have shown that plasma GGT and AST activities increase in cows with severe fatty livers [34], and that cows with fatty livers exhibit high blood NEFA and BHBA concentrations [35–37]. In addition, blood AST activity is higher in cows with mildly fatty livers than in healthy cows, even though their blood NEFA and BHBA concentrations are similar [38]. Moreover, serum TG concentrations decrease during the peripartum period [39–41], since greater fat mobilization from

Fig. 1. Comparisons of nutritional and metabolic from the far-off dry period to the early postpartum period in ovulatory and anovulatory cows (open, ovulatory cows [n = 9]; solid, anovulatory cows [n = 11]). Values are presented as means ± SDs. Data were analyzed separately for the far-off dry period, the close-up dry period, and the early postpartum period, using the repeated-measures ANOVA procedure, which included time (week in each period), group (ovulatory and anovulatory cows), and individual cows as the repeated subjects. Moreover, the significant effect of time (week) within each group was analyzed using the Tukey-Kramer test. P values indicate differences between ovulatory and anovulatory cows or significant time and group effect. The letters a and b indicate differences of $P < 0.05$ among ovulatory cows in each period, whereas x, y, and z indicate differences of $P < 0.05$ among anovulatory cows in each period.

adipose tissue to the liver is induced to compensate for negative energy balance. This led to the accumulation of TG in the liver and the secretion of VLDL, which transports TG from the liver, which is low compared with TG synthesis in the liver [31, 42].

In addition, serum NEFA and BHBA concentrations did not differ during the dry period for either ovulatory or anovulatory cows, and TG concentrations tended to be higher during the far-off dry period in anovulatory cows, whereas serum AST activities tended to be higher during the close-up dry period and early postpartum period. Thus, the present study indicates that the high GGT activity observed in anovulatory cows may not have been caused by fatty liver. However, liver disorders, including hepatitis, degeneration, and telangiectasia, are all more common in cows with fatty livers [43]. Therefore, anovulatory cows with higher GGT activity may be more likely to acquire such disorders. Further studies are needed to investigate the causes of continuous high GGT activity and to determine strategies for improving liver function.

Postpartum reproductive performance is greater in ovulatory cows than in anovulatory cows, and the ratio of abnormal cycles is higher in anovulatory cows than in ovulatory cows [4]. Furthermore, abnormal ovarian cycles before service negatively affect reproductive performance by increasing the interval until first AI and by lowering the pregnancy rate [44]. In addition, early resumption of ovarian activity and increased ovarian cycles before AI may be related to reproductive performance [45].

In the present study, ovulation by 3 weeks postpartum was associated with high fertility, supporting the results of previous work [2–4]. Moreover, cows with liver disorders exhibited higher GGT activity in follicular fluid, as well as a negative correlation between the GGT activity of the follicular fluid and the rate of blastocyst formation after *in vitro* fertilization [43]. Sarentonglaga *et al.* [43] concluded that higher GGT activity in follicular fluid causes a reduced glutathione concentration in oocytes and embryos, which results in a decrease in the anti-oxidative capability of the resultant embryos. Thus, oocytes from cows with damaged livers exhibit reduced ability to reach the blastocyst stage [46].

Moreover, Tanaka *et al.* [47] showed that delayed meiotic maturation of oocytes in dairy cows with damaged livers is induced by slow gap junctional communication closure between oocytes and cumulus cells, which results in lower fertility. Furthermore, cows with damaged livers exhibit lower concentrations of growth factors in their follicular fluid, compared with those of cows with healthy livers [48]. Therefore, in the present study, the higher GGT activity during the far-off dry period could have continued until AI, although we did not measure the serum GGT activity before AI.

In conclusion, continuously high GGT activities in the serum, which may be induced by liver disorders, prevent ovulation in the latter time frame and affect subsequent fertility, even if cows obtain sufficient ovulation-related energy and β -carotene. It is clear that sufficient energy is a major factor necessary for the improvement of reproductive function in peripartum dairy cows; however, once a cow acquires a liver disorder, reproductive performance might be negatively affected, even if the energy status is improved.

Acknowledgments

We thank Dr Kiyoshi Okuda (Okayama University, Japan; currently of Obihiro University of Agriculture and Veterinary Medicine, Japan) for the P₄ antiserum and Dr Parlow (National Institute of Diabetes and Digestive Kidney Diseases) for the GH standard and the GH and IGF-I antisera. This study was supported by a Grant-in-Aid for Scientific Research (C; Grant number: 19580320) and the Global COE program from the Japan Society for the Promotion of Science.

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