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FULL PAPER

Theriogenology

Association between liver failure and hepatic UDP-glucuronosyltransferase activity in dairy cows with follicular cysts

Kouichi TANEMURA[1)], Tadatoshi OHTAKI[1)]*, Yasushi KUWAHARA[1)] and Shigehisa TSUMAGARI[1)]

[1)]Laboratory of Theriogenology, College of Bioresouce Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan

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ABSTRACT. Uridine 5′-diphospho-glucuronosyltransferase (UGT) liver activity was measured using estradiol-17β as a substrate in dairy cows with follicular cysts. The activity was significantly lower than that in dairy cows with normal estrous cycles ($P<0.01$). Liver disorders, such as fatty liver and hepatitis, were observed in half cows with follicular cysts, and liver UGT activity was lower than that in cows with normal estrus cycles. In addition, the liver UGT activity was significantly lower in dairy cows with follicular cysts without liver disorders than in dairy cows with normal estrous cycles. Therefore, the cows were divided into those with low, middle and high liver UGT activities, and liver disorder complication rates were investigated. The complication rate was significantly higher in the low- (78.1%) than in the middle- (22.2%) and high-level (8.3%) groups, suggesting that liver disorders are closely associated with the development of follicular cysts in dairy cows and that steroid hormone metabolism is delayed because of reduced liver UGT activity, resulting in follicular cyst formation. We conclude that reduced estradiol-17β glucuronidation in the liver and liver disorders are associated with follicular cyst occurrence in dairy cows.

KEY WORDS: dairy cows, fatty liver, follicular cyst, liver disorder, UGT activity

More than 50% of dairy cows develop fatty liver in the periparturient period, because of metabolic changes resulting from the transition to the lactation period and a moderate or severe negative energy balance [11, 12, 31]. Fatty liver is the major cause of periparturient diseases [4, 29, 30] and reproductive disorders [5, 20, 25]. We have previously demonstrated that dairy cows with moderate or severe fatty liver after parturition tend to develop various secondary diseases, including follicular cysts, and many become disused and are culled within a year [26]. It has been reported that approximately 23% of the first follicular waves after parturition [2, 3] or in the early postpartum period [21] become follicular cysts in dairy cows.

Reportedly, there are two peaks of follicular cyst development in the early postpartum period [21]: 30–40 days [1] and 190–220 days [1, 15] after parturition, and it has also been reported that 70% of follicular cysts develop within 3 months after parturition [16]. This timing is consistent with the period of fatty liver development (peaks at 1–5 weeks after parturition) [7]. Some follicular cysts spontaneously resume an estrous cycle, but approximately half repeat turnover [6, 23]. In follicular cysts, the estrogen level increases with turnover of coexisting follicles [13, 34], and the estrogen level in follicular cysts is higher than that in the estrous cycle [9, 15, 27, 28]. Accordingly, it was suggested that before follicular cyst development, estrogen increases progesterone receptor expression in the hypothalamus of rats [24]. In ewes, it has been reported that estradiol enhances progesterone receptor mRNA concentrations within all uterine cells [10].

The clinical incidence of follicular cysts has been reported to be 6–19% [23], and approximately half of untreated follicular cysts repeat turnover of coexisting follicles [6, 22, 23]. The cause and mechanism of turnover-repeating follicular cysts have not been clarified, and the association with liver disorders, which frequently develop during the same period, has not been investigated. In this study, focusing on steroid-metabolizing liver function, we investigated the association between the activity of uridine 5′-diphospho (UDP)-glucuronosyltransferase (UGT), the main conjugating enzyme in the liver, and follicular cyst development in dairy cows.

*Correspondence to: OHTAKI, T., Laboratory of Theriogenology, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan. e-mail: ohtaki.tadatoshi@nihon-u.ac.jp

MATERIALS AND METHODS

Animals

Seventy Holstein dairy cows (62 with follicular cysts and eight with normal estrous cycles) maintained by 23 dairy farms near Mito City, Japan, were investigated. All cows were maintained in tie-stall barns, fed dry grasses (such as timothy, oat and Italian ryegrass) and corn silage before parturition, and fed concentrated feed from 1 week before parturition. After parturition, cows were fed mainly dry grasses, such as lucerne, timothy and oat, and corn silage, and concentrated feed was increased depending on the milk yield. All herds received regularly scheduled visits from veterinarians one or two times a month. Follicular cysts were diagnosed by only rectal palpation of 3–4 times at weekly intervals. When a fluctuant follicle with a diameter of at least 2.5 cm persisted for 10 days or longer without a corpus luteum, it was defined as a follicular cyst [14], based on follicle size, fluid content, follicular wall thickness and behavioral changes. Follicular cystic conditions persisted for at least 3–4 weeks.

Follicular cysts were diagnosed in 62 dairy cows at 94.4 ± 7.6 days after parturition; comprising the first medical examination, and normal estrous cycles were observed in eight dairy cows at 89.3 ± 14.6 days after parturition. Measurements of the peripheral blood steroid hormone in these cows were not performed. The cows of the normal estrous cycle at the time of reproductive examinations did a sampling. The authors confirmed that there was no difference in UGT activity within an estrous cycle.

Liver biopsy

Liver specimens (approximately 1 g; 4 × 4 ×30 mm) were collected in a blinded manner from dairy cows diagnosed with follicular cysts at the first medical examination and those with normal estrous cycles using a puncture device for cattle liver (Fujihira Industry, Tokyo, Japan) following the method of Ono *et al*. [18]. The collected liver specimens were immediately placed in a serum tube for freezing, stored in liquid nitrogen (−196°C) and subjected to UGT activity measurement. Tissues stored in formol-calcium solution were subjected to histopathological examination. Liver biopsy specimens collected from 62 dairy cows with follicular cysts and eight dairy cows with normal estrous cycles were subjected to liver UGT activity measurements using the substrate metabolism method.

Pathological examination

Pathological diagnoses were made based on findings of hematoxylin eosin (H-E), Sudan III and Nile blue staining. When infiltration of inflammatory cells, such as lymphocytes and neutrophils, was noted on H-E staining, the animal was diagnosed with hepatitis. Liver tissue with vacuolation on H-E staining was subjected to fat staining to definitively diagnose fatty liver. A hepatic fat deposition rate of >10% was diagnosed as fatty liver. Cows with hepatitis and fatty liver with hepatocellular degeneration were classified with liver disorder.

Hepatic UGT assay

Liver UGT was measured after partially modifying a previously reported method [19]. Approximately 0.25 g wet weight of half-thawed liver tissue was placed in a tube for ultracentrifugation and minced in four volumes (1 m*l*) of ice-cooled 0.15 M potassium chloride using ophthalmic scissors. This suspension was transferred into a Teflon glass vessel (5 m*l*) and homogenized using a homogenizer (B. Braun Melsungen, Wendelsteinstr, Germany) at 1,000 rpm for 60 sec with ice-cooling. The whole suspension was transferred into a tube for ultracentrifugation and centrifuged at 9,000 ×*g*, 4°C for 15 min, and the supernatant was further centrifuged at 105,000 ×*g*, 4°C for 1 hr (Beckman Coulter Inc., Brea, CA, U.S.A.). The supernatant was removed, and the precipitate was combined with 250 *µl* of 0.25 M sucrose-0.1 M Tris-HCl buffer (pH 7.4) and stirred. This microsomal fraction was used as the sample. In a preliminary test with 0, 5, 10, 15 and 20 *µl* of normal liver microsomal fraction, the reaction was linear within the 0 to 10 *µl* range. Therefore, the sample volume was set at 5 *µl*. When a time-course test was performed with 5 *µl* of microsomal fraction, linearity was observed from 0 to 20 min. Consequently, the reaction time was set at 10 min. Each sample measurement was performed in duplicate.

The sample-containing test tubes were immersed in ice-cold water, and 250 *µl* of 0.25 M sucrose-0.1 M Tris-HCl buffer (pH 7.4), 20 *µl* of 1 mg/ m*l* bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.), 50 *µl* of 0.1 M magnesium chloride, 100 *µl* of 10 mM UDP-glucuronic acid (Sigma Chemical Co.), 5 *µl* of microsomal fraction and 1.88×10^5 dpm (100 *µl*) of [4-^{14}C]-estradiol-17β (specific activity: 58.2 Ci mmol^{-1}, New England Nuclear Co., Boston, MA, U.S.A.) were added to each tube and stirred, followed by incubation with shaking 120 times/min at 37°C for 10 min. The reaction was stopped by adding 200 *µl* of ethanol and rapid vortexing, to which 1 m*l* of distilled water was added and stirred, followed by 5 m*l* of chloroform. The mixture was shaken for 2 min using a shaker and centrifuged at 3,000 rpm for 15 min. The supernatant (500 *µl*) was combined with 2 m*l* of liquid scintillator, and the radioactivity was measured using a liquid scintillation counter (Hitachi Aloka Medical, Ltd., Tokyo, Japan). The protein concentration in the samples was measured following the method of Lowry *et al*. [17] using bovine serum albumin as a standard protein. The enzyme activity level was presented as formed pM glucronide/min/mg protein, and the mean of two measurements was adopted. The within-measurement coefficient of variation was 8%.

Statistical analyses

Statistical analyses were performed using statistical analysis software, JMP® 11.2.0 (SAS INS, Tokyo, Japan). For between-group comparison of means, nonparametric Wilcoxon's test was used, and the ratio was compared using the chi-square test. The results were presented as the mean ± standard error. A significance level of 5% or lower was regarded as significant.

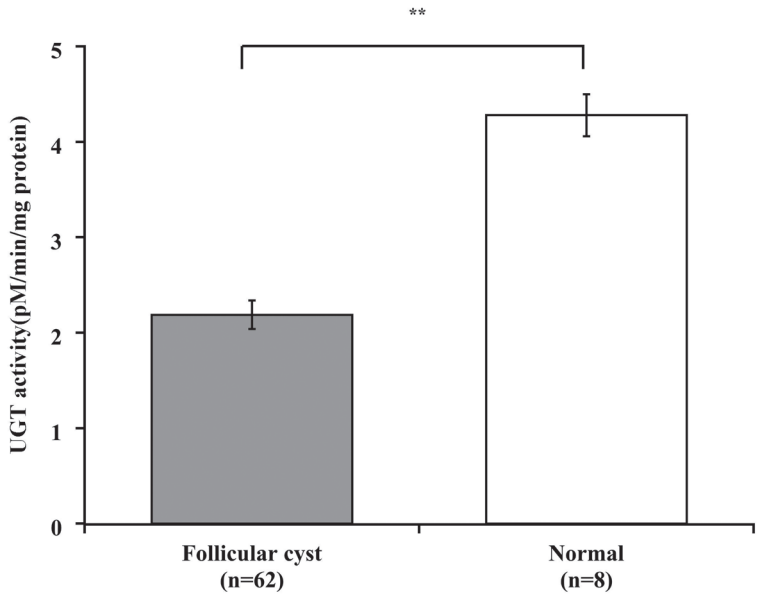

Fig. 1. Comparison of hepatic UDP-glucuronosyltransferase activity in dairy cows with follicular cysts and normal estrous cycles. Vertical lines of the bar graph show the standard error. Differences in mean values between two groups were examined by Wilcoxon test; **: Asterisks indicate significant differences of $P<0.01$.

RESULTS

Comparison of the liver UGT activity level between dairy cows with follicular cysts and those with normal estrous cycles

Liver UGT activity levels were 2.19 ± 0.15 and 4.28 ± 0.22 pM/min/mg protein in the follicular cyst (n=62) and normal estrous cycle groups (n=8), respectively (Fig. 1), showing a significantly lower level in the follicular cyst group ($P<0.01$). When the animals were limited to those at 60–90 days after parturition, the same stage of the cows with normal estrous cycles, the UGT activity level was 2.30 ± 0.36 pM/min/mg protein in the follicular cysts group (n=21), significantly lower than that in the normal estrous cycle group (n=8) ($P<0.05$).

Liver UGT activity level in cows with pathological liver disorders

The association between liver disorder and liver UGT activity level was investigated in the dairy cows with follicular cysts. Liver tissues collected from the 62 dairy cows with follicular cysts were pathologically examined using H-E, Sudan III and Nile blue staining. Hepatitis (n=13), fatty liver (n=11), and concomitant fatty liver and hepatitis (n=6) were recorded. These 30 cows were designated as the liver disorder group, and the remaining 32 cows with follicular cysts without a pathological finding were designated as the non-liver disorder group. The liver UGT activity levels in the groups with and without liver disorder were 1.57 ± 0.12 and 2.78 ± 0.23 pM/min/mg protein, respectively, and were significantly lower than that (4.28 ± 0.22 pM/min/mg protein) in the normal estrous cycle group ($P<0.01$). The UGT activity level was lower in the cows with follicular cysts without liver pathological abnormality than in the cows with normal estrous cycles, showing that the liver UGT activity level was reduced in dairy cows with follicular cysts regardless of the presence or absence of liver disorder (Fig. 2). Such clear differences by days postpartum were not observed (Fig. 3). When the analysis was limited to cows at 60–90 days after parturition (reflecting normal estrous cycles), liver UGT activity levels in the groups with and without liver disorder were 1.27 ± 0.20 and 3.10 ± 0.51 pM/min/mg protein, respectively. The UGT activity level of the liver disorder group (n=9/30) was significantly lower than that in the normal estrous cycle group (n=8) ($P<0.01$) and non-liver disorder group (n=12/32) ($P<0.05$), but there was no significant difference between the normal estrous cycle group and non-liver disorder group.

Incidence of follicular cyst and liver disorder complication rate by liver UGT activity level

The cows were divided into three groups based on the measured liver UGT activity level, and the incidence of follicular cysts and liver disorder complication rate were investigated in each group. When cows with a liver UGT activity level of 2.0 pM/min/mg protein or lower were designated as the low-level group (n=32), the mean UGT activity level was 1.37 ± 0.08 pM/min/mg protein. In cows with a level of 2.0–3.0 pM/min/mg protein, designated as the middle-level group (n=18), the mean level was 2.35 ± 0.06 pM/min/mg protein. In cows with a level of 3.0 pM/min/mg protein, designated as the high-level group (n=12), the mean level was 4.15 ± 0.31 pM/min/mg protein. The rates of cows with follicular cysts in the low-, middle- and high-level groups were 51.6 (32/62), 29.0 (18/62) and 19.4% (12/62), respectively, with a higher incidence in the low-level group. The liver disorder complication rates in the low-, middle- and high-level groups were 78.1 (25/32), 22.2 (4/18) and 8.3% (1/12), respectively, with a

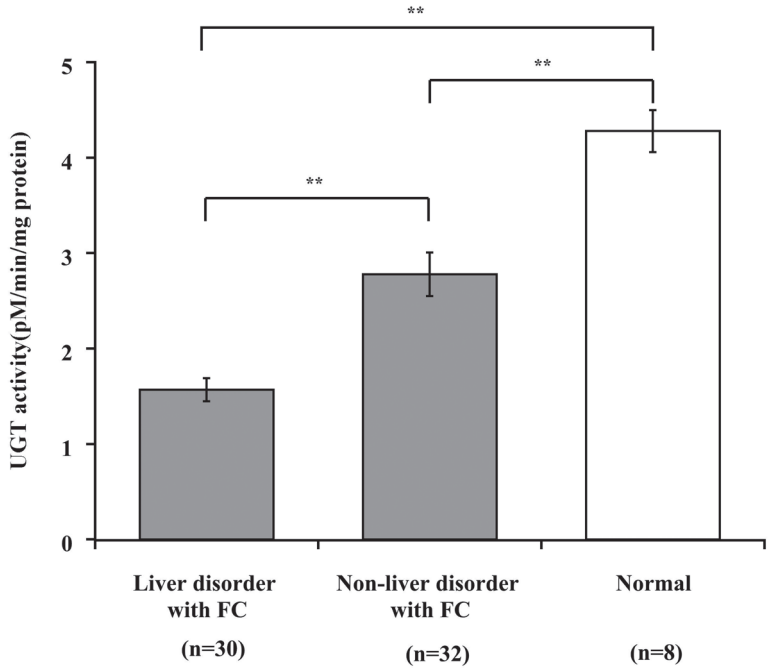

Fig. 2. Comparison of hepatic UDP-glucuronosyltransferase activity in dairy cows with follicular cyst concomitant with liver disorder and non-liver disorder, and those with normal estrous cycles. Normal means normal estrous cycle. Vertical lines of bar graph show the standard error. Differences in mean values between two groups were examined by Wilcoxon test; **: Asterisks indicate significant differences of $P<0.01$.

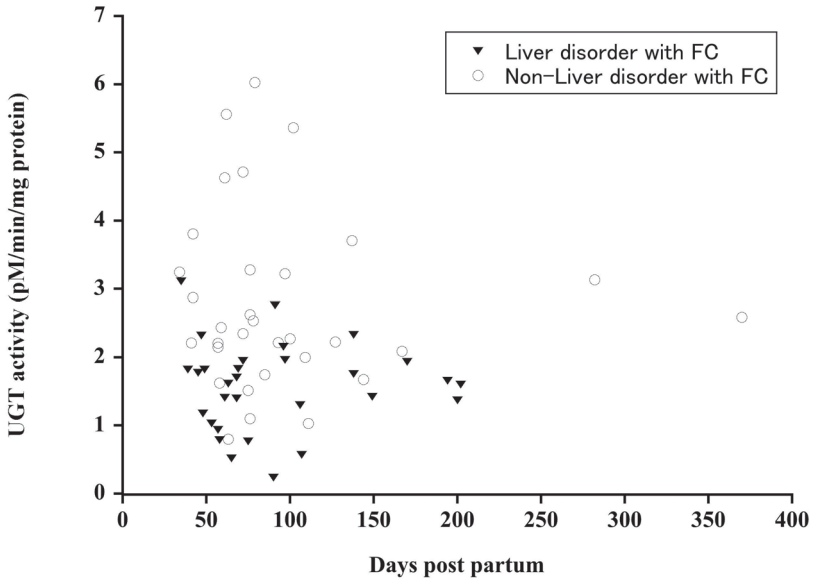

Fig. 3. Comparison of hepatic UDP-glucuronosyltransferase activity in dairy cows with follicular cyst concomitant with liver disorder and non-liver disorder during postpartum.

significantly higher rate in the low-level group than in the other groups (Fig. 4: $P<0.01$).

DISCUSSION

In this study, we measured uridine 5′-diphospho-glucuronosyltransferase (UGT) activity in the liver using estradiol-17β as a substrate in dairy cows with follicular cysts. Estrogen is conjugated by the second-phase metabolic enzyme in the liver and hepatic UGT, and is excreted into urine and feces through the enterohepatic circulation [33]. Rao *et al.* [19] observed that estrone glucuronosyltransferase activity in ovariectomized rats increased after 6 days of estradiol-17β daily treatment; the specific activity

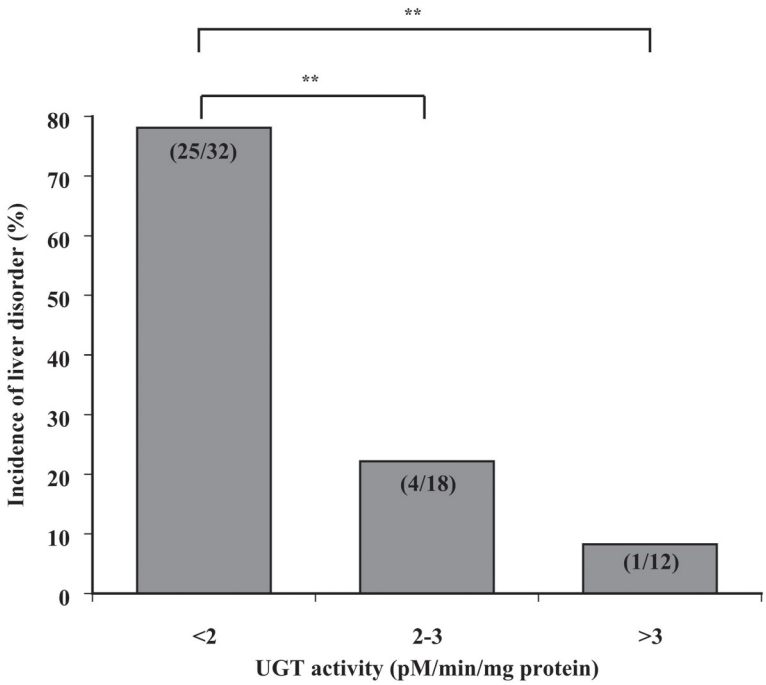

Fig. 4. Incidence of concomitant liver disorder at low (<2), middle (2–3) and high (>3) levels of hepatic UDP-glucuronosyltransferase activity in dairy cows with follicular cysts. **: Asterisks indicate significant differences of $P<0.01$.

continued to rise over the following 6 days. However, non-ovariectomized rats did not react to estradiol-17 treatment. In the past experiments of the authors, although the measurement method was different, the UGT activity in the cows was not significantly different between 0, 7, 14 and 21 days of the estrus cycle. Therefore, fluctuations in the concentration of estrogen during the estrus cycle apparently do not influence the activity of glucuronosyltransferase (unpublished data).

In our study, the liver UGT activity level, measured using estradiol-17β as a substrate, was significantly lower in the dairy cows with follicular cysts than in those with normal estrous cycles (60–90 days after parturition; $P<0.05$), suggesting delayed metabolic conjugation of blood estrogen. In addition, when dairy cows with follicular cysts were divided into the low, middle- and high-liver UGT activity groups, the proportion of cows with follicular cysts was higher in the low-level group (51.6%), and the liver disorder complication rate in the three groups was 78.1% (25/32), 22.2% (4/18) and 8.3% (1/12), respectively, showing a significantly higher rate in the low-level group. These results suggest that liver disorders are closely involved with the development of follicular cysts. Veenhuizen *et al*. [29] performed a ketosis development test in dairy cows and observed a transition to severe fatty liver before the manifestation of ketosis symptoms, in addition to a reduction in liver UGT activity level, consistent with our findings. However, there was no significant difference between the normal estrous cycle group and the non-liver disorder group in cows at 60–90 days after parturition. This indicates that the incidence of follicles cysts in this period did not cause a reduction in liver UGT activity.

Although the relationship between ovarian cysts and liver blood flow rate is unclear, Wiltbank *et al*. [32] reported that the liver blood flow in high-yield dairy cows is higher than that in low-yield dairy cows, increasing the amount of steroid hormones metabolized in the liver and that this was the underlying reason for reduced estrous behavior in high-yield dairy cows. In contrast, Ono *et al*. [18] reported that the progesterone and estrogen levels in estrus immediately after a short 4-day fasting period in dairy cows were significantly higher than those in the free-feeding group and that this may have been due to a reduced liver blood flow-induced delay in steroid hormone metabolism.

Furthermore, during starvation, estradiol-17β is elevated, hepatic lipid and triglyceride accumulate, and plasma has a very low density lipoprotein concentration [8]. Silvia *et al*. [23] reported that when the blood progesterone level is suprabasal, or 0.1–1 *n*g/m*l*, the hypothalamic surge center becomes inhibited, leading to follicular cyst formation. Vanholder *et al*. [27] also reported a suprabasal progesterone level (intermediate levels) in the presence of follicular cysts; however, they did not mention the potential cause of such an elevation. Since the degree of the state of steroid metabolism of peripheral blood is unclear, these results need a warrants examination. Since the liver UGT activity of cows with follicular cysts is low, a utilization of a hepatic activation medicine is considered to be useful as a treatment of follicular cysts.

In conclusion, reduced estradiol-17β glucuronidation in the liver and the liver disorders are associated with follicular cyst occurrence in dairy cows.

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