The Journal of Veterinary Medical Science

FULL PAPER

Theriogenology

Effect of high-concentrate corn grain diet-induced elevated ruminal lipopolysaccharide levels on dairy cow liver function

Tadatoshi OHTAKI[1)]*, Kanae OGATA[1)], Hiroshi KAJIKAWA[2)], Toshiaki SUMIYOSHI[3)], Sanae ASANO[2)], Shigehisa TSUMAGARI[1)] and Tetsuya HORIKITA[1)]

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

[2)]Laboratory of Animal Nutrition, Department of Animal Science and Resources, College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252-0880, Japan

[3)]Laboratory of Large Animal Clinical Sciences, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan

ABSTRACT. A high-concentrate diet destroys gram-negative bacteria in the cattle rumen, leading to elevated ruminal lipopolysaccharide (LPS) levels. LPS causes liver inflammation through the hepatic portal vein but little is known about the effects of rumen-derived LPS on liver function and the reproductive organs. In this study, we determined the effect of increasing rumen fluid LPS levels on liver function and genital LPS levels. Cows were assigned to control (CON; n=5) and high-concentrate diet (HC; n=7) groups. We observed that the ruminal LPS and haptoglobin (Hp) levels were significantly higher and albumin levels were lower in the HC group than in the CON group. In the HC group, The Hp levels and aspartate transaminase (AST) activity were significantly higher and the total cholesterol levels were significantly lower after high-concentrate diet feeding than before feeding. No differences were observed in LPS levels in the peripheral veins, hepatic veins, hepatic portal vein, uterine perfusate, and follicular fluids between the groups. In all samples, the LPS level in the hepatic portal vein blood positively correlated with the AST activity and serum amyloid A level. In conclusion, our results indicate that high-concentrate diets do not have a direct effect on the reproductive organs upon a moderate ruminal LPS level increase. However, an increased ruminal LPS influx into the liver might affect negatively liver function.

KEY WORDS: high-concentrate diet, lipopolysaccharide, liver, reproductive organ, rumen

J. Vet. Med. Sci.
82(7): 971–977, 2020
doi: 10.1292/jvms.20-0117

Received: 27 February 2020
Accepted: 4 May 2020
Advanced Epub: 27 May 2020

In order to compensate for the energy shortage in high-lactating cows, the amount of their concentrated feed has been increased over the years. However, feeding large amounts of a high-concentrate diet leads to rapid fermentation in the rumen and a lower pH due to an increase in hydrogen ions caused by elevated levels of volatile fatty acids (VFAs) and lactic acid [27]. The decreasing pH changes the microbiota in the rumen and kills gram-negative bacteria, resulting in the release of large amounts of the endotoxin lipopolysaccharide (LPS) from the surface of the dead cells [16, 17, 26, 27]. In high-concentrate dietary trials, subacute ruminal acidosis (SARA) reportedly caused an increase in ruminal LPS levels [10–12, 16, 17, 19]. The LPS in the rumen fluid is mainly absorbed by the rumen wall, then it passes into the ruminal veins and reaches the liver via the portal vein [12, 16, 17]. However, the intestinal environment may also affect LPS translocation into the bloodstream [16].

A previous study focusing on postpartum cows with uterine *Escherichia coli* infection reported on increased circulating prostaglandin E_2 (PGE_2) but not prostaglandin F metabolite (PGFM), and that the uterine LPS shifted endometrial epithelial cell secretion from prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to PGE_2, which prolonged the luteal phase [14]. However, Lüttgenau *et al.* [20] reported that repeated intrauterine LPS infusions at the onset of the estrous cycle promoted premature luteolysis and altered gene expression in the endometrium and corpus luteum. Previous studies also reported on high LPS levels in the follicular fluid of repeat breeder cows [24] and follicular cyst cows [34], and that LPS in the follicular fluid suppresses steroidogenesis in the follicle granulosa cells *in vitro* [21].

In addition, LPS administration to cattle before ovulation altered the pulsatile secretion of luteinizing hormone, decreased the blood estradiol-17β (E_2) levels, and suppressed follicular development [36]. These findings also suggested that the transfer of LPS or tumor necrosis factor-α (TNF-α) into the bloodstream might directly affect the genital tract. However, it is still unclear to what extent

*Correspondence to: Ohtaki, T.: ohtaki.tadatoshi@nihon-u.ac.jp

rumen fluid-derived LPS migrates into the portal vein and affects the genital tract. Hence, in this study, we investigated the effect of increased LPS concentration in the rumen on the LPS level in the genital tract of high-concentrate diet-fed cows.

MATERIALS AND METHODS

Animals, experimental design, diet

A total of 12 non-pregnant, dry Holstein cows were used for the study. The animals were kept at the Department of Veterinary Medicine, Nihon University. Their average body weight was 650 kg. The cows were tied in a stall during the study period. All experiments were conducted according to the Nihon University Guidelines for Management of Animal Experiments (AP17B048-1).

Table 1. Ingredient dry matter content (%) in each treatment week (weeks 1–4) in the high-concentrate diet group

Ingredients	Week 1	Week 2	Week 3	Week 4
Timothy	40.0	30.0	25.0	20.0
Corn	55.2	64.4	69.0	73.6
Soybean	4.8	5.6	6.0	6.4

The dry matter amount was calculated assuming that any feed comprised of 89% of it, and that 8% of the concentrated feed consisted of soybean meal. The concentration of roughage in the feed was reduced in a stepwise manner by substituting it with corn and soybean meal-based feed in the HC group (weekly fed proportion of roughage from Week 1 to 4: 40, 30, 25, and 20%, respectively).

The cows were allocated to a control group (CON; n=5) and a high-concentrate diet group (HC; n=7). The CON group was mainly raised on timothy hay. The HC group received a reduced roughage (timothy hay)-content feed in the dry matter, lowered weekly in consecutive steps (Week 1 to 4: 40, 30, 25, and 20%, respectively); the high-concentrate portion of the diet included compressed corn and soybean meal-based feed (Table 1). The feeding regimens ensured that all cows received 100% of the total digestible nutrient and crude protein requirement according to the Nutrient Requirements of Dairy Cattle [29]. All cows were fed twice per day at 8:00 and 16:00, and water was given *ad libitum*.

To synchronize the estrous cycle, all cows received 100 μg of gonadotropin-releasing hormone (GnRH, fertirelin acetate; Conceral®, MSD Animal Health, Tokyo, Japan) through intramuscular injection, and a controlled intravaginal drug-releasing device (CIDR device, containing 1.9 g of P_4; CIDR® 1900, Zoetis Japan, Tokyo, Japan) was inserted into their vagina. Seven days later, the CIDR device was removed, and the animals were administered 25 mg of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, dinoprost, Pronalgon® F, Zoetis Japan) through intramuscular injection. A 2nd GnRH dose was administered 2 days after the CIDR device removal and $PGF_{2\alpha}$ administration. All samples were collected one day after the 2nd GnRH injection (after 4 weeks of high-concentrate diet feeding in the HC group), when the dominant follicle was present, right before ovulation in order to prevent follicular fluid blood contamination during sampling. Furthermore, in the HC group, peripheral blood was collected before feeding the high-concentrate diet in order to compare blood parameters before and after feeding.

Peripheral blood samples

Peripheral blood was collected from the coccygeal vein. Samples were collected into vacuum tubes with glass granules coated with a clot activator for serum collection, and into heparinized vacuum tubes for plasma collection. The serum separator tubes were left to clot, and plasma separator tubes were placed in a cooler box. The tubes were then centrifuged at 1,700 × g at 4°C for 15 min, and the serum and plasma samples were stored frozen at −20°C until the assay.

Blood samples from the portal vein and hepatic veins

After collecting peripheral blood, samples were taken from the portal vein and the hepatic veins, according to the method described by Ono *et al.* [30]. Briefly, sterile 14-G catheters (ZENOAQ, Fukushima, Japan) were inserted near to one side of the ultrasonography probe, and blood was collected under ultrasonographic guidance. The portal vein was distinguished from the hepatic veins based on the vessel wall thickness and confirmed by the ammonia levels in the collected blood [25]. The portal blood samples from 1 cow in the CON group and 4 cows in the HC group could not be obtained, excluding these cows from the hormone analysis.

Rumen fluid samples

The ruminal fluid collection was performed by transcutaneous direct puncture 3 hr after feeding, using the procedure reported by Garrett *et al.* with modifications [9]. Briefly, a 5-cm square area was identified, located 12 to 15 cm caudoventral to the costochondral junction of the last rib on a line parallel with the top of the stifle. The area was clipped, scrubbed with a povidone-iodine scrub, and wiped with 70% alcohol. A sterile 14-G catheter (ZENOAQ was inserted into the ventral rumen, and a 20-m*l* syringe was used to aspirate a minimum of 3 m*l* of fluid. The collected rumen solution was immediately filtered through double gauze to prepare a sample stock solution, and after pH measurements, stored frozen at −20°C.

Uterine perfusate collection

The uterine perfusate was collected by a simplified method described by Kaneko *et al.* [15]. A balloon catheter (16 Fr; Fujihira Industry Co., Ltd., Tokyo, Japan) was advanced into the uterus using the recta-vaginal method, then the balloon was inflated. Sterile physiological saline (50 m*l*) was infused into the uterus through the balloon catheter and recovered by gently massaging the uterus. The collected uterine perfusate samples were transferred to sterile tubes and stored at −20°C until the assay.

Follicular fluid collection

Follicular fluid aspiration was performed using an ultrasound device (MyLab OneVET, Esaote, Genova, Italy) equipped with a transvaginal micro-convex 6 to 10 MHz transducer connected to a needle guide system (SC3123 with ovum pick up attachment, Esaote). The dominant follicle was confirmed by ultrasound, the egg collection needle (Disposable cow ova needle, 17G × 600 mm, Misawa Medical Industry Co., Kasama, Japan) was punctured into the follicle, and aspirated with a 5 m*l* syringe. The follicular fluid samples were stored frozen at −20°C until the assay. Follicular fluid from 1 cow in the CON group and 3 follicles in the HC group were excluded from the analysis as the follicular fluid was lost due to rupture during collection, and the samples could not be collected.

Sample analysis

LPS concentrations in the peripheral blood, hepatic portal vein blood, uterine perfusate, and follicular fluid were measured using the QCL-1000 Chromogenic Limulus Amebocyte Lysate Endpoint Assay Kit (Lonza Walkersville, Inc., Walkersville, MD, USA) as previously described by Magata *et al.* [21]. Samples were thawed, diluted in endotoxin-free distilled water, and tested for non-specific limulus amebocyte lysate (LAL) inhibition by comparing samples spiked with a known LPS concentration to non-spiked samples. Samples with evidence of LAL inhibition were subjected to proteinase K digestion, as described by Petsch *et al.* [31]. Briefly, samples were treated with proteinase K at a final concentration of 0.4 mg/m*l* for 18 hr at 37°C. The reaction was stopped by heat inactivation at 80°C for 10 min using a heat block, and the measurement was performed according to the according to the manufacturer's instructions.

Furthermore, commercial kits were used to measure peripheral blood LPS binding protein (LBP, multi-species, HK503 ELISA kit; Hycult Biotech, Uden, Netherlands) and TNF-α (Duoset ELISA Development bovine TNF-α; R&D Systems, Minneapolis, MN, USA) levels. In addition, peripheral blood and follicular fluid estradiol-17β concentrations were measured using a commercial kit (# 501890; Estradiol ELISA Kit, Cayman Chemical, Ann Arbor, MI, USA). The serum samples were submitted to the Health Sciences Research Institute (Yokohama, Japan) for albumin (ALB), aspartate transaminase (AST), γ-glutamyl transpeptidase (GGT), total cholesterol (T-Chol), and non-esterified fatty acid (NEFA) analysis. VFAs were measured using a gas chromatograph (7890A; Agilent) equipped with a flame ionization detector and a capillary column (Nukol, 15 m, 0.25 mm ID, 0.25 μm df; Supelco / Sigma-Aldrich, St. Louis, MO, USA) as previously described by Asano [4]. Serum amyloid A (SAA) was measured using an automated latex agglutination turbidimetric immunoassay kit (LAT: SAA-1, Eiken Chemical Co., Tokyo, Japan) and an automated clinical chemical analyzer (Hitachi 7170S, Hitachi Ltd., Tokyo, Japan) according to the method described by Tsukano *et al.* [37]. Furthermore, peripheral blood haptoglobin (Hp) levels were measured using a modified hemoglobin binding assay according to the method described by Nakamura *et al.* [28].

Statistical analysis

All values are expressed as mean ± standard error. The means of two groups were compared using a significance test, specifically, Student's *t*-test for normally distributed parameter values and Wilcoxon rank-sum test for non-normally distributed parameter values. Normality was tested using the Shapiro-Wilk W-test. In order to compare the corresponding samples in the HC group before (week 0) and after (week 4) high-concentrate diet feeding, the Wilcoxon signed-rank test for paired comparisons was performed. The differences between LPS concentrations at different sites were compared using the Kruskal-Wallis test. Univariate correlation analysis among inflammatory markers, blood biochemical parameters indicating the liver function, and LPS concentrations at various sites was performed using the Spearman rank correlation coefficient. A difference was considered significant when the probability value (P) was less than the significance level of 5% (P<0.05). Statistical analyses were performed using the JMP® 13.2.0 software (SAS Institute Inc., Tokyo, Japan).

RESULTS

Rumen properties

The rumen fluid pH, VFA composition, and LPS concentration of each group are shown in Table 2. The rumen pH was significantly lower in the HC group than that in the CON group (6.12 ± 0.27 vs. 7.21 ± 0.27, P<0.05). The assessment of the VFA (mol%) in the rumen fluid showed that acetic acid was significantly lower in the HC group than that in the CON group, whereas butyric acid and isovaleric acid were significantly higher in the HC group. The acetic-acid-to-propionic-acid ratio (A/P ratio) was significantly lower in the HC group than that in the CON group (2.6 ± 0.3 vs. 3.6 ± 0.2, P<0.05). The rumen LPS concentration was significantly higher in the HC group than in the CON group (36.5 ± 2.4 vs. 21.7 ± 2.7 EU/*l*, P<0.05).

Table 2. Rumen fluid pH, volatile fatty acid composition (mol%), and lipopolysaccharide (LPS) concentrations in each group

Item	CON (n=5)	HC (n=7)
pH	7.21 ± 0.27	6.12 ± 0.27 [a)]
Acetic acid (mol %)	69.0 ± 1.5	54.5 ± 2.2 [b)]
Propionic acid (mol %)	19.4 ± 0.8	23.1 ± 2.6
A/P ratio	3.6 ± 0.2	2.6 ± 0.3 [a)]
Butyric acid (mol %)	8.7 ± 0.8	16.2 ± 2.0 [a)]
Isovaleric acid (mol %)	1.1 ± 0.1	3.3 ± 0.4 [b)]
Valeric acid (mol %)	1.4 ± 0.3	1.9 ± 0.3
LPS (EU/*l*)	21.7 ± 2.7	36.5 ± 2.4 [a)]

The data are expressed as mean ± SEM. Asterisks indicate significant differences between the high-concentrate diet (HC) and control (CON) groups (a) P<0.05, b) P<0.01).

Peripheral blood LBP, TNF-α, Hp, SAA, and blood biochemical properties

The LBP, TNF-α, Hp, SAA, and blood biochemical properties of each group are shown in Table 3. The Hp concentration was significantly higher in the HC group than that in the CON group (421.8 ± 130.3 vs. 86.9 ± 28.3 μg/m*l*, $P<0.05$) but the LBP, SAA, and TNF-α did not significantly differ between the two groups. The blood biochemical property analysis showed that ALB was significantly lower in the HC group than that in the CON group (2.63 ± 0.11 vs. 2.90 ± 0.03 g/d*l*, $P<0.05$). The AST, GGT, T-Chol, and NEFA values did not significantly differ between the two groups. In the HC group, the Hp level and AST activity were significantly increased after high-concentrate diet feeding compared with those before feeding (Fig. 1; 421.8 ± 130.3 vs. 69.5 ± 11.3 μg/m*l*, $P<0.05$ and 96.9 ± 24.7 vs. 56.9 ± 5.5 U/*l*, $P<0.05$, respectively). In contrast, the T-Chol level was significantly lower (60.4 ± 7.8 vs. 75.0 ± 6.7 mg/d*l*, $P<0.05$) and the ALB level showed a trend to be lower after treatment (2.63 ± 0.11 vs. 2.79 ± 0.09 g/d*l*, $P=0.09$) than before treatment (Fig. 1).

Table 3. Blood parameter concentrations in control (CON) and high-concentrate diet (HC) cows

Item	CON (n=5)	HC (n=7)
LBP (*n*g/m*l*)	128.3 ± 4.2	129.7 ± 6.9
TNF-α (*p*g/m*l*)	81.4 ± 49.5	275.3 ± 214.4
Hp (μg/m*l*)	86.9 ± 28.3	421.8 ± 130.3 [a)]
SAA (μg/m*l*)	1.42 ± 0.53	1.44 ± 0.13
ALB (g/d*l*)	2.90 ± 0.03	2.63 ± 0.11 [a)]
AST (U/*l*)	54.2 ± 3.1	96.9 ± 24.7
GGT (U/*l*)	26.6 ± 0.7	33.6 ± 9.5
T-Chol (mg/d*l*)	75.0 ± 5.6	60.4 ± 7.8
NEFA (mEq/*l*)	0.24 ± 0.10	0.15 ± 0.02

The blood lipopolysaccharide-binding protein (LBP), tumor necrosis factor-α (TNF-α), haptoglobin (Hp), serum amyloid A (SAA), albumin (ALB), aspartate transaminase (AST), γ-glutamyl transpeptidase (GGT), total cholesterol (T-Chol), and non-esterified fatty acids (NEFA) levels were evaluated. The data are expressed as mean ± SEM. Asterisks indicate significant differences between the HC and CON groups (a) $P<0.05$).

Estradiol concentration in follicular fluid

The follicular fluid E_2 concentrations did not significantly vary between the HC group and the CON group (26.8 ± 2.5 vs. 28.7 ± 7.3 *n*g/m*l*).

LPS concentration in peripheral blood, hepatic portal vein blood, uterine perfusate, and follicular fluid

The LPS concentrations in the peripheral vein blood, hepatic portal vein blood, hepatic vein blood, uterine perfusate, and follicular fluid are presented in Table 4. The LPS concentrations (EU/m*l*) in the CON and HC groups were 0.28 ± 0.00 (n=5) and 0.26 ± 0.01 (n=7) in peripheral vein blood, 0.35 ± 0.10 (n=4) and 0.31 ± 0.06 (n=3) in hepatic portal vein blood, 0.20 ± 0.05 (n=4) and 0.21 (n=1) in hepatic vein blood, 0.27 ± 0.03 (n=5) and 0.27 ± 0.00 (n=7) in uterine perfusate, and 0.31 ± 0.02 (n=4) and 0.31 ± 0.03 (n=4) in follicular fluid, respectively. We did not observe statistically significant differences between the two groups. In a comparison among different sampling sites, we did not observe statistically significant differences in the LPS concentration between the CON and the HC groups.

Correlations among inflammatory markers, liver function indicators, and LPS concentrations

Table 5 shows the Spearman correlation coefficient (ρ) of ALB, AST, Hp, SAA, and TNF-α relative to the LPS concentration. A significant negative correlation was detected between the LPS concentration in the rumen fluid and the ALB concentration ($\rho=-0.82$, $P<0.01$). The LPS concentration in the rumen fluid showed a trend to positively correlate with the AST ($\rho=0.51$, $P=0.09$) and Hp concentration ($\rho=0.51$, $P=0.09$). In contrast, the LPS concentration in the hepatic portal vein blood showed a trend to positively correlate with the AST ($\rho=0.67$, $P=0.10$) and SAA concentration ($\rho=0.87$, $P=0.10$). A significant positive correlation was observed between LPS in the uterine perfusate and SAA ($\rho=0.64$, $P<0.05$), and LPS in the follicular fluid showed a trend to positively correlate with TNF-α ($\rho=0.67$, $P=0.07$). However, no significant correlation was observed between other LPS levels, inflammatory markers, and blood biochemical parameters indicating liver function.

DISCUSSION

In our study, the pH and A/P ratio in the rumen fluid were significantly lower in the HC group than those in the CON group. We further determined that the LPS concentration in the rumen fluid was significantly higher in the HC group than that in the CON group. These observations were consistent with previous reports [12, 16, 26, 27] describing that a high-concentrate diet lowers the rumen pH and kills gram-negative bacteria, releasing LPS, a proinflammatory component of the gram-negative cell envelope, into the rumen fluid supernatant. Nearly 60% of rumen LPS is reportedly generated by the rapid growth of gram-negative bacteria [17]. LPS released in the rumen is absorbed by the rumen wall, released into the ruminal veins, and carried to the liver via the portal vein. Importantly, increased peripheral blood LPS levels could be observed when the endotoxin inflow into the liver exceeds the hepatic detoxification capacity [12, 17]. In addition, it has been reported that a high-concentrate diet-triggered increase in ruminal LPS concentration induces inflammatory gene expression in the uterus of cows [5] and goats [6]. The uterine inflammation-induced intrauterine LPS production was also reported to accumulate in the follicular fluid [14, 22, 23]. For these reasons, in the case of increased concentration in the rumen, LPS could translocate into the blood and affect the reproductive organs. Nevertheless, in our study, the LPS levels in the peripheral vein blood, hepatic portal vein blood, follicular fluid, and uterine perfusate did not significantly vary between the two dietary groups. This result was likely due to the slight ruminal LPS level increase in the HC group. In a previous study, without feed treatment, the rumen LPS concentrations in the healthy and SARA groups in 13 farms were 34,179 and 42,206 EU/m*l*, respectively [35], which is consistent with the level recorded in our study. In contrast, the ruminal LPS level in grain-induced SARA cows was 12,589–168,391 EU/m*l* (3.4 to 16.2 times higher than that in the control) [10–12,

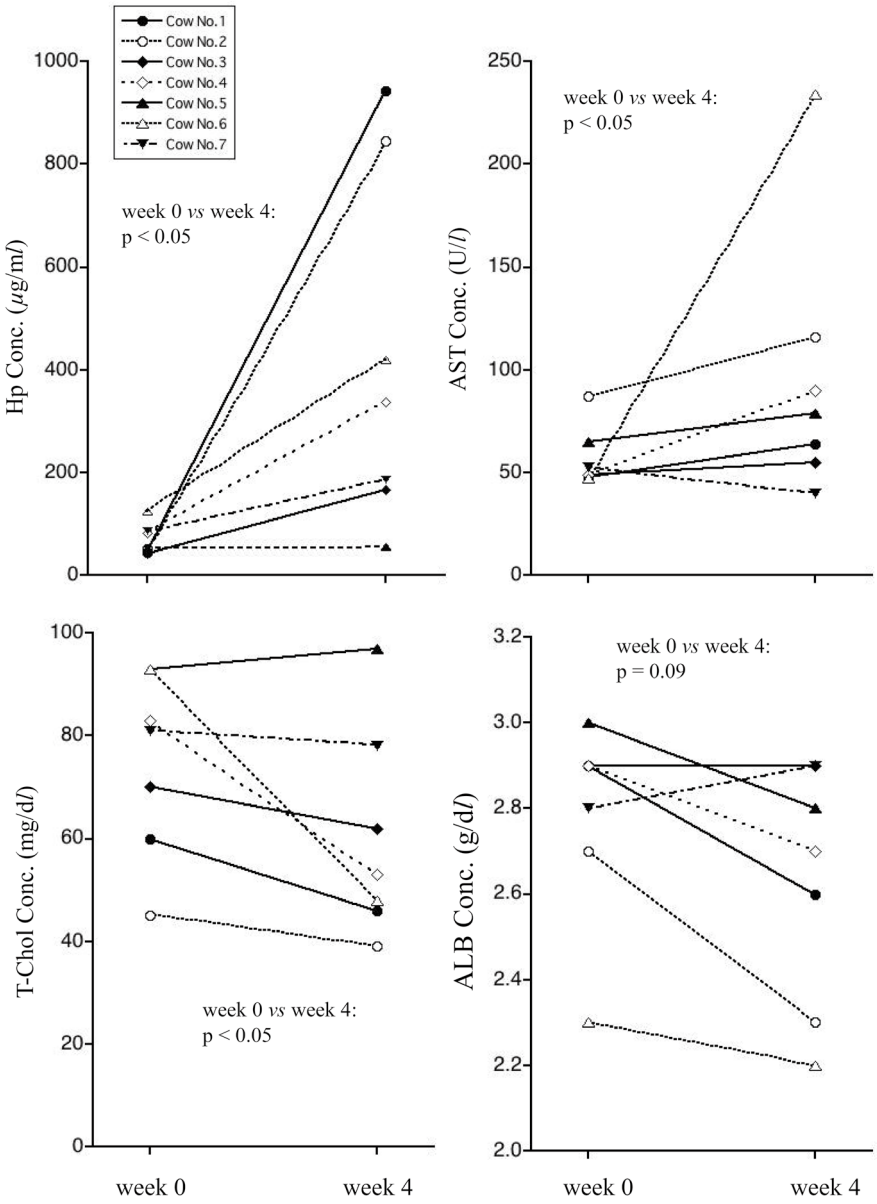

Fig. 1. Haptoglobin (Hp), albumin (ALB), aspartate transaminase (AST) and total cholesterol (T-Chol), before (week 0) and after treatment (week 4) in high-concentrate diet (HC) cows.

Table 4. Lipopolysaccharide (LPS) concentration in peripheral vein blood, hepatic portal vein blood, uterine perfusate, and follicular fluid

Sample type	CON	HC
Peripheral vein (EU/m*l*)	0.28 ± 0.00 (n=5)	0.26 ± 0.01 (n=7)
Portal vein (EU/m*l*)	0.35 ± 0.10 (n=4)	0.31 ± 0.06 (n=3)
Hepatic vein (EU/m*l*)	0.20 ± 0.05 (n=4)	0.21 (n=1)
Uterine perfusate (EU/m*l*)	0.27 ± 0.03 (n=5)	0.27 ± 0.00 (n=7)
Follicular fluid (EU/m*l*)	0.31 ± 0.02 (n=4)	0.31 ± 0.03 (n=4)

CON: control, HC: high-concentrate diet. The data are expressed as the mean ± SEM.

Table 5. Albumin (ALB), aspartate transaminase (AST), haptoglobin (Hp), serum amyloid a (SAA), and tumor necrosis factor-α (TNF-α) spearman correlation coefficients (ρ) relative to the lipopolysaccharide (LPS) concentration

LPS palameter	indicces	ρ	*P*
Rumen LPS	ALB	−0.82	<0.01
Rumen LPS	AST	0.51	0.09
Rumen LPS	Hp	0.51	0.09
Portal vein LPS	SAA	0.87	0.10
Portal vein LPS	AST	0.67	0.10
Uterine perfusate LPS	SAA	0.64	<0.05
Follicular fluid LPS	TNF-α	0.67	0.07

33]. In addition, the LPS concentration in the hepatic portal vein of grain-induced SARA cows was reportedly 0.95–1.42 EU/m*l* (1.8–3.2 times higher than that in the control) [1, 18]. The SARA-induced reduction in rumen pH increases gram-negative bacterial lysis and the LPS level but such a rumen LPS level increase suggests that it depends on the diet fed before the induction of SARA, and thus, the rumen bacterial population at the time of induction [32]. In our study, it might be due to the fact that the test cows were fed dent corn silage in both groups before the start of the study.

Hp, SAA, and LBP are representative bovine inflammatory markers [8, 40]. Many previous studies [10, 11, 13, 16, 17, 41] described significantly increased blood Hp, SAA, and LBP levels in SARA cows that were associated with high levels of inflammation. In our study, Hp levels were significantly higher in the HC group than those in the CON group, but no significant variations could be detected in the SAA and LBP levels. The discrepancy in the findings between our study and previous ones was probably influenced by the slight ruminal LPS level increase in the HC group and the differences in the SAA and LBP assay methods. However, our results still suggest that increased LPS concentrations in the rumen induce Hp due to inflammation. Khafipour *et al.* [16] reported that a grain-based SARA challenge increased the LPS to 0.52 EU/m*l*. Our study did not show elevated levels of peripheral blood LPS in the HC group, and this discrepancy might be due to the varying degrees of ruminal wall damage. It has been reported that ruminal acidosis, induced by lowering the effective neutral digestive fiber in the feed, increases ruminal LPS but cows without colonic acidosis do not have elevated blood LPS levels [19]. If inflammation does not occur in the rumen wall or the mucosa of the large intestine, LPS absorption is blocked. However, if the intestinal mucosa is damaged, LPS absorption will likely occur at this site. In addition, ruminal LPS that enter the hepatic portal circulation can be detoxified in the liver before reaching the peripheral blood circulation [2]. However, the majority of cytokine (TNF-α and interleukin-6) receptors that induce inflammatory proteins such as Hp and SAA are found in liver Kupffer cells [7], and therefore, proinflammatory cytokines might be expressed prior to detoxification. Some studies have shown that experimentally induced SARA increases inflammatory markers without increasing LPS in the peripheral blood [12].

LPS induces cytokine production, such as TNF-α [39], which causes liver damage [38]. Hepatic lipidosis is associated with a decreased capacity for endotoxin clearance in the liver, as demonstrated by Andersen *et al.* [3]. By measuring the liver function biochemical indicators, T-Chol, ALB, and AST, we found that the ALB level was lower and AST activity was higher in the HC group than those in the CON group. Similarly, in the HC group, the AST activity increased, and the T-Chol and ALB levels decreased after feeding the high-concentrate diet compared with those before feeding. Furthermore, in our feeding study, increased rumen fluid LPS levels were associated with reduced ALB levels, and higher portal blood LPS levels were linked to increased blood AST activity and SAA level. These results suggested that a large LPS influx into the liver might reduce liver function.

In conclusion, these results indicate that high-concentrate diets do not have a direct effect on the reproductive organs following a moderate rumen LPS level increase. However, an increased ruminal LPS influx into the liver might affect negatively liver function. Further studies, including analyses in cows with a high LPS influx into the liver, are needed to clarify the effect of increased ruminal LPS on the genital tract.

ACKNOWLEDGMENTS. This work was supported by JSPS KAKENHI Grant Number 17K08052. We are grateful to Zoetis Japan for providing the CIDR device and $PGF_{2\alpha}$ for estrus synchronization.

REFERENCES

1. Abaker, J. A., Xu, T. L., Jin, D., Chang, G. J., Zhang, K. and Shen, X. Z. 2017. Lipopolysaccharide derived from the digestive tract provokes oxidative stress in the liver of dairy cows fed a high-grain diet. *J. Dairy Sci.* **100**: 666–678. [Medline] [CrossRef]
2. Andersen, P. H. 2000. Bovine endotoxicosis: aspects of relevance to ruminal acidosis. Dr.Vet. Sci. Thesis, The Royal Veterinary and Agricultural University, Copenhagen.
3. Andersen, P. H., Jarløv, N., Hesselholt, M. and Baek, L. 1996. Studies on in vivo endotoxin plasma disappearance times in cattle. *Zentralbl. Veterinärmed. A* **43**: 93–101. [Medline] [CrossRef]
4. Asano, S., Aoki, K., Kaizuka, Y., Kobayashi, Y., Watanabe, K., Kato, C., Saito, C., Nishimura, S., Kato, T., Takagi, M. and Kajikawa, H. 2019. In situ ruminal degradation and in vitro fermentation characteristics, and antioxidative activities of the lotus rhizome. *Anim. Sci. J.* **90**: 1453–1459. [Medline] [CrossRef]
5. Bilal, M. S., Abaker, J. A., Ul Aabdin, Z., Xu, T., Dai, H., Zhang, K., Liu, X. and Shen, X. 2016. Lipopolysaccharide derived from the digestive tract triggers an inflammatory response in the uterus of mid-lactating dairy cows during SARA. *BMC Vet. Res.* **12**: 284. [Medline] [CrossRef]
6. Bilal, M. S., Abaker, J. A., ul Aabdin, Z., Waheed, U., Xu, T., Dai, H., Guo, J. and Shen, X. 2017. High grain diet triggers inflammation in the goat uterus: A comprehensive regulation diet modulates the immune response. *Int. J. Agric. Biol.* **19**: 34–40. [CrossRef]
7. Bode, J. G. and Heinrich, P. C. 2001. Interleukin-6 signaling during the acute-phase response of the liver. pp. 565–580. *In*: The Liver: Biology and Pathobiology, 4th ed. (Arias, I. M., Boyer, J. L., Chisari, F. V., Fausto, N., Schachter, D. and Shafritz, D. A. eds.), Lippincott Williams and Wilkins, Philadelphia.
8. Eckersall, P. D. and Bell, R. 2010. Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. *Vet. J.* **185**: 23–27. [Medline] [CrossRef]
9. Garrett, E. F., Pereira, M. N., Nordlund, K. V., Armentano, L. E., Goodger, W. J. and Oetzel, G. R. 1999. Diagnostic methods for the detection of subacute ruminal acidosis in dairy cows. *J. Dairy Sci.* **82**: 1170–1178. [Medline] [CrossRef]
10. Gozho, G. N., Plaizier, J. C., Krause, D. O., Kennedy, A. D. and Wittenberg, K. M. 2005. Subacute ruminal acidosis induces ruminal lipopolysaccharide endotoxin release and triggers an inflammatory response. *J. Dairy Sci.* **88**: 1399–1403. [Medline] [CrossRef]
11. Gozho, G. N., Krause, D. O. and Plaizier, J. C. 2006. Rumen lipopolysaccharide and inflammation during grain adaptation and subacute ruminal acidosis in steers. *J. Dairy Sci.* **89**: 4404–4413. [Medline] [CrossRef]
12. Gozho, G. N., Krause, D. O. and Plaizier, J. C. 2007. Ruminal lipopolysaccharide concentration and inflammatory response during grain-induced

subacute ruminal acidosis in dairy cows. *J. Dairy Sci.* **90**: 856–866. [Medline] [CrossRef]
13. Heegaard, P. M., Godson, D. L., Toussaint, M. J., Tjørnehøj, K., Larsen, L. E., Viuff, B. and Rønsholt, L. 2000. The acute phase response of haptoglobin and serum amyloid A (SAA) in cattle undergoing experimental infection with bovine respiratory syncytial virus. *Vet. Immunol. Immunopathol.* **77**: 151–159. [Medline] [CrossRef]
14. Herath, S., Lilly, S. T., Fischer, D. P., Williams, E. J., Dobson, H., Bryant, C. E. and Sheldon, I. M. 2009. Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F2alpha to prostaglandin E_2 in bovine endometrium. *Endocrinology* **150**: 1912–1920. [Medline] [CrossRef]
15. Kaneko, K., Kawakami, S., Miyoshi, M., Abukawa, T., Yamanaka, S., Mochizuki, M. and Yoshihara, S. 1996. Bacteriological and cytological examination of the uterine perfusate in cows. *Nippon Juishikai Zasshi* **49**: 435–438.
16. Khafipour, E., Krause, D. O. and Plaizier, J. C. 2009. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J. Dairy Sci.* **92**: 1060–1070. [Medline] [CrossRef]
17. Khafipour, E., Krause, D. O. and Plaizier, J. C. 2009. Alfalfa pellet-induced subacute ruminal acidosis in dairy cows increases bacterial endotoxin in the rumen without causing inflammation. *J. Dairy Sci.* **92**: 1712–1724. [Medline] [CrossRef]
18. Li, L., Cao, Y., Xie, Z. and Zhang, Y. 2017. A high-concentrate diet induced milk fat decline via glucagon-mediated activation of AMP-activated protein kinase in dairy cows. *Sci. Rep.* **7**: 44217. [Medline] [CrossRef]
19. Li, S., Khafipour, E., Krause, D. O., Kroeker, A., Rodriguez-Lecompte, J. C., Gozho, G. N. and Plaizier, J. C. 2012. Effects of subacute ruminal acidosis challenges on fermentation and endotoxins in the rumen and hindgut of dairy cows. *J. Dairy Sci.* **95**: 294–303. [Medline] [CrossRef]
20. Lüttgenau, J., Lingemann, B., Wellnitz, O., Hankele, A. K., Schmicke, M., Ulbrich, S. E., Bruckmaier, R. M. and Bollwein, H. 2016. Repeated intrauterine infusions of lipopolysaccharide alter gene expression and lifespan of the bovine corpus luteum. *J. Dairy Sci.* **99**: 6639–6653. [Medline] [CrossRef]
21. Magata, F., Horiuchi, M., Echizenya, R., Miura, R., Chiba, S., Matsui, M., Miyamoto, A., Kobayashi, Y. and Shimizu, T. 2014. Lipopolysaccharide in ovarian follicular fluid influences the steroid production in large follicles of dairy cows. *Anim. Reprod. Sci.* **144**: 6–13. [Medline] [CrossRef]
22. Magata, F., Ishida, Y., Miyamoto, A., Furuoka, H., Inokuma, H. and Shimizu, T. 2015. Comparison of bacterial endotoxin lipopolysaccharide concentrations in the blood, ovarian follicular fluid and uterine fluid: a clinical case of bovine metritis. *J. Vet. Med. Sci.* **77**: 81–84. [Medline] [CrossRef]
23. Magata, F., Kubota, R. and Shimizu, T. 2019. Association among endometrial hyperemia, uterine bacterial infection, and characteristics of large ovarian follicles in dairy cows. *J. Vet. Med. Sci.* **81**: 1313–1317. [Medline] [CrossRef]
24. Mogheiseh, A., Kafi, M., Golestani, N., Roshan-Ghasrodashti, A., Nazifi, S. and Mirzaei, A. 2019. Follicular fluid composition of ovulatory follicles in repeat breeder Holstein dairy cows. *Asian Pac. J. Reprod.* **8**: 124–131. [CrossRef]
25. Mohamed, T., Sato, H., Kurosawa, T. and Oikawa, S. 2002. Echo-guided studies on portal and hepatic blood in cattle. *J. Vet. Med. Sci.* **64**: 23–28. [Medline] [CrossRef]
26. Mochizuki, M., Kamata, N., Itoh, T., Shimizu, G., Yamada, Y., Kadosawa, T., Nishimura, R., Sasaki, N. and Takeuchi, A. 1996. Postparturient change in endotoxin levels of ruminal fluid and serum in dairy cows. *J. Vet. Med. Sci.* **58**: 577–580. [Medline] [CrossRef]
27. Motoi, Y., Oohashi, T., Hirose, H., Hiramatsu, M., Miyazaki, S., Nagasawa, S. and Takahashi, J. 1993. Turbidimetric-kinetic assay of endotoxin in rumen fluid or serum of cattle fed rations containing various levels of rolled barley. *J. Vet. Med. Sci.* **55**: 19–25. [Medline] [CrossRef]
28. Nakamura, M., Koyama, T., Matsui, Y., Hirai, T., Minamihashi, A., Nakada, K., Yokota, H. and Miyamoto, T. 2012. Modification of hemoglobin binding assay and measurement of bovine haptoglobin in postpartum period. *Nippon Juishikai Zasshi* **65**: 682–688.
29. National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed., Natl. Acad. Sci., Washington, D.C.
30. Ono, M., Ohtaki, T., Tanemura, K., Ishii, M., Watanabe, G., Taya, K. and Tsumagari, S. 2011. Effect of short-term fasting on hepatic steroid hormone metabolism in cows. *J. Vet. Med. Sci.* **73**: 1145–1149. [Medline] [CrossRef]
31. Petsch, D., Deckwer, W. D. and Anspach, F. B. 1998. Proteinase K digestion of proteins improves detection of bacterial endotoxins by the Limulus amebocyte lysate assay: application for endotoxin removal from cationic proteins. *Anal. Biochem.* **259**: 42–47. [Medline] [CrossRef]
32. Plaizier, J. C., Krause, D. O., Gozho, G. N. and McBride, B. W. 2008. Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. *Vet. J.* **176**: 21–31. [Medline] [CrossRef]
33. Rodríguez-Lecompte, J. C., Kroeker, A. D., Ceballos-Márquez, A., Li, S., Plaizier, J. C. and Gomez, D. E. 2014. Evaluation of the systemic innate immune response and metabolic alterations of nonlactating cows with diet-induced subacute ruminal acidosis. *J. Dairy Sci.* **97**: 7777–7787. [Medline] [CrossRef]
34. Shimizu, T., Ishizawa, S., Magata, F., Kobayashi, M., Fricke, P. M. and Miyamoto, A. 2018. Involvement of lipopolysaccharide in ovarian cystic follicles in dairy cow: Expressions of LPS receptors and steroidogenesis-related genes in follicular cells of cystic follicles. *Anim. Reprod. Sci.* **195**: 89–95. [Medline] [CrossRef]
35. Stefanska, B., Człapa, W., Pruszynska-Oszmałek, E., Szczepankiewicz, D., Fievez, V., Komisarek, J., Stajek, K. and Nowak, W. 2018. Subacute ruminal acidosis affects fermentation and endotoxin concentration in the rumen and relative expression of the CD14/TLR4/MD2 genes involved in lipopolysaccharide systemic immune response in dairy cows. *J. Dairy Sci.* **101**: 1297–1310. [Medline] [CrossRef]
36. Suzuki, C., Yoshioka, K., Iwamura, S. and Hirose, H. 2001. Endotoxin induces delayed ovulation following endocrine aberration during the proestrous phase in Holstein heifers. *Domest. Anim. Endocrinol.* **20**: 267–278. [Medline] [CrossRef]
37. Tsukano, K., Shimamori, T., Fukuda, T., Nishi, Y., Otsuka, M., Kitade, Y. and Suzuki, K. 2019. Serum iron concentration as a marker of inflammation in young cows that underwent dehorning operation. *J. Vet. Med. Sci.* **81**: 626–628. [Medline] [CrossRef]
38. Valatas, V., Kolios, G., Manousou, P., Xidakis, C., Notas, G., Ljumovic, D. and Kouroumalis, E. A. 2004. Secretion of inflammatory mediators by isolated rat Kupffer cells: the effect of octreotide. *Regul. Pept.* **120**: 215–225. [Medline] [CrossRef]
39. Williams, E. J., Sibley, K., Miller, A. N., Lane, E. A., Fishwick, J., Nash, D. M., Herath, S., England, G. C., Dobson, H. and Sheldon, I. M. 2008. The effect of *Escherichia coli* lipopolysaccharide and tumour necrosis factor alpha on ovarian function. *Am. J. Reprod. Immunol.* **60**: 462–473. [Medline] [CrossRef]
40. Zebeli, Q. and Ametaj, B. N. 2009. Relationships between rumen lipopolysaccharide and mediators of inflammatory response with milk fat production and efficiency in dairy cows. *J. Dairy Sci.* **92**: 3800–3809. [Medline] [CrossRef]
41. Zhao, C., Liu, G., Li, X., Guan, Y., Wang, Y., Yuan, X., Sun, G., Wang, Z. and Li, X. 2018. Inflammatory mechanism of Rumenitis in dairy cows with subacute ruminal acidosis. *BMC Vet. Res.* **14**: 135. [Medline] [CrossRef]