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NOTE

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Increased serum malondialdehyde concentration in cows with subclinical ketosis

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ABSTRACT. The purpose of this study is to compare the assessment of pre- and postpartum oxidative stress-related causal indicators and other metabolites in cows with postpartum subclinical ketosis (SCK). The prepartum serum malondialdehyde concentration and body condition score (BCS) were elevated in the SCK cows (n=17) compared to healthy controls (n=12), while the insulin sensitivity check index was lower in the SCK cows than in the controls. Oxidative stress is enhanced in cows with prepartum higher BCS, causing decreased insulin sensitivity, and may be associated with onset of postpartum SCK. However, paraoxonase alone might be insufficient to assess the antioxidant state because of no difference in pre- and postpartum activities between the two groups.

KEY WORDS: cow, malondialdehyde, paraoxonase, subclinical ketosis

Oxidative stress results from an imbalance between lipid peroxides and the antioxidant enzymes that neutralize them [24]. This imbalance alters the cell membrane and organelles, leading to oxidation of lipids and micromolecules [26]. When such a condition persists, the organism's metabolism is impeded, resulting in various diseases [18].

Malondialdehyde (MDA) is a product of lipid peroxidation and a major lipid peroxide indicator [5]. Polyunsaturated fatty acids are easily oxidized by reactive oxygen species (ROS) and free radicals. For example, they react readily with hydroxyl radicals to form lipid peroxyl radicals. These radicals react in turn with other polyunsaturated fatty acids to form lipid hydroperoxide and more lipid peroxyl radicals. Lipid peroxyl radicals also react with intramolecular double bonds to form cyclic endoperoxide, which is further broken down to give MDA [6].

Paraoxonase (PON) is a major antioxidant enzyme. In humans, PON is reduced in severe diseases such as atherosclerosis and coronary heart disease [16]. PON is synthesized in hepatocytes, where it accumulates until it is associated with high-density lipoproteins (HDL) and then released from the liver [16]. It is a calcium-dependent esterase-type hydrolytic enzyme responsible for the hydrolysis of various xenobiotic substances [9] that impedes the progression of oxidative stress by hydrolyzing the lipid peroxides generated in lipoproteins [17].

In cows with higher body condition scores (BCS) over the dry period to lactation, blood concentrations of non-esterified fatty acids (NEFA) and β-hydroxybutyric acid (BHBA) are elevated and oxidative stress is increased from 7 days prepartum until 25 days postpartum compared to cows with lower BCS [2]. Dairy cows often experience dystocia, retained placenta, and metritis after parturition. Oxidative stress status worsens with the occurrence of these problems [4]. That is, proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and IL-1β are released in these postpartum conditions, enhancing the production of lipid peroxides such as ROS and MDA [10]. These cytokines increase hepatic production of haptoglobin and serum amyloid A and decrease apolipoprotein B-100 (ApoB-100) production.

Subclinical ketosis (SCK) is a typical disease in cows during early lactation. As cattle with SCK display high blood levels of NEFA and BHBA [1, 22, 29], increased oxidative stress is suspected. However, little is known about oxidative condition in SCK. Therefore, this study aimed to perform comparative analysis of the prepartum and postpartum oxidative statuses in cows diagnosed with SCK after parturition.

This study was conducted on a single dairy farm (approximately 200 cows) in Ebetsu City, Hokkaido, Japan, where we regularly perform clinical examinations for keeping the herd healthy. The study period was between November 2014 and May

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2015. Lactating cows on this farm consisted of fresh cows, high-producing cows, first-calf heifers, and low-producing cows, which were kept in a free-stall system. Dry period cattle consisted of a close-up period group, kept in free stalls, and a far-off period group, kept in a loose housing system. All animals were fed based on the National Research Council recommendations and treated appropriately, following the Laboratory Animal control Guidelines of Rakuno Gakuen University, which essentially conform to the guide for the Care and Use of Laboratory Animals of the National Institute of Health in the U.S.A. (NIH publication No. 86-23, revised 1996).

Two clinical examinations were performed for 105 cows, the first in the close-up period (2–14 days antepartum), and the second at 3 days postpartum. Diagnosis of SCK was done at the second examination by measuring the blood BHBA concentration on-site using a portable device (Precision Xtra Meter, Abbott Laboratories, Abbott Park, IL, U.S.A.). According to a previous report [15], those cows with BHBA concentrations of 1.0 mM or more and no other diagnosed clinical diseases between parturition and blood collection were diagnosed with SCK. Those cows with BHBA concentrations below 1.0 mM that remained without clinical symptoms between parturition and blood collection became the controls. Thirty cows that received veterinary treatments before 3 days postpartum, 25 cows that received propylene glycol, and 21 first-calf heifers were excluded from the study. The final numbers of cows were 17 in the SCK group (parity: 3.1 ± 1.2) and 12 in the control group (2.5 ± 0.9).

Blood was collected from the caudal vein between 9 a.m. and 10 a.m. in the close-up period, and between 2 p.m. and 3 p.m. at 3 days postpartum, before feeding total mixed rations. Plain collection tubes were used to collect sera for measurement of the concentrations of MDA, PON, insulin, total cholesterol (T-Cho) and ApoB-100, as well as for BHBA measurement. Collection tubes with sodium fluoride were used for measuring the glucose concentration, and EDTA-containing tubes were used for NEFA [25]. Collected blood was stored immediately at 4°C, and serum and plasma were stored at −20°C until measurement.

MDA was measured using a commercial kit (MDA-586, Oxis Research, Portland, OR, U.S.A.), as an indicator of serum lipid peroxides. PON was measured as an indicator of serum antioxidant enzymes using a commercial kit (PON-1, Japan Institute for the Control of Aging, Nikken Seil Co., Ltd., Shizuoka, Japan). The concentrations of glucose, NEFA, BHBA, and T-Cho as well as the aspartate transaminase activity value (AST) were measured with an automatic analyzer (Bio Majesty JCA-BM2250, JEOL Ltd., Tokyo, Japan). The concentrations of insulin were evaluated by radioimmunoassay [13]. The ApoB-100 concentrations were measured using a commercial kit based on single-radial immunodiffusion assay (bovine ApoB plate, Metabolic Ecosystem Laboratory, Sendai, Japan).

Insulin sensitivity was calculated using the revised quantitative insulin sensitivity check index (RQICKI)=$1/[\log_{10}(\text{Glu}) + \log_{10}(\text{Ins}) + \log_{10}(\text{NEFA})]$, according to a previous report [12].

The prepartum BCS was assessed according to Ferguson *et al.* [8], together with blood collection. The score was taken again at 3 weeks postpartum.

The occurrence of periparturient diseases was investigated using the farm's clinical data obtained within 30 days postpartum.

Measurements were statistically analyzed using SPSS ver. 21.0 software (IBM Japan Inc., Tokyo, Japan). Normality of data was verified by the Shapiro-Wilk test. Concentrations of NEFA and BHBA were log-transformed before data analysis [20]. The difference in mean values between SCK and healthy control groups was tested with Student's *t*-test and the Mann-Whitney *U*-test for each sampling point.

Follow-up study of the SCK group up to 30 days postpartum revealed that 5 of the 17 cows developed periparturient disease after the sampling at 3 days postpartum and thereafter received veterinary care. Their specific conditions were left displacement of the abomasum (onset at 4 days and 6 days postpartum), clinical ketosis and downer (onset at 6 days postpartum), fatty liver (onset at 7 days postpartum), and mastitis (onset at 22 days postpartum). During this entire period, no disease occurred in the 12 control cows, revealing a clear difference in the disease occurrence rates of the two groups.

Table 1 shows the results of BCS and blood analyses. Prepartum BCS was higher in the SCK group than in the controls ($P<0.05$), but the postpartum BCS scores were similar in the SCK group and controls. Both prepartum and postpartum MDA concentrations were elevated in the SCK group compared to the control group ($P<0.05$); in contrast, no difference in PON activities was found. There was no marked difference in the MDA concentration or PON activity before and after parturition in either group. There was no difference in prepartum NEFA and BHBA concentrations between the two groups; however, both postpartum values were significantly elevated in the SCK group. The prepartum insulin concentration was higher in the SCK group than in the controls, but the postpartum insulin concentrations were equally lower in both groups. Prepartum RQUICKI was lower in the SCK group than in the control, but there was no postpartum difference between the groups. No difference was found between the groups for either antepartum and postpartum AST activities or T-Cho concentrations. The pre- and postpartum ApoB-100 concentrations were similar in both groups, but a significant postpartum decrease was observed in both groups compared to prepartum.

In this study, a clear elevation of the MDA concentration was observed in the prepartum SCK group. This was considered to be due to a higher BCS in the SCK group than in the control group because MDA and reactive oxygen metabolites (ROM) are known to increase in cattle with higher prepartum BCS [3]. In this mechanism, macrophages infiltrate the adipose tissue and TNF-α produced by these macrophages and adipocytes enhances the production of MDA and ROM [3]. The postpartum MDA concentration was significantly higher in the SCK group than in controls, and higher blood NEFA and BHBA concentrations have been also confirmed in such animals [1, 22, 29]. This may explain that higher postpartum blood NEFA and BHBA concentrations were the primary causes of the elevated MDA concentration because lipid peroxides such as ROM and thiobarbituric acid reactive substances are increased and anti-oxidant enzymes are decreased in this condition [3]. Treatment of bovine hepatocytes with high NEFA and BHBA results in the activation of the gene expression of NF-κB, which regulates proinflammatory cytokines such as TNF-α, IL-6, and IL-1β [23]. Increases in these proinflammatory cytokines enhance the production of lipid peroxides

such as ROS and MDA. Thus, the postpartum rise in the MDA concentration could be caused by either a direct effect of the rise in the concentrations of NEFA and BHBA in the blood or by an indirect effect of increased NEFA and BHBA via the release of proinflammatory cytokines. Considering the above findings, we propose that those cows that develop postpartum SCK experience some level of prepartum oxidative stress. In addition, it seems that this oxidative stress is further extended by high blood concentrations of NEFA and BHBA caused by the negative energy balance after parturition.

The primary factors causing reduced PON production are: insults to the liver caused by postpartum lipid mobilization and triglyceride accumulation, and subsequent dysfunction; postpartum reduction of HDL cholesterols; postpartum increases of oxidative stress; and combination of these three factors [27, 28]. In humans, PON production is lowered in severe inflammatory diseases such as atherosclerosis, coronary heart disease [16], and clinical liver diseases [9]. In previous studies on bovine fatty liver, blood PON activities dropped to as low as 40 U/*l* due to reduced PON production or alteration of the HDL composition in the liver [14], as well as inhibition of PON synthesis caused by increased proinflammatory cytokines in the blood and liver [7]. However, no difference in pre- and postpartum PON activities was observed between our experimental groups. This may have been because our cases of SCK did not have decreased HDL enough to reduce PON; indeed, compared to this study, the concentrations of NEFA and BHBA were clearly higher [7] and ApoB-100 values were much lower [19] than those in prior reports on bovine fatty liver. Therefore, it might be insufficient to investigate blood PON alone as the antioxidant assessment in these SCK cows.

Oxidative stress status may be one of the causes of the higher insulin concentration and lower RQUICKI (decreased insulin sensitivity) observed in the present study in the prepartum SCK group compared to the control. In human diabetes, augmented ROS production under oxidative stress leads to the induction of serine/threonine phosphorylation, which disturbes cellular redistribution of insulin signaling components and gives rise to insulin resistance [21]. Undergoing parturition with enhanced insulin resistance causes an extreme boost of body fat mobilization [2] and increased blood NEFA. When the liver is flooded with NEFA, the acetyl-CoA produced cannot enter the TCA cycle of hepatocyte mitochondria easily because of accelerated gluconeogenesis, which results in leading to massive production of ketone bodies [11] and facilitating onset of SCK.

Table 1. Body condition score (BCS) and blood concentrations of malondialdehyde (MDA), paraoxonase (PON), non-esterified fatty acids (NEFA), β-hydroxybutyric acid (BHBA), glucose, insulin, revised quantitative insulin sensitivity check index (RQUICKI), aspartate transaminase (AST), total cholesterol (T-Cho) and apolipoprotein B-100 (ApoB-100) for subclinical ketosis (SCK) and control cows at pre- and postpartum sampling

Variables	Control (n=12)	SCK (n=17)
BCS		
Prepartum[1]	3.35 ± 0.04	3.51 ± 0.05[a)]
Postpartum[3]	3.20 ± 0.07	3.32 ± 0.04
MDA (μM)		
Prepartum[1]	3.26 ± 0.53	4.21 ± 1.26[a)]
Postpartum[2]	3.44 ± 0.76	4.17 ± 0.73[a)]
PON (U/*l*)		
Prepartum[1]	72.6 ± 19.3	70.4 ± 24.8
Postpartum[2]	68.0 ± 18.4	79.2 ± 23.1
NEFA (mEq/*l*)		
Prepartum[1]	0.22 ± 0.10	0.24 ± 0.12
Postpartum[2]	0.41 ± 0.09	0.89 ± 0.45[b)]
BHBA (mM)		
Prepartum[1]	0.65 ± 0.15	0.63 ± 0.27
Postpartum[2]	0.68 ± 0.19	1.54 ± 0.90[b)]
Glucose (mg/d*l*)		
Prepartum[1]	64.6 ± 4.0	62.3 ± 2.9
Postpartum[2]	57.8 ± 6.4	47.8 ± 9.0[b)]
Insulin (μU/m*l*)		
Prepartum[1]	8.3 ± 5.9	9.7 ± 4.3
Postpartum[2]	3.4 ± 2.4	2.5 ± 1.6
RQUICKI		
Prepartum[1]	0.52 ± 0.04	0.47 ± 0.05[a)]
Postpartum[2]	0.56 ± 0.09	0.51 ± 0.05
AST (U/*l*)		
Prepartum[1]	54.4 ± 9.8	59.7 ± 14.4
Postpartum[2]	105.8 ± 31.4	116.3 ± 23.0
T-Cho (mg/d*l*)		
Prepartum[1]	102.4 ± 14.4	84.0 ± 37.2
Postpartum[2]	82.7 ± 15.8	79.3 ± 15.3
Apo-B (μg/m*l*)		
Prepartum[1]	208 ± 133	207 ± 118
Postpartum[2]	117 ± 57	69 ± 43

Date are expressed as mean ± standard error. Postpartum[1], −2 to −14 days; Postpartum[2], day 3; Postpartum[3], 3 weeks. a) $P<0.05$, b) $P<0.01$; compared with control at each sampling.

In conclusion, we have shown evidence of mounting oxidative stress from before parturition in cows diagnosed as having SCK. We presume that this condition elicits insulin resistance, which is connected to subsequent onsets of SCK and other periparturient diseases. This is a preliminary study on the phenomena involved with oxidative stress in cows with SCK. For further elucidation of the mechanisms, it will be necessary to evaluate the expression states of oxidative stress-related cytokines such as the ones mentioned above.

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