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Changes in peripheral blood oxidative stress markers and hepatic gene expression related to oxidative stress in Holstein cows with and without subacute ruminal acidosis during the periparturient period

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ABSTRACT. We investigated changes in peripheral blood metabolites, oxidative stress markers (malondialdehyde, potential antioxidant capacity, and glutathione peroxidase [GPX]), and hepatic gene expression related to oxidative stress in Holstein cows with and without subacute ruminal acidosis (SARA) during the periparturient period. Eighteen multiparous Holstein cows were categorized into SARA (n=9) or non-SARA (n=9) groups depending on whether they developed SARA; reticulo-ruminal pH was <5.6 for more than 3 hr per day, during the 2 weeks after parturition. Blood and liver tissue samples were collected 3 weeks prepartum and 2 and 6 weeks postpartum, with an additional blood sample collected 0 and 4 weeks postpartum. Blood aspartate transaminase (AST) and nonesterified fatty acid (NEFA) increased significantly ($P<0.05$) after parturition in both groups. GPX activity decreased gradually after parturition in the SARA group. In the SARA group, gene expression of *GPX 1* and microsomal glutathione S-transferase 3 (*MGST3*) decreased significantly ($P<0.05$), and expression of metallothionein 2A increased significantly ($P<0.05$) after parturition in the SARA group. Superoxide dismutase 1 and *MGST3* decreased significantly ($P<0.05$) 2 weeks postpartum in the non-SARA group. Gene expression related to oxidative stress was negatively correlated with AST, NEFA and total ketone body levels. Therefore, the hepatic gene expression related to oxidative stress might change associated with a negative energy balance, and might relate the high oxidative stress in the SARA group during periparturient period.

KEY WORDS: hepatic gene expression, Holstein cow, oxidative stress, periparturient period, subacute ruminal acidosis

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Major physiological, nutritional, metabolic, and immunological changes occur during the transition period defined as 3 weeks before and after parturition to adapt to parturition and the onset of lactation [29]. A high-grain postpartum diet is necessary after parturition to meet the energy requirements for lactation while increasing the risk of subacute ruminal acidosis (SARA) [3, 16]. SARA reduces productivity by decreasing dry matter intake and milk production, and is involved in various disease occurrences by translocation of ruminal lipopolysaccharides (LPS) into the bloodstream [16, 22].

Oxidative stress, which reflects an imbalance between oxidant and antioxidant status, causes oxidative damage to macromolecules, such as lipids, proteins, and DNA [21]. Oxidative stress develops during early lactation in cows and increases the risk of various diseases by causing dysfunction in the inflammatory response [3, 29]. Furthermore, oxidative stress can be measured using biological markers, including malondialdehyde (MDA), glutathione peroxidase (GPX), and potential antioxidant

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capacity (PAO). Among these, MDA is the end product of lipid peroxidation caused by the generation of reactive oxygen species (ROS) [32]. GPX is an antioxidant enzyme involved in lipid peroxidation that produces MDA and converts hydrogen peroxide to water in the presence of glutathione [32]. PAO is a measure of the total antioxidant capacity using the reduction reaction of copper ions [23]. In addition, Gessner *et al.* [11] reported that upregulation of the nuclear factor E2-related factor 2 (*NEF2L2*) gene controls the transcription of genes encoding various antioxidant enzymes and antioxidant material in the liver after parturition in Holstein cows.

Serum L-lactate concentration, a marker of ruminal activity, correlates with serum oxidative stress markers in early postpartum cows [2]. In addition, it has reported that there is a relationship between increased oxidative stress and occurrence of SARA in Holstein cows. SARA-induced cows fed a high-grain diet show increased oxidative stress [1, 13], and exhibit changes in oxidative stress markers in the liver and plasma and hepatic *NEF2L2* target gene expression with increasing LPS translocated into the bloodstream [1]. However, the relationships among the severity of SARA, peripheral blood metabolites, and peripheral blood oxidative stress markers, and hepatic gene expression relative to oxidative stress are unclear in periparturient Holstein cows. Therefore, the objective of this study was to investigate changes in peripheral blood metabolites, peripheral blood oxidative stress markers, and expression of hepatic genes involved in oxidative stress and metabolism in Holstein cows with and without postpartum SARA. We re-analyzed our previously published data regarding blood measurements [30] to evaluate their relationships with oxidative stress markers and liver gene expression related to oxidative stress and metabolism.

MATERIALS AND METHODS

Animals and group assignment

This experimental protocol was approved by the Iwate University Laboratory Animal Care and Use Committee (A201452-2; Morioka, Japan). Eighteen multiparous Holstein cows were obtained from Farm A and B, and were fed dry and lactation period diets *ad libitum* before and after parturition. Feed composition and amounts were based on the requirements of the Japanese Feeding Standard for Dairy Cattle. Nutrient compositions (% of dry matter) of the diets fed lactation period in Farm A and B, total digestible nutrient was 66.0% and 74.7%, crude protein was 16.1% and 15.0%, neutral detergent fiber was 41.1% and 37.4%, acid detergent fiber was 25.1% and 18.4%, starch was 16.7% and 23.3%, respectively. SARA was diagnosed when reticulo-ruminal pH was <5.6 for more than 3 hr per day [12] during the 2 weeks following parturition. Based on these criteria, cows were assigned to the SARA (n=9) or non-SARA (n=9) group. The 7 day mean reticulo-ruminal pH decreased after parturition in both groups and decreased significantly 1 week after parturition in the SARA group compared with the non-SARA group. Cows were milked twice daily at 0830 and 1600 hr, and the quantity of milk was determined and recorded at each milking using a milk meter installed in the milking parlor. Dry matter intake (DMI) were measured daily throughout the experiment period.

Blood sampling, biochemical components, and oxidative stress parameter analyses

Blood samples were collected 3 weeks before and 0, 2, 4, and 6 weeks after parturition. Samples were collected from the jugular vein into 10 m*l* evacuated tubes containing heparin (BD Vacutainer, Franklin Lakes, NJ, USA). Samples were centrifuged (1,500 ×*g*, 15 min, 4°C) immediately to separate the plasma and stored at −80°C until analysis. For blood biochemical analysis, triglyceride (TG), total cholesterol (T-CHO), total protein (TP), albumin (ALB), blood urea nitrogen (BUN), aspartate transaminase (AST), γ-glutamyl transpeptidase (GGT), total calcium (Ca), phosphate (IP), glucose (GLU), non-esterified fatty acid (NEFA), and total ketone body (T-KB) were measured using an automated biochemistry analyzer (Accute, Toshiba Ltd., Tokyo, Japan). For blood oxidative stress parameter analysis, plasma MDA was measured using a commercially available kit (Malondialdehyde Assay; Northwest Life Science Specialties LCC, Vancouver, WA, USA) as described elsewhere [6]. Plasma PAO was measured using a commercially available kit (PAO antioxidant capacity measurement kit; Nikken Zile Japan Aging Control Laboratory, Shizuoka, Japan). GPX was measured using a commercially available kit (Glutathione Peroxidase Assay; Northwest Life Science Specialties LCC).

Liver biopsy and RNA extraction

The liver tissue was biopsied from intercostal space 9–11 under ultrasound guidance by a skilled veterinarian as 3 weeks prepartum and 2 and 6 weeks postpartum. A sterile, percutaneous needle biopsy (Acecut; TSK Laboratory, Tochigi, Japan) was used under local anesthesia to biopsy the tissue. Samples were collected three times from each cow during each sample collection in the 2 hr after the morning feeding (1100 hr). Then, the liver tissue samples were temporarily stocked in a liquid nitrogen tank and stored at −80°C until use. Total RNA was extracted from liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as described previously [15]. The purity of the extracted RNA was improved using an RNeasy RNA Clean-up Kit (Qiagen, Valencia, CA, USA). Total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA samples were treated with TURBO DNase (Applied Biosystems, Foster City, CA, USA) to remove contaminating DNA. Total RNA (600 *n*g) was converted to first-strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the StepOne™ Plus Real-Time PCR system (Applied Biosystems) as described previously [15]. Primers designed for the genes involved in oxidative

Table 1. Gene symbols and sequences of the primers used for quantitative real-time PCR

Gene symbol [a)]	Primer Sequences (5′-3′)		Amplicon Size, bp
	Forward	Reverse	
Oxidative stress			
GPX3	TAGCCACCCTCAAGTATGTTCGA	CCCATTCACATCGCCTTTCT	80
GPX1	CCCCTGCAACCAGTTTGG	CGCCTGGTCGGACGTACTT	80
SOD1	CACGATGGTGGTCCATGAAA	TTCCAGCGTTGCCAGTCTTT	80
CAT	TCCAAGGCGAAGGTGTTTG	CCCGATTCTCCAGCAACAGT	80
MT1A	CCCACTGGCGGCTCCT	ACTTGGCACAGCCCACAG	80
MT2A	CACCGCGGGTGAATCCT	GAGCAGCAGCTCTTCTTGCA	80
MGST3	TTTTTTCTAGCTGTCGGAGGTGTT	AGGACTCGTCCAACGATCCA	80
NFE2L2	CAAGTTTGGGAGGAACTATTATCCA	GTACTAGTCTCAGCCAGCTTGTCATT	80
Metabolism			
PC	CAAAGCAGGTGGGCTACGAGAAC	CAGGTCCACATCTGTGATCTCCTC	137
PCK1	CCTGTTGGTGTCCCTCTGGTCTAC	CATGATGACTTTGCCCTTGTACTCC	117
IGF1R	TTAAAATGGCCAGAACCTGAG	ATTATAACCAAGCCTCCCAC	314
SLC2A4	CTCTTCTGGAGCAGGAAGTGAAA	CCTCTGTGGCCCTCAGTCAT	80
ACADVL	ATCATTGCTAAGGCGGTGGAT	TCCTGGATCAGCCCAAAGTT	80
ACSL1	CAACCCCAAAGGAGCAATGAT	ACGTGTTCTCTGTCATTTTCACAAA	80
CPT1A	GAGGAATGTCAGGAGGTCATTGA	AAGTGCGGAATGGGAAGGA	80
PPARA	CCCATAACGCGATTCGTTTT	CATGCTCACACGTAAGGATTTCTG	80
Negative energy balance			
FGF21	CGGATCGCTGCACTTTGAC	CCGACTGGTAGACGTTGTATCCA	80
KLB	CGCTTGGCATGGGTATGG	TCGAATGAGCCTTGATCAGGTT	80
Housekeeping gene			
GAPDH	GCCGATGCCCCCATGT	CAGGAGGCATTGCTGACAATC	80
ACTB	GGCCGAGCGGAAATCG	GCCATCTCCTGCTCGAAGTC	80
RPL27	GCCCGACGAGAGGCAA	AACCGCAGCTTCTGGAAGAA	80

a) *GPX3*; glutathione peroxidase 3, *GPX1*; glutathione peroxidase 1, *SOD1*; superoxide dismutase 1, *CAT*; catalase, *MT1A*; metallothionein 1A, *MT2A*; metallothionein 2A, *MGST3*; microsomal glutathione S-transferase 3, *NFE2L2*; nuclear factor E2-related factor 2, *PC*; pyruvate carboxylase, *PCK1*; cytosolic phosphoenolpyruvate carboxykinase, *IGF1R*; insulin like growth factor 1 receptor, *SLC2A4*; solute carrier family 2 member 4, *ACADVL*; acyl-CoA dehydrogenase very long chain, *ACSL1*; acyl-CoA synthetase long-chain family member 1, *CPT1A*; carnitine-palmitoyl-transferase 1A, *PPARA*; peroxisome proliferator-activated receptor α, *FGF21*; fibroblast growth factor 21, *KLB*; klotho beta, *GAPDH*; glyceraldehyde-3-phosphate dehydrogenase, *ACTB*; β-actin, *RPL27*; ribosomal protein L27.

stress, metabolism, and negative energy balance (NEB) are shown in Table 1. The results were recorded as relative changes in gene expression normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L27 (*RPL27*), and β-actin (*ACTB*) by the $2^{-\Delta\Delta Ct}$ method [18]. We examined *GAPDH*, *RPL27*, and *ACTB* to evaluate their use as reference genes for qPCR, and there were no significant differences among them.

Statistical analyses

Outliners were excluded by the outliner test (ROUT method, Q=1.0%). Data were assessed for normality using the Shapiro–Wilk test. Significant differences between the SARA and non-SARA groups at the same sampling time were evaluated using the unpaired *t*-test for normally distributed variables and Mann–Whitney *U*-test for non-normallly distributed variables. A mixed-model repeated measures analysis of variance, using time as a fixed effect, followed by Dunnett's multiple comparison method, was used to determine within-group differences. Correlation coefficients were calculated using Pearson's correlation coefficient analysis for normally distributed variables and Spearman's correlation coefficient analysis for non-normally distributed variables. A heatmap was constructed using Prism software (version 8.21; GraphPad Software Inc., La Jolla, CA, USA) based on the Pearson's or Spearman's correlation coefficient. Additionally, all numerical data were analyzed using Prism software (version 8.21). A P value <0.05 was considered significant.

RESULTS

DMI and milk yield

DMI increased significantly (P<0.05) at 1–6 weeks postpartum compared with 3 weeks prepartum in both groups. Milk yield increased significantly (P<0.05) at 1–6 weeks postpartum compared with 0 week postpartum in both groups. No significant difference was observed in DMI or milk yield between the SARA and non-SARA groups during the periparturient period. For example, at 4 weeks postpartum, DMI in the SARA and non-SARA group were 25.7 kg and 23.6 kg, and milk yield in the SARA and non-SARA group were 45.1 kg and 45.9 kg, respectively.

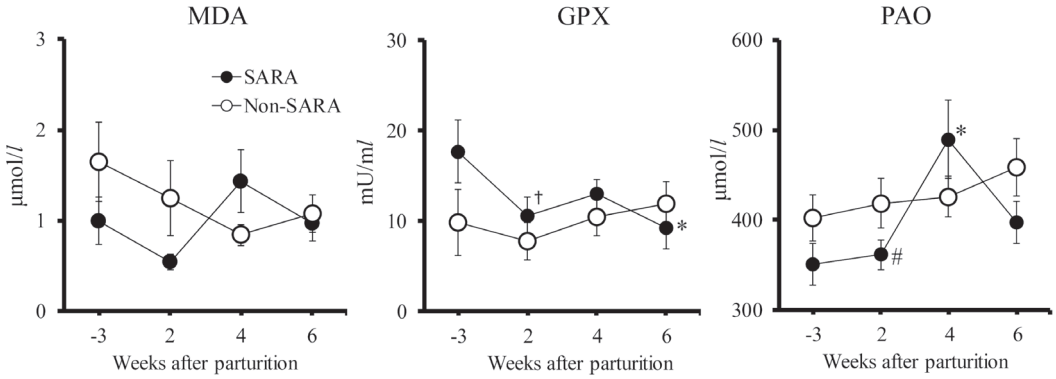

Fig. 1. Changes in malondialdehyde (MDA), glutathione peroxidase (GPX), and potential antioxidant capacity (PAO) levels in Holstein cows with subacute ruminal acidosis (SARA group, n=8) and without SARA (non-SARA group, n=8) during the periparturient period. *, † Denote significant (P<0.05) and tendency (P<0.1) within-group differences compared to 3 weeks prepartum. # Denotes a tendency (P<0.1) difference between the SARA and non-SARA groups. Values represent mean ± SE.

Biochemical components

Mean values at 0–6 weeks postpartum in the SARA and non-SARA group, TG (mg/d*l*) was 2.4–6.5 and 3.3–6.4, T-CHO (mg/d*l*) was 67.0–175.1 and 65.4–174.0, TP (g/d*l*) was 6.4–6.9 and 6.4–7.4, ALB (g/d*l*) was 4.5–5.2 and 4.1–4.6, BUN (mg/d*l*) was 9.6–12.5 and 9.5–11.7, AST (IU/*l*) was 76.8–99.1 and 66.8–100.3, GGT (IU/*l*) was 22.6–27.4 and 18.6–26.9, Ca (mg/d*l*) was 8.4–10.0 and 8.5–9.4, IP (mg/d*l*) was 2.8–3.4 and 1.8–2.9, GLU (mg/d*l*) was 57.8–68.3 and 55.2–81.5, NEFA (μmol/*l*) was 143–549 and 160–569, T-KB (μmol/*l*) was 639–806 and 640–922, respectively. T-CHO and NEFA levels, as well as AST activity, increased significantly (P<0.05), and TG concentrations decreased significantly (P<0.05) after parturition in both groups. GLU concentration significantly (P<0.05) decreased after parturition in the SARA group, and T-KB concentration increased significantly (P<0.05) after parturition in the non-SARA group. TG concentration at 0 weeks and T-KB concentration at 2 weeks postpartum were significantly lower (P<0.05) in the SARA group compared with the non-SARA group.

Oxidative stress parameters

The MDA level did not change significantly (P<0.05) at any point during the periparturient period in the SARA and non-SARA groups (P=0.103 and 0.253, Fig. 1). GPX activity tended to decrease (P<0.1) 2 weeks postpartum, and decreased significantly (P<0.05) 6 weeks postpartum compared with 3 weeks prepartum in the SARA group. No significant differences were observed in MDA level or GPX activity between the SARA and non-SARA groups during the periparturient period. PAO level increased significantly (P<0.05) 4 weeks postpartum compared with 3 weeks prepartum in the SARA group. The SARA group tend to have a lower PAO level (P=0.090) 2 weeks postpartum than that of the non-SARA group.

Relative gene expression

In the SARA group, glutathione peroxidase 1 (*GPX1*) and microsomal glutathione S-transferase 3 (*MGST3*) expression levels decreased were significantly (P<0.05) after parturition and glutathione peroxidase 3 (*GPX3*) expression 2 weeks postpartum and metallothionein 2A (*MT2A*) expression 6 weeks postpartum increased significantly (P<0.05) (Fig. 2). In the non-SARA group, superoxide dismutase 1 (*SOD1*) and *MGST3* expression levels were significantly (P<0.05) lower 2 weeks postpartum. Expression of metallothionein 1A (*MT1A*) tended to be higher 2 and 6 weeks postpartum in the SARA group than that in the non-SARA group (P=0.054, and P=0.055, respectively). Pyruvate carboxylase (*PC*) and cytosolic phosphoenolpyruvate carboxykinase (*PCK1*) expression levels increased significantly (P<0.05) after parturition in both groups. Expression of solute carrier family 2 member 4 (*SLC2A4*) in both groups and insulin like growth factor 1 receptor (*IGF1R*) in the SARA group decreased significantly (P<0.05) after parturition. Acyl-CoA dehydrogenase very long chain (*ACADVL*) and carnitine-palmitoyl-transferase 1A (*CPT1A*) expression levels in the SARA group, and *ACADVL* and acyl-CoA synthetase long-chain family member 1 (*ACSL1*) expression levels in the non-SARA group were significantly higher (P<0.05) 2 weeks postpartum. Expression of *IGF1R* and *ACADVL* was significantly (P<0.05) lower 6 weeks postpartum in the SARA group compared with the non-SARA group.

Correlation analyses of relative gene expression

The expression levels of *SOD1*, catalase (*CAT*), and *MT1A* were negatively correlated (P<0.05; r= −0.356, −0.350, and −0.305, respectively) with NEFA level (Fig. 3). The expression levels of *GPX3*, *CAT*, *MT1A*, *MT2A*, and *NFE2L2* were negatively correlated (P<0.05; r= −0.305, −0.317, −0.407, −0.432, and −0.380, respectively) with T-KB concentration. The expression levels of *GPX1*, *MGST3*, and *NFE2L2* were negatively correlated (P<0.05; r= −0.344, −0.410, and −0.316, respectively) with AST level. The expression levels of *PC*, *PCK1*, *ACADVL*, *ACSL1*, and *CPT1A* were positively correlated with AST (r=0.689, 0.575, 0.497, 0.391, and 0.445, respectively) and NEFA levels (r=0.510, 0.215, 0.577, 0.385, and 0.263, respectively), and negatively correlated

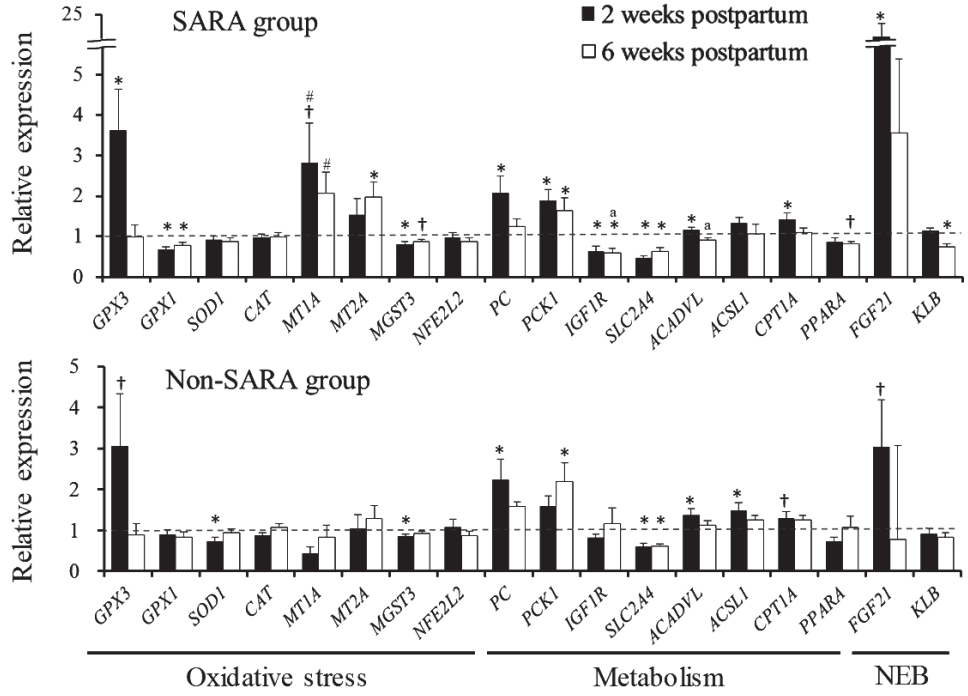

Fig. 2. Relative gene expression involved in oxidative stress, metabolism, and negative energy balance (NEB) relative to 3 weeks prepartum in Holstein cows with subacute ruminal acidosis (SARA group, n=9) and without SARA (non-SARA group, n=9) during the periparturient period. *, † Denote significant (P<0.05) and tendency (P<0.1) within-group differences compared to 3 weeks prepartum. [a], [#] Denote significant (P<0.05) and tendency (P<0.1) differences between the SARA and non-SARA groups. Values represent mean ± SE.

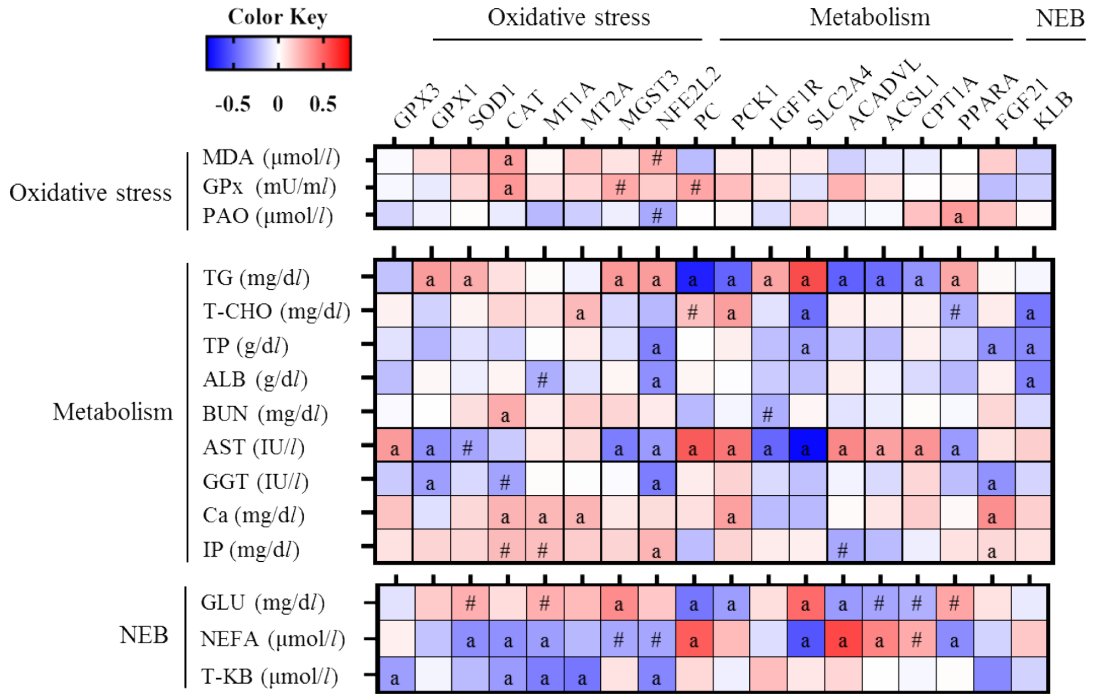

Fig. 3. Heatmaps of the correlation coefficients for Holstein cows during the periparturient period. Cells are colored based on Pearson's or Spearman's correlation coefficient analyses. Blue and red represent negative and positive correlations, respectively. [a], [#] Denote significant (P<0.05) and tendency (P<0.1) correlations.

with TG (r= −0.697, −0.488, −0.497, −0.464, and −0.338, respectively) and GLU levels (r= −0.431, −0.310, −0.314, −0.283, and −0.248, respectively). By contrast, the expression levels of *IGF1R* and *SLC2A4* were positively correlated with TG (r=0.374 and 0.743) and GLU levels (r=0.101 and 0.467), and negatively correlated with AST (r= −0.479 and −0.773) and NEFA levels (r= −0.109 and −0.534).

DISCUSSION

Previous studies have shown that peripheral blood oxidative stress parameters increase in SARA-induced cows fed a high-grain diet [1, 13]. Plasma MDA levels increase and GPX activity decreases in SARA-induced cows compared with those in non-SARA cows [1]. However, in the present study, MDA level and GPX activity were not different at any point between the SARA and non-SARA groups. Abaker *et al.* [1] compared SARA and non-SARA cows after an 18 week long-term SARA induced challenge, which differed in the duration of SARA from our present study. Therefore, the lack of significant differences in MDA level and GPX activity between the groups was likely due to the lack of a severe long-term challenge compared to the previous report [1]. GPX activity decreased gradually after parturition in the SARA group. In addition, the PAO level was lower than that in the non-SARA group 2 weeks postpartum. Therefore, antioxidant capacity decreased after parturition in the SARA group. The PAO levels increased significantly, and the MDA level and GPX activity increased numerically at 4 weeks postpartum in the SARA group compared with that prepartum. Bernabucci *et al.* [5] reported that the antioxidant enzyme transiently increases during the early stage of the oxidative stress response in dairy cows during the periparturient period. Therefore, we assumed that the PAO level increased because of the compensatory effects of the antioxidants accompanying increased oxidative stress. However, it was not possible to clarify why oxidative stress increased 4 weeks postpartum in the SARA group.

Oxidative stress is associated with high NEFA values and hyperketonemia, and NEFAs are mobilized from adipose tissue and increase β-oxidation in the liver during NEB, enhancing the production of ROS [3, 29]. High NEFA and β-hydroxybutyrate levels increase ROS production in hepatocytes, leading to increased oxidative stress [27, 28]. In the present study, AST and NEFA increased after parturition in both groups, and GLU decreased in the SARA group, suggesting that NEB occurred, and β-oxidation of NEFA in the liver was enhanced after parturition in both groups. In addition, the expression of many of the hepatic antioxidative genes was negatively correlated with AST, GGT, NEFA, and T-KB levels, and positively correlated with the GLU level. Therefore, reduced hepatic antioxidative gene expression was associated with NEB, such as increases in NEFA and T-KB and decreases in GLU and liver function by lipid β-oxidation in periparturient dairy cows.

Expression of the hepatic *NFE2L2* target gene with antioxidative properties is enhanced during the transition period [11] when oxidative stress increases [3, 29]. In addition, the expression of several hepatic *NFE2L2* target genes decrease with increased blood MDA and decreased blood GPX in SARA-induced cows [1]. These reports [1, 11] suggest that hepatic gene expression related to oxidative stress is upregulated or downregulated in response to increased oxidative stress. In the present study, *GPX1* and *MGST3* expression levels decreased, *GPX3* and *MT2A* expression levels increased after parturition in the SARA group, and *SOD1* and *MGST3* decreased 2 weeks postpartum in the non-SARA group. In addition, significant intragroup differences were identified 2 and 6 weeks postpartum in the SARA group, but only at 2 weeks postpartum in the non-SARA group. These results suggest that the different hepatic reactions to oxidative stress are induced and show greater and more longitudinal fluctuations in hepatic gene expression related to oxidative stress in the SARA group than those in the non-SARA group.

MT1A encodes the metallothionein 1A protein, which controls oxidative stress, inflammation, and hormone signaling [17]. Furthermore, *MT1A* is one of the *NFE2L2* target genes [11], and relative *NFE2L2* mRNA and protein expression decreased in SARA-induced cows [1]. *MT1A* expression was higher in the SARA group 2 and 6 weeks postpartum compared with the non-SARA group. These results suggest that the expression of *MT1A* might be associated with the response to oxidative stress in SARA cows. NFE2L2 controls the transcription of genes related to antioxidative functions [11]. In the present study, *NEF2L2* expression did not change significantly after parturition in either group, but was positively correlated with blood MDA level and negatively correlated with blood PAO level. In addition, *CAT* expression was positively correlated with MDA and expression of many of the hepatic *NFE2L2* target genes was positively correlated with MDA and negatively correlated with PAO, although not statistically significant. These results suggest that changes in peripheral blood oxidative stress markers might be associated with the status of hepatic gene expression related to oxidative stress.

PC and *PCK1* are involved in the gluconeogenic pathway [26], and *ACSL*, *CPT1A*, and *ACADVL* are involved in β-oxidation of lipids [19, 25, 31]. In the present study, expression of these genes increased after parturition in both groups and was positively correlated with NEFA and AST, and negatively correlated with GLU, suggesting that NEFA increased and GLU decreased after parturition in response to NEB [29], and gluconeogenesis and β-oxidation of lipids in the liver were enhanced. In addition, *FGF21* expression increased at 2 weeks and 6 weeks postpartum in the SARA group compared with the non-SARA group. *FGF21* expression increases with NEB development, and enhances fatty acid oxidation [9, 14]. Therefore, these results suggested that SARA group might respond more strongly to NEB than non-SARA group. Further, *ACADVL* expression decreased 6 weeks postpartum in the SARA group compared with the non-SARA group. *ACADVL* expresses an enzyme that converts acyl CoA to acetyl CoA in mitochondria during β-oxidation of lipids [31]. Therefore, we assumed that while it was necessary to enhance fatty acid oxidation, as shown by the increased expression of *FGF21*, β-oxidation of lipids in the liver was suppressed 6 weeks postpartum in the SARA group compared to the non-SARA group.

IGF1R expression decreased after parturition in the SARA group, and *SLC2A4* expression decreased after parturition in both groups. In contrast to the correlations between blood metabolites and gene expression related to gluconeogenesis and β-oxidation

of lipids, *IGF1R* and *SLC2A4* expression levels were positively correlated with GLU, and negatively correlated with NEFA. *IGF1R* and *SLC2A4* are involved in insulin function [8, 10]. These results suggest that the insulin response decreased in both groups accompanying NEB after parturition. Insulin sensitivity and responsiveness are attenuated during the early lactation period [4], and the expression of hepatic *IGF1* mRNA decreases [24]. The action of IGF1 is mainly controlled by IGF1 receptor signaling [8]. In the present study, the expression of *IGF1R*, which encodes the IGF1 receptor, decreased 6 weeks postpartum in the SARA group compared with the non-SARA group. The peripheral tissue response to insulin remains low during the early lactation period but gradually recovers [4]. We speculated that recovery of the hepatic insulin response might be delayed in the SARA group compared to the non-SARA group. SARA induces metabolic endotoxemia and systemic inflammation [1, 12], and metabolic endotoxemia results in insulin resistance [7]. Therefore, it has been suggested that SARA may cause insulin resistance [20], as supported by our results.

In conclusion, hepatic antioxidative gene expression decreased in association with an increase of NEFA and enhanced β-oxidation of lipids accompanied by NEB in Holstein cows during the periparturient period. In addition, the decrease in peripheral blood GPX activity and fluctuations in hepatic gene expression related to oxidative stress were larger in the SARA group than those in the non-SARA group. The SARA group was considered high oxidative stress status. Moreover, SARA cows might develop glycometabolic changes such as suppressed β-oxidation in the liver and a reduced insulin reaction compared to non-SARA cows.

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