Oxidative stress, NF- κ B signaling, NLRP3 inflammasome, and caspase apoptotic pathways are activated in mammary gland of ketotic Holstein cows

Xudong Sun,^{1*} Yan Tang,^{1*} Chunhui Jiang,¹ Shengbin Luo,¹ Hongdou Jia,¹ Qiushi Xu,¹ Chenxu Zhao,¹ Yusheng Liang,² Zhijun Cao,³ Guang Shao,⁴ Juan J. Lóor,² and Chuang Xu^{1†}

¹Heilongjiang Provincial Key Laboratory of Prevention and Control of Bovine Diseases, College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang Province 163319, China

²Mammalian NutriPhysioGenomics, Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana 61801

³State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China

⁴Branch of Animal Husbandry and Veterinary of Heilongjiang Academy of Agricultural Sciences, Qiqihar, Heilongjiang Province 161000, China

ABSTRACT

Ketosis is a serious metabolic disorder characterized by systemic and hepatic oxidative stress, inflammation, and apoptosis, as well as reduced milk yield. Because of the paucity of data on mammary responses during ketosis, the aim of this study was to evaluate alterations in oxidative stress, NF- κ B signaling, NLRP3 inflammasome, and caspase apoptotic pathways in mammary gland of dairy cows with ketosis. Blood, mammary gland tissue, and milk samples were collected from healthy cows [Control, blood concentration of β -hydroxybutyrate (BHB) <0.6 mM, $n = 10$] and cows with subclinical ketosis (SCK, blood concentration of BHB >1.2 mM and <3 mM, $n = 10$) or clinical ketosis (CK, blood concentration of BHB >3 mM, $n = 10$) at median 8 d in milk (range = 6–12). Compared with Control, serum concentration of glucose was lower (3.91 vs. 2.86 or 2.12 mM) in cows with SCK or CK, whereas concentrations of fatty acids (0.25 vs. 0.57 or 1.09 mM) and BHB (0.42 vs. 1.81 or 3.85 mM) were greater. Compared with Control, the percentage of milk fat was greater in cows with SCK or CK. In contrast, the percentage of milk protein was lower in cows with SCK or CK. We detected no differences in milk lactose content across groups. Compared with Control, activities of glutathione peroxidase, superoxide dismutase, and catalase were lower in mammary gland tissue of cows with SCK or CK. In contrast, concentrations of hydrogen peroxide and malondialdehyde were greater in cows with SCK or CK. Compared with Control, mRNA abundances of *TNFA*, *IL6*, and *IL1B* were greater in mammary tissues of cows with SCK or CK. In addition,

activity of IKK β and the ratio of phosphorylated inhibitor of κ B α to I κ B α , and of phosphorylated NF- κ B p65 to NF- κ B p65, were also greater in mammary tissues of cows with SCK or CK. Subclinical or clinical ketosis also led to greater activity of caspase 1 and protein abundance of caspase 1, NLRP3, Bax, caspase 3, and caspase 9. In contrast, abundance of the anti-apoptotic protein was lower in SCK or CK cows. The data indicate that the mammary gland of SCK or CK cows undergoes severe oxidative stress, inflammation, and cell death.

Key words: ketosis, mammary gland, oxidative stress, inflammation, apoptosis

INTRODUCTION

Ketosis is a common metabolic disorder in periparturient high-yielding dairy cows (Itle et al., 2015). Fatty acids released from adipose before and during onset of ketosis help compensate for the lack of energy and nutrients from the decrease in DMI but also could trigger liver dysfunction (Gross et al., 2013; Du et al., 2018). Depending on the severity of negative energy balance, blood concentrations of BHB can be used to assess subclinical ketosis (**SCK**) or clinical ketosis (**CK**) in dairy cows (Vanholder et al., 2015). In addition to its negative effect on hepatic function, ketosis may decrease milk production, increase culling, decrease reproductive efficiency, and increase the risk of other disorders (displaced abomasum and mastitis), resulting in economic losses to farmers (Duffield et al., 2009; Gross and Bruckmaier, 2019).

Metabolic challenges during periods of negative energy balance increase reactive oxygen species (**ROS**) production (Bernabucci et al., 2005). Imbalances between increased levels of ROS and low activity of antioxidant mechanisms trigger oxidative stress, which results in tissue and cellular damage (Kruk and Duchnik, 2014). The positive correlations between the level of oxidative

Received April 25, 2020.

Accepted August 13, 2020.

*These authors contributed equally to this study.

†Corresponding author: xuchuang7175@163.com

stress and concentrations of fatty acids and BHB in plasma are often observed in dairy cows with SCK and CK (Li et al., 2016). Whether redox systems of the mammary gland respond to metabolic challenges such as high concentrations of fatty acids, BHB, or both, in dairy cows with SCK or CK is unknown.

Oxidative stress is a crucial determinant of the mechanisms by which various stressors might injure cells and cause production disorders in dairy cows during early lactation (Putman et al., 2018). This is attributed to the links among oxidative stress, inflammation, and apoptosis, all of which impair the structure and function of cells. Excessive oxidative stress results in activation of redox-sensitive signal transduction pathways, such as the NF- κ B pathway and the NLRP3 inflammasome pathway, all of which trigger transcription of various proinflammatory genes such as *IL1B*, *IL6*, and *TNFA* (Abais et al., 2015; Zhang et al., 2016). In fact, work with ketotic cows has suggested that elevated concentrations of *TNFA*, *IL6*, and *IL1B* in the blood are associated with systemic inflammation and impaired immune function (Abuajamieh et al., 2016; Zhou et al., 2016). Inflammatory responses in mammary gland tissue impaired lactation capacity, which contributed to reduced milk synthesis and quality (Seegers et al., 2003). In addition to inflammation, oxidative stress also triggers cell apoptosis by activating the caspase signaling pathway. In ruminants, cellular antioxidant status may modulate apoptosis and proliferation of mammary epithelial cells under oxidative stress (Miranda et al., 2011). As such, a decrease in numbers of mammary epithelial cells due to enhanced apoptosis would contribute to a decline in milk production (Capuco et al., 2001). Thus, inflammation and apoptosis may compromise normal mammary gland function during ketosis.

The increased metabolic rate of the mammary gland after calving enhances its susceptibility to metabolic stress and an altered redox state, with onset of ketosis potentially compromising normal coordination of these processes. Thus, we hypothesized that enhanced oxidative stress, inflammation, and apoptosis triggered by metabolic stress may contribute to dysfunction of mammary gland in dairy cows with ketosis. The purpose of this study was to investigate alterations in pathways associated with oxidative stress, inflammation, and apoptosis in mammary gland of dairy cows with SCK and CK.

MATERIALS AND METHODS

All animal procedures were approved by the Animal Ethics Committee for the Use and Care, Heilongjiang Bayi Agricultural University (Daqing, China).

Animals

Lactating Holstein cows (parity: median = 3, range = 2–4; DIM: median = 8 d, range = 6–12) were selected from a commercial dairy herd located in Heilongjiang Province, China. Although we did not perform a power test, it was deemed based on previous similar studies that 10 cows would provide sufficient power for detecting statistical differences in terms of production and molecular analyses (Dann et al., 2005; Loor et al., 2007; Sun et al., 2019b). To ensure that cows did not have any other comorbidities, they were subjected to a routine physical examination by farm veterinarians. Clinical signs of mastitis (swelling, redness, pain in the udder, or abnormal milk color, visible clots or flakes in milk) were not observed in any of these cows or quarters. Veterinarians classified cows as suspected SCK if results for a nitroprusside-based milk ketone test were positive in absence of clinical symptoms, and classified cows as suspected CK if results for a nitroprusside-based milk ketone test were positive in the presence of clinical symptoms including substantial and rapid loss of live weight, decreased feed intake and milk yield, severely decreased appetite, and excessively dry feces. Then we preselected 57 cows with suspected SCK, 46 cows with suspected CK, and 42 healthy cows. To diagnose SCK and CK, the blood concentration of BHB in these cows was measured. According to the serum BHB concentration and clinical symptoms (Zhu et al., 2019), 10 healthy cows with serum BHB concentrations <0.6 mM were selected randomly among 42 healthy cows, 10 SCK cows with serum BHB concentrations in the range of 1.2 to 3 mM without clinical symptoms were selected randomly among 57 cows with suspected SCK, and 10 CK cows with serum BHB concentrations >3 mM with clinical symptoms were selected randomly among 46 cows with suspected CK. To reduce environmental effects, cows were housed in a climate-controlled barn in individual tiestalls. Cows were fed a TMR consisting of 49.3% corn silage, 4.5% hay (alfalfa hay and oat hay), and 46.2% concentrate and mineral mix on a DM basis (DM: $46.4 \pm 1.3\%$). Individual cow feed intake was recorded by the dairy cattle breeder every day. The DM percentages of TMR were used to calculate daily DMI. The BCS was assigned based on a 1- to 5-point scale according to previous reports (emaciated cows are scored 1; thin cows, 2; average cows, 3; fat cows, 4; and obese cows, 5; Edmonson et al., 1989).

Milk Sample Collection and Milk Composition

Cows were milked 3 times daily at 0400, 1200, and 2000 h, and milk yield was recorded via an automatic

milking system. Samples were collected at each milking from 3 d before until 2 d after biopsy, and 1 mg/mL potassium dichromate was added as a preservative for storage at 4°C. Considering the effects of biopsy on milk yield and to account for daily variation in milk yield (Farr et al., 1996), data from 3 d before the biopsy were averaged with data from 2 d after the biopsy, disregarding production data from both the day of biopsy and the first day after the biopsy. Milk lactose, protein, and fat analyses were performed according to published procedures (Sun et al., 2019b) by infrared analysis with a 4-channel spectrophotometer (Multispec, Foss Food Technology Corp., Eden Prairie, MN). Each sample ($n = 10$ cows per group) was run in triplicate.

Blood Collection and Blood Biomarkers

Before feeding, blood samples were collected via coccygeal venipuncture without anticoagulant between 0630 and 0730 h on 3 consecutive days. Serum was harvested after centrifugation at $3,500 \times g$ at 4°C for 15 min. Serum concentrations of BHB, fatty acids, and glucose were measured using commercially available kits (fatty acids: cat. no. FA115; BHB: cat. no. RB1008; glucose: cat. no. GL3815; Randox Laboratories, Crumlin, UK) with Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan). The limit of quantification of fatty acids was 0.04 mM, and the intra- and interassay coefficients of variation for Control, SCK, and CK were 2.5, 2.6, and 2.3%, and 2.2, 2.3, and 2.8%, respectively. The limit of quantification of BHB was 0.1 mM, and the intra- and interassay coefficients of variation for Control, SCK, and CK were 2.3, 2.7, and 2.5%, and 2.2, 2.6, and 2.6%, respectively. The limit of quantification of glucose was 0.2 mM, and the intra- and interassay coefficients of variation for Control, SCK, and CK were 3.5, 2.7, and 3.2%, and 3.8, 4.5, and 3.6%, respectively. Each sample ($n = 10$ cows per group) was run in triplicate.

Mammary Biopsy

Mammary gland tissue biopsies from the right or left rear quarter of the mammary gland ($n = 10$) were carried out as described by Bionaz and Looor (2008). Cows received an intramuscular injection of 2% lidocaine (0.05 mg/kg of BW; 2% Seractal, Bayer, Leverkusen, Germany) for local anesthesia. Then, the skin of the gland was washed and sterilized with 70% alcohol and iodine tincture (ANNJET Pharma, Shandong, China). A 1- to 2-cm incision was made with the scalpel through the skin and gland capsule, avoiding any large subcutaneous blood vessels. Mammary parenchyma was then obtained via blunt dissection of the mammary capsule. To control bleeding, pressure was applied to the entry

point after removal of the biopsy instrument from the capsule. The skin incision was closed with 4 or 5 Michel clips (11 mm; Henry Schein, Melville, NY). Topical antiseptic (povidone iodine ointment, 10%; Taro Pharmaceuticals, Hawthorne, NY) was applied to the incision site. Michel clips were removed 7 to 10 d after the biopsy. Tissue samples were washed with normal saline and were frozen immediately in liquid nitrogen and stored at -80°C until analysis.

Quantitative Reverse-Transcription PCR

Quantitative reverse-transcription PCR was performed according to published procedures (Sun et al., 2019b). Total RNA was extracted from mammary tissue (30 mg) using RNAiso Plus (TaKaRa Biotechnology Co. Ltd., Dalian, China) following manufacturer's instructions. The RNA concentration and purity were measured using K5500 MicroSpectrophotometer (Beijing Kaiao Technology Development Ltd., Beijing, China). In the present study, the optical density ratio at 260 and 280 nm ($\text{OD}_{260}/\text{OD}_{280}$) of the total RNA was determined to be 1.85 to 1.95 and met the minimum information for publication of quantitative real-time PCR experiment guidelines. (The adequate threshold for determining the purity of RNA upon extraction of biologic samples is an $\text{OD}_{260}/\text{OD}_{280}$ ratio of 1.8–2.0; Heiko and Christopher, 2011). The RNA was then reverse-transcribed into cDNA using a reverse-transcription kit (RR047A, TaKaRa Biotechnology Co. Ltd.) in accordance with the manufacturer's protocol. Relative mRNA abundance was measured with the 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) using a SYBR green plus reagent kit (Roche, Norwalk, CT). The mRNA abundances of target genes were normalized to the mean of GAPDH and ubiquitin B, and calculated by the $2^{-\Delta\Delta\text{CT}}$ method. The PCR reaction was performed

Table 1. Primer sequences (F, forward; R, reverse) of the genes analyzed

Gene ¹	Primer sequences (5'-3')	Length (bp)
<i>TNFA</i>	F: GCCTCCCTCTCATCAGTTCTA R: GGCAGCCTTGTCCTTG	246
<i>IL6</i>	F: AGTTGTGCAATGGCAATTCTGA R: CCCCAGCATCGAAGGTAGA	223
<i>ILB</i>	F: ACCTGTGTCTTTCCCGTGG R: TCATCTCGGAGCCTGTAGTG	162
Ubiquitin B	F: AGATCCAGGATAAGGAAGGCAT R: GCTCCACCTCCAGGGTGAT	198
<i>GAPDH</i>	F: GTCTTCACTACCATGGAGAAGG R: TCATGGATGACCTTGCCAG	197

¹*TNFA* = tumor necrosis factor α ; *IL6* = interleukin-6; *IL1B* = interleukin-1 β .

in triplicate for each sample ($n = 10$ cows per group). Primer sets are shown in Table 1.

Protein Extraction and Western Blotting

Radio immunoprecipitation assay lysis buffer (P0013, Beyotime Institute of Biotechnology, Jiangsu, China) was used to extract total protein from mammary tissue (40 mg). The bicinchoninic acid assay (P1511, Applygen Technologies, Beijing, China) was used to measure protein concentrations. Western blotting was performed as described previously (Zhu et al., 2019). The protein samples (30 μ g per lane) were separated using SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membrane (45 μ m). The membranes were then blocked in Tris-HCL (0.05 M Tris, pH 7.4, 0.2 M NaCl) buffer solution containing 3% bovine serum albumin and 0.1% Tween-20 in for 4 h at room temperature. Subsequently, membranes were incubated with specific antibodies for ACTB (1:1000, sc-47778, Santa Cruz Biotechnology, Dallas, TX), phosphorylated inhibitor of κ B α (**p-I κ B α** ; 1:5,000, sc-271980, Santa Cruz Biotechnology), I κ B α (1:1,000, sc-166588, Santa Cruz Biotechnology), NLRP3 (1:1,000, ab210491, Abcam, Cambridge, MA), caspase 1 (1:5,000, ab201476, Abcam), NF- κ B p65 (1:1,000, sc-8008, Santa Cruz Biotechnology), phosphorylated-NF- κ B p65 (**p-NF- κ B p65**; 1:1,000, sc-166748, Santa Cruz Biotechnology), Bax (1:1000, ab32503, Abcam), Bcl-2 (1:500, ab201566, Abcam), caspase 3 (1:500, ab4051, Abcam), and caspase 9 (1:1,000, sc-56076, Santa Cruz Biotechnology) at 4°C overnight. Membranes were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Boster, Wuhan, China) at room temperature for 45 min. Immunoreactive bands were visualized via protein imager (ProteinSimple, San Jose, CA) using an enhanced chemiluminescence solution (Pierce Biotechnology Inc., Chicago, IL). Bands were quantified with Image-Pro Plus 6.0 (Media Cybernetics Inc., Rockville, MD). Individual samples ($n = 10$ cows per group) were run in triplicate.

IKK β Activity

The IKK β activity was measured as reported previously (Shen et al., 2019) using IKK β Kinase Assay Kit (GMS50162.4; GenMed Scientifics Inc., Wilmington, DE). Briefly, 30 mg of mammary tissue was washed and homogenized in an ice bath for 30 min. Samples were then centrifuged at $10,000 \times g$ for 10 min at 4°C. A bicinchoninic acid assay kit (P1511, Applygen Technologies) was used to measure protein concentrations. Subsequently, total IKK β and nonspecific IKK β activity were measured by detecting absorbance values

using a spectrophotometer (Thermo Fisher Scientific) at 450 nm. The specific IKK β activity is reported as total IKK β activity minus nonspecific activity. Each sample ($n = 10$ cows per group) was run in triplicate.

Caspase 1 Activity

Caspase 1 activity was measured using a commercial kit (C1101, Beyotime) following the manufacturer's instructions. Briefly, mammary tissue samples (50 mg) were homogenized and lysed using radio immunoprecipitation assay buffer (P0013, Beyotime). Then, samples were centrifuged at $16,000 \times g$ for 15 min at 4°C. Protein concentration was measured with the bicinchoninic acid assay kit (P1511, Applygen Technologies). Then 10 μ L of protein (3 mg/mL) was incubated with 10 μ L of Ac-YVAD-pNA (2 mmol/mL) at 37°C for 2 h. Absorbance at 405 nm was measured with a spectrophotometer (Thermo Fisher Scientific). Caspase 1 activity was normalized to the protein concentration of each sample, and results are reported as the relative activity of caspase 1 in SCK and CK versus the Control group. Each sample ($n = 10$ cows per group) was run in triplicate.

Detection of Oxidative Stress Indicators

Activity of glutathione peroxidase (**GSH-Px**), superoxide dismutase (**SOD**), and catalase (**CAT**), as well as concentrations of hydrogen peroxide and malondialdehyde (**MDA**) in mammary tissue (200 mg) were measured using commercially available kits (GSH-Px: S0058; SOD: S0109; MDA: S0131; Beyotime Institute of Biotechnology, Jiangsu, China; H₂O₂: A064-1; CAT: A007; Nanjing Jian Cheng Institute of Bioengineering, Nanjing, China), following the manufacturer's instructions. Mammary tissue samples were homogenized in ice-cold PBS using a microthermal (4°C) tissue grinder (Shanghai Gino Tech and Trading Co. Ltd., Shanghai, China). Subsequently, samples were centrifuged at $12,000 \times g$ for 10 min at 4°C. The 722N spectrophotometer (Scientific Instrument Co. Ltd., Shanghai, China) was used to monitor absorbance of GSH-Px, SOD, CAT, H₂O₂, and MDA at 340 nm, 560 nm, 405 nm, 405 nm, and 532 nm, respectively. Each sample ($n = 10$ cows per group) was run in triplicate.

Statistical Analysis

Data were analyzed using GraphPad Prism 7 (GraphPad InStat Software, San Diego, CA). All data were tested for normality of distribution with the Shapiro-Wilk test. The data for BW, milk yield, DMI, BCS, milk lactose, protein, and fat, and serum levels of fatty

acids, and BHB and glucose were not normally distributed and were analyzed with a nonparametric test using the Kruskal–Wallis test followed by Dunn’s test for multiple comparisons. These data are reported as the median and interquartile range. Other data were normally distributed and analyzed with 2-way ANOVA followed by a Bonferroni correction and are reported as means $\pm$ standard error of the mean (mean $\pm$ SEM). To present the data for mRNA abundance of cytokines, activity of IKK β and caspase 1, and abundance of target proteins, we set the relative abundance of the Control group to 1 and showed differences for the SCK and CK groups relative to the Control group, rather than for each group relative to expression of reference genes or protein content. This approach has been used by others (Pi et al., 2010; Dong et al., 2020). We divided each cow’s result by the mean value of the outcome for cows in the Control group to present fold differences relative to the Control group. Each experiment was repeated at least 3 times; $P < 0.05$ was considered statistically significant.

RESULTS

Changes of Content of Protein and Fat in Milk, and Fatty Acids, BHB, and Glucose in Serum of Cows with Ketosis

Milk yield, milk protein percentage, and DMI were greater for cows in the Control and SCK groups compared with the CK group, whereas BCS was lower for Control and SCK cows compared with the CK group ($P < 0.05$, Table 2). Compared with the Control group, milk fat percentage was greater for the SCK and CK groups, and milk fat percentage increased with

Table 2. Basic description of Holstein cows classified as healthy (Control, $n = 10$) or having subclinical ketosis (SCK, $n = 10$) or clinical ketosis (CK, $n = 10$)

Item	Median (IQR) ¹		
	Control	SCK	CK
BW (kg)	623 (20.32) ^a	632 (23.75) ^a	645 (26.48) ^a
Milk yield (kg)	31.3 (2.36) ^a	28.8 (2.27) ^a	24.3 (1.69) ^b
Milk fat (%)	4.13 (0.14) ^a	4.77 (0.25) ^b	5.25 (0.23) ^c
Milk protein (%)	3.31 (0.17) ^a	3.12 (0.22) ^a	2.82 (0.34) ^b
Milk lactose (%)	4.58 (0.11) ^a	4.46 (0.13) ^a	4.38 (0.21) ^a
DMI (kg/d)	22.1 (1.48) ^a	20.7 (1.68) ^a	19.2 (1.75) ^b
BCS	2.93 (0.25) ^a	3.17 (0.21) ^a	3.84 (0.31) ^b

^{a–c}Different superscripts within a row denote differences ($P < 0.05$).

¹IQR = interquartile range.

increased degree of ketosis ($P < 0.05$, Table 2). We detected no differences in BW and milk lactose content across groups ($P > 0.05$, Table 2).

Compared with the Control group, serum levels of fatty acids and BHB were greater for the SCK and CK groups, and serum levels of fatty acids and BHB increased with increased degree of ketosis ($P < 0.01$, Figure 1 A and B). In contrast, the concentration of glucose was lower in cows with SCK ($P = 0.02$) and CK ($P < 0.01$) compared with the Control group (Figure 1 C), and the concentration of glucose decreased with increased degree of ketosis ($P = 0.02$).

Redox State in Mammary Tissue of Cows with SCK or CK

Contents of H₂O₂ and MDA were greater in mammary tissue of cows with SCK and CK compared with Control ($P < 0.01$, Figure 2 A and B). Contents of H₂O₂ and MDA increased with increased degree of ketosis (P

Figure 1. Serum concentrations of fatty acids (A), BHB (B), and glucose (C) in healthy Control ($n = 10$), subclinical ketotic (SCK, $n = 10$), and clinical ketotic (CK, $n = 10$) cows. Dots represent results of individual cows. Horizontal bars represent median and interquartile range. Different lowercase letters in bar charts indicate significant differences ($P < 0.05$); letters that are the same denote lack of significance ($P > 0.05$).

Figure 2. Oxidative stress status of mammary gland in mammary gland tissue of healthy Control (n = 10), subclinical ketotic (SCK, n = 10), and clinical ketotic (CK, n = 10) cows. Concentrations of H_2O_2 (A) and malondialdehyde (MDA, B), and activities of superoxide dismutase (SOD, C), glutathione peroxidase (GSH-Px, D), and catalase (CAT, E) in mammary gland tissue. Comparisons among groups were calculated using a 2-way ANOVA with subsequent Bonferroni correction. Data are mean $\pm$ SEM. Different lowercase letters in bar charts indicate significant differences ($P < 0.05$).

< 0.01). However, the activities of SOD, GSH-Px, and CAT were lower in mammary tissue of cows with SCK and CK compared with Control ($P < 0.01$, Figure 2 C–E). Activities of SOD, GSH-Px, and CAT decreased with increased degree of ketosis ($P < 0.01$).

Expression of Proinflammatory Cytokines in Mammary Tissue of Cows with SCK or CK

The mRNA abundances of *TNFA*, *IL6*, and *IL1B* were greater in mammary tissue of cows with SCK and CK compared with Control (Figure 3), and mRNA abundances of *TNFA*, *IL6*, and *IL1B* increased with increased degree of ketosis.

NF- κ B and NLRP3 Pathways in Mammary Tissue of Cows with SCK or CK

The IKK β activity and the ratio of p-I κ B α to I κ B α were lower in mammary tissues of cows in the Control and SCK groups compared with the CK group (Figure 4 A–C). Activity of IKK β and the ratio of p-I κ B α to I κ B α were not different in the SCK compared with the

Control group (Figure 4 A–C). The ratio of p-NF- κ B p65 to NF- κ B p65 was greater in mammary tissues of cows with SCK and CK compared with Control (Figure 4 B and D). The ratio of p-NF- κ B p65 to NF- κ B p65 increased with increased degree of ketosis.

Activity of caspase 1 was greater in mammary tissues of cows with SCK and CK compared with Control (Figure 5 A). Caspase 1 activity increased with increased degree of ketosis. Protein abundance of caspase 1 and NLRP3 was greater in mammary tissue of cows with SCK and CK compared with Control (Figure 5 B–D). Protein abundance of caspase 1 and NLRP3 increased with increased degree of ketosis.

Caspase Apoptosis Pathway in Mammary Tissue of Cows With SCK or CK

Protein abundances of Bax, caspase 3, and caspase 9 were greater in mammary tissues of cows with SCK and CK compared with Control (Figure 6 A, B, D, and E). Protein abundances of Bax, caspase 3, and caspase 9 increased with increased degree of ketosis. However, protein abundance of Bcl-2 was lower in mammary tis-

sue of cows with CK and SCK compared with Control (Figure 6 A and C). Protein abundance of Bcl-2 decreased with increased degree of ketosis.

DISCUSSION

The high blood concentrations of fatty acids and BHB as well as low concentrations of glucose detected in the present study underscored the metabolic challenges experienced by cows. In response to metabolic stress, changes in H_2O_2 and MDA production, as well as in antioxidant enzyme activities, indicated that the mammary gland of ketotic cows is susceptible to oxidative stress. More importantly, ketotic cows displayed activation of NF- κ B signaling, NLRP3 inflammasome, and the Bcl-2/caspase 9 apoptotic pathway, along with greater mRNA abundance of proinflammatory cytokines (*TNFA*, *IL6*, and *IL1B*) in the mammary gland. Together, these data suggest that ketosis can induce oxidative stress in the mammary gland, which in turn activates inflammation and apoptotic pathways, all of which may contribute to dysfunctional responses.

Metabolic challenges in dairy cows during periods of negative energy balance alter the intracellular reduction-oxidation (redox) balance, which results in oxidative stress and decreased milk yield. Previous studies have revealed that dairy cows with ketosis display systemic oxidative stress (Sahoo et al., 2009; Youssef and El-Ashker, 2017). In the present study, the greater H_2O_2 and MDA contents highlighted the effects of metabolic stress on free radical production and levels of lipid peroxidation products. This might have been due to excessive fatty acids and ketone body

absorption by mammary gland from plasma and to an intracellular increase in substrates for oxidation. In fact, increased circulating ketone bodies (BHB and acetoacetate) and fatty acids were reported to enhance production of ROS (Li et al., 2015, 2016; Song et al., 2016). The lower activities of SOD, GSH-Px, and CAT, coupled with greater H_2O_2 and MDA content, in cows with SCK or CK confirmed the greater degree of oxidative stress within the mammary gland, particularly of cows with CK. Work with ruminants has demonstrated that fatty acids (Song et al., 2014) and BHB (Song et al., 2016) induced hepatocyte oxidative stress in a dose-dependent manner. Thus, we speculated that the degree of oxidative stress in the mammary gland may be related to the severity of metabolic stress in cows, as evidenced by the greater serum levels of fatty acids and BHB in cows with CK. Because enhancing the antioxidant capacity of periparturient dairy cows can increase milk production (Garavaglia et al., 2015), finding strategies to maintain the redox balance might provide the foundation for future research on their feasibility for practical use in dairy cow management.

Proinflammatory cytokines (*TNFA*, *IL6*, and *IL1B*) act as intercellular chemical messengers involved in the regulation of mammary immune response (Moyes et al., 2011), and their mRNA abundance in bovine mammary epithelial cells is known to increase in response to inducers of oxidative stress (Sun et al., 2019a). Along with higher oxidative stress, the greater mRNA abundance of proinflammatory cytokines in cows with CK than in those with SCK underscored the possibility that the degree of inflammation in the mammary gland of ketotic cows is related to the degree of oxidative

Figure 3. Inflammation status of mammary gland in mammary gland tissue of healthy Control (n = 10), subclinical ketotic (SCK, n = 10), and clinical ketotic (CK, n = 10) cows: mRNA abundance of *TNFA* (A), *IL6* (B), and *IL1B* (C). Comparisons among groups were calculated using a 2-way ANOVA with subsequent Bonferroni correction. To present the data for mRNA abundance of cytokines, we set the relative abundance of the Control group to 1, and show differences of the SCK and CK groups relative to the Control group, rather than of each group relative to expression of reference genes or protein content. We divided each cow's result by the mean value of the outcome for cows in the Control group to present fold differences relative to the Control group. Data are mean ± SEM. Different lowercase letters in bar charts indicate significant differences ($P < 0.05$).

Figure 4. Abundance of molecules involved in NF- κ B signaling in mammary gland tissue of healthy Control (n = 10), subclinical ketotic (SCK, n = 10), and clinical ketotic (CK, n = 10) cows. (A) IKK β activity; (B) Western blot analysis of phosphorylated inhibitor of κ B α (p-I κ B α), inhibitor of κ B α (I κ B α), phosphorylated NF- κ B p65 (p-NF- κ B p65), and NF- κ B p65; (C) protein abundance of ratio of p-I κ B α to I κ B α ; (D) protein abundance of ratio of p-NF- κ B p65 to NF- κ B p65. Comparisons among groups were calculated using a 2-way ANOVA with subsequent Bonferroni correction. To present the data for activity of IKK β and abundance of target proteins, we set the relative abundance of the Control group to 1, and show differences of the SCK and CK groups relative to the Control group, rather than of each group relative to expression of reference genes or protein content. We divided each cow's result by the mean value of the outcome for cows in the Control group, to present fold differences relative to the Control group. Data are mean $\pm$ SEM. Different lowercase letters in bar charts indicate significant differences ($P < 0.05$); letters that are the same denote lack of significance ($P > 0.05$).

stress. In addition to oxidative stress, palmitic acid, one of the major components of fatty acids, could directly activate proinflammatory signaling pathways that promote proinflammatory cytokine expression (Lee et al., 2001). In addition, intramammary stressors, such as those causing mastitis, lead to increased secretion of proinflammatory cytokines, including *TNFA*, *IL6*, and *IL1B* (Miao et al., 2007). The increase in IL-6 concentration in quarter milk is believed to be an indicator of mammary gland inflammation (Sakemi et al., 2011). In the present study, the greater mRNA abundances of *TNFA*, *IL6*, and *IL1B* in the mammary tissue of ketotic cows partly explained the higher susceptibility to mastitis during oxidative stress conditions. In fact, Brunner et al. (2018) reported that risk of ketosis also enhances the likelihood of mastitis. In turn, mastitis increases the

production of free radicals (e.g., lipoperoxidation) that could cause lesions in mammary tissue (Eslami et al., 2015). Clearly, a limitation of the present study is that analyses of biomarkers of mastitis were not performed (e.g., SCC, leucocyte counts, bacterial cultures). However, the present data strongly suggest the existence of a link between those biomarkers and tissue oxidative stress and inflammation.

Work in nonruminants has demonstrated that NF- κ B signaling plays vital roles in regulating inflammatory responses, with intracellular redox status having a major role in the control of NF- κ B activity (Zhao et al., 2017; Lecoeur et al., 2020). As a vital cell signaling messenger, ROS can activate the NF- κ B signaling pathway, which in turn increases abundance of proinflammatory cytokines (Nakajima and Kitamura, 2013).

Figure 5. Abundance of molecules involved in NLRP3 inflammasome pathway in mammary gland tissue of healthy Control ($n = 10$), sub-clinical ketotic (SCK, $n = 10$), and clinical ketotic (CK, $n = 10$) cows. (A) Caspase 1 activity; (B) Western blot analysis of caspase 1 and NLRP3; (C) protein abundance of caspase 1; (D) protein abundance of NLRP3. Comparisons among groups were calculated using a 2-way ANOVA with subsequent Bonferroni correction. To present the data for activity of caspase 1 and abundance of target proteins, we set the relative abundance of the Control group to 1, and show differences of the SCK and CK groups relative to the Control group, rather than of each group relative to expression of reference genes or protein content. We divided each cow's result by the mean value of the outcome for cows in the Control group to present fold differences relative to the Control group. Data are mean $\pm$ SEM. Different lowercase letters in bar charts indicate significant differences ($P < 0.05$).

When cells are stimulated, IKK β is rapidly activated, leading to phosphorylation and proteasome-mediated degradation of I κ B α (Zandi et al., 1997). The resultant NF- κ B is then phosphorylated and translocated into the nucleus, which induces transcription of cytokines (Barnes and Karin, 1997). In HeLa cells (human cervical cancer cells), hypoxia and reoxygenation induced ROS-mediated phosphorylation of I κ B α and activated NF- κ B (Fan et al., 2003). Thus, the greater activity of IKK β , phosphorylation of I κ B α and phosphorylation of NF- κ B p65 in cows with SCK or CK likely promoted the transcription of proinflammatory cytokines. We speculate that such a response was partly due to increased ROS production in response to an overload of fatty acids and BHB in the mammary gland. In fact, palmitic acid and BHB trigger inflammatory responses

in bovine endometrial (Li et al., 2019b) or kidney (Bionaz et al., 2012) cells via activation of oxidative stress-mediated NF- κ B signaling. The specific mechanisms whereby activated NF- κ B travels to the nucleus and activates the transcriptional program in mammary gland of ketotic cows remains to be elucidated.

At least in nonruminants, in addition to NF- κ B, NLRP3 inflammasome is another ROS-sensitive pathway responsible for activating the inflammatory response (Tschopp and Schroder, 2010). Changes in the redox microenvironment regulate NLRP3 inflammasome activation, resulting in cleaved caspase 1 (Rubartelli, 2012). Thus, the greater activity of caspase 1, upregulation of NLRP3, and caspase 1 protein abundance in the present study are suggestive of NLRP3 inflammasome activation in mammary gland of ketotic

Figure 6. Abundance of molecules involved in the Bcl-2/caspase9 apoptotic pathway in mammary gland tissue of healthy Control ($n = 10$), subclinical ketotic (SCK, $n = 10$), and clinical ketotic (CK, $n = 10$) cows. (A) Western blot analysis of Bax, Bcl-2, caspase 3, and caspase 9; (B) protein abundance of Bax; (C) protein abundance of Bcl-2; (D) protein abundance of caspase 3; (E) protein abundance of caspase 9. Comparisons among groups were calculated using a 2-way ANOVA with subsequent Bonferroni correction. To present the data for abundance of target proteins we set the relative abundance of the Control group to 1, and show differences of the SCK and CK groups relative to the Control group, rather than of each group relative to expression of reference genes or protein content. We divided each cow's result by the mean value of the outcome for cows in the Control group to present fold differences relative to the Control group. Data are mean $\pm$ SEM. Different lowercase letters in bar charts indicate significant differences ($P < 0.05$).

cows. Work among nonruminants has demonstrated that palmitic acid triggers activation of the NLRP3 inflammasome, which promotes IL1B production (Wen et al., 2011). During the transition period, dairy cows with high BCS are more susceptible to metabolic challenges and oxidative stress (Bernabucci et al., 2005; Akbar et al., 2015). Thus, in the present study, high concentrations of fatty acids and BHB in ketotic cows, especially in CK cows with high BCS, were likely the major cause of altered redox balance and activation of the inflammasome. These data underscore that metabolic stress could induce systemic inflammation in dairy cows during early lactation (Esposito et al., 2014).

In addition to inflammation, metabolic stress alters mammary epithelial cell component synthesis and secretory capacity (Gross and Bruckmaier, 2019). The percentage of fat and protein in the milk has also been suggested as an indication of ketosis (Duffield et al.,

1997). In the present study, along with greater concentrations of fatty acids and BHB, the greater milk fat percentage and lower milk protein percentage in ketotic cows underscored that metabolic stress alters milk composition (Duffield et al., 2009). Excessive absorption of long-chain fatty acids and ketone bodies by mammary gland of ketotic cows increases availability of precursors for milk fat synthesis and secretion (Zhang et al., 2015; Sun et al., 2019b). Furthermore, Shu et al. (2020) reported that high levels of long-chain fatty acids inhibit milk protein synthesis through suppression of the JAK2/STAT5 and mTOR signaling pathways in mammary epithelial cells of cows with CK. Thus, it is likely that high levels of fatty acids and BHB absorbed by the mammary gland contribute to altering the percentage of milk fat and milk protein in ketotic cows.

A shortfall in the supply of nutrients and energy to meet the metabolic demands of the mammary gland

clearly contributes to the decrease in milk yield of ketotic cows (Gross and Bruckmaier, 2019). However, from a strict mammary gland standpoint, a greater rate of cell apoptosis relative to cell renewal is a key contributor to decreased milk yield (Capuco et al., 2001). In addition to inflammation, oxidative stress induces bovine mammary epithelial cell apoptosis, which decreases milk yield (Hadsell et al., 2007). Activation of the cysteine protease family (caspase) apoptotic pathway contributes to this process. Caspase 9 is an important intracellular amplifier of caspase signaling downstream of the mitochondria apoptosis pathway. Excessive ROS can activate caspase 9, which in turn leads to activation of a caspase 3-mediated cascade reaction and results in apoptosis (Li et al., 2019a). Furthermore, Bcl-2 family proteins (Bcl-2 and Bax) are also involved in the regulation of cell apoptosis by controlling the release of cytochrome C from mitochondria. The antiapoptotic protein Bcl-2 can inhibit oxyradical-mediated membrane damage and prevent mitochondrial cytochrome C release (Adams and Cory, 1998), whereas Bax, a proapoptotic protein, can change the permeability of the mitochondrial membrane and release cytochrome C, leading to cellular apoptosis (Eskes et al., 1998). In the present study, the lower protein abundance of Bcl-2 and greater protein abundance of Bax, caspase 9, and caspase 3 all provide evidence that the Bcl-2/caspase9 apoptotic pathway was activated in the mammary glands of ketotic cows. Song et al. (2014) reported that fatty acids induced bovine hepatocyte apoptosis by increasing ROS production, altering the ratio of Bax to Bcl-2, and activating the caspase 9/caspase 3 pathway. Thus, we propose that high levels of fatty acids absorbed by the mammary gland contributed to activation of the Bcl-2/caspase 9 apoptotic pathway in ketotic cows.

CONCLUSIONS

The mammary glands of ketotic cows display oxidative stress and activation of NF- κ B signaling, NLRP3 inflammasome, and Bcl-2/caspase 9. These molecular responses to excessive concentrations of fatty acids and BHB may contribute to dysfunction of the mammary gland and declines in milk yield. Thus, strategies to maintain redox balance might provide the foundation for future research on their feasibility for practical use in dairy cow management.

ACKNOWLEDGMENTS

This work was supported by the project funded by Heilongjiang Postdoctoral Science Foundation (Heilongjiang, China; grant no. LBH-Z19090), the National

Natural Science Foundation of China (Beijing, China; grant no. 31672622), the China Postdoctoral Science Foundation (Beijing, China; grant no. 2019M661316), and the Personnel Foundation in Heilongjiang Bayi Agricultural University (Daqing, China; grant no. XYB201909). Authors XS, YT, and CX conceived of the study; XS, YT, CJ, SL, HJ, QX, CZ, and CX carried out experiments and data analysis; XS, JJJ, and CX interpreted the data; XS, JJJ, and CX wrote the manuscript. All authors approved the final version. The authors declare that no conflicts of interest exist.

REFERENCES

- Abais, J. M., M. Xia, Y. Zhang, K. M. Boini, and P. L. Li. 2015. Redox regulation of NLRP3 inflammasomes: ROS as trigger or effector? *Antioxid. Redox Signal.* 22:1111–1129. <https://doi.org/10.1089/ars.2014.5994>.
- Abuajamieh, M., S. K. Kvidera, M. V. Fernandez, A. Nayeri, N. C. Upah, E. A. Nolan, S. M. Lei, J. M. DeFrain, H. B. Green, K. M. Schoenberg, W. E. Trout, and L. H. Baumgard. 2016. Inflammatory biomarkers are associated with ketosis in periparturient Holstein cows. *Res. Vet. Sci.* 109:81–85. <https://doi.org/10.1016/j.rvsc.2016.09.015>.
- Adams, J. M., and S. Cory. 1998. The Bcl-2 protein family: Arbiters of cell survival. *Science* 281:1322–1326. <https://doi.org/10.1126/science.281.5381.1322>.
- Akbar, H., T. M. Grala, M. Vailati Riboni, F. C. Cardoso, G. Verkerk, J. McGowan, K. Macdonald, J. Webster, K. Schutz, S. Meier, L. Matthews, J. R. Roche, and J. J. Loo. 2015. Body condition score at calving affects systemic and hepatic transcriptome indicators of inflammation and nutrient metabolism in grazing dairy cows. *J. Dairy Sci.* 98:1019–1032. <https://doi.org/10.3168/jds.2014-8584>.
- Barnes, P. J., and M. Karin. 1997. Nuclear factor- κ B: A pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* 336:1066–1071. <https://doi.org/10.1056/NEJM199704103361506>.
- Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of body condition score on the relationship between metabolic status and oxidative stress in periparturient dairy cows. *J. Dairy Sci.* 88:2017–2026. [https://doi.org/10.3168/jds.S0022-0302\(05\)72878-2](https://doi.org/10.3168/jds.S0022-0302(05)72878-2).
- Bionaz, M., and J. J. Loo. 2008. Gene networks driving bovine milk fat synthesis during the lactation cycle. *BMC Genomics* 9:366. <https://doi.org/10.1186/1471-2164-9-366>.
- Bionaz, M., B. J. Thering, and J. J. Loo. 2012. Fine metabolic regulation in ruminants via nutrient-gene interactions: Saturated long-chain fatty acids increase expression of genes involved in lipid metabolism and immune response partly through PPAR- α activation. *Br. J. Nutr.* 107:179–191. <https://doi.org/10.1017/S0007114511002777>.
- Brunner, N., S. Groeger, J. Canelas Raposo, R. M. Bruckmaier, and J. J. Gross. 2018. Prevalence of subclinical ketosis and production diseases in dairy cows in Central and South America, Africa, Asia, Australia and New Zealand, and Eastern Europe. *Transl. Anim. Sci.* 3:84–92. <https://doi.org/10.1093/tas/txy102>.
- Capuco, A. V., D. L. Wood, R. Baldwin, K. Mcleod, and M. J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: Relation to milk production and effect of bST. *J. Dairy Sci.* 84:2177–2187. [https://doi.org/10.3168/jds.S0022-0302\(01\)74664-4](https://doi.org/10.3168/jds.S0022-0302(01)74664-4).
- Dann, H. M., D. E. Morin, G. A. Bollero, M. R. Murphy, and J. K. Drackley. 2005. Prepartum intake, postpartum induction of ketosis, and periparturient disorders affect the metabolic status of dairy cows. *J. Dairy Sci.* 88:3249–3264. [https://doi.org/10.3168/jds.S0022-0302\(05\)73008-3](https://doi.org/10.3168/jds.S0022-0302(05)73008-3).
- Dong, J., G. Bohe, Y. Guan, G. Li, R. Zuo, X. Shu, Y. Wang, X. Sun, X. Chen, and X. Li. 2020. Mitochondrial membrane protein

- Mitofusin 2 as a potential therapeutic target for treating free fatty acid-induced hepatic inflammation in dairy cows during early lactation. *J. Dairy Sci.* 103:5561–5574. <https://doi.org/10.3168/jds.2019-17652>.
- Du, X., Y. Zhu, Z. Peng, Y. Cui, Q. Zhang, Z. Shi, Y. Guan, X. Sha, T. Shen, Y. Yang, X. Li, Z. Wang, X. Li, and G. Liu. 2018. High concentrations of fatty acids and β -hydroxybutyrate impair the growth hormone-mediated hepatic JAK2-STAT5 pathway in clinically ketotic cows. *J. Dairy Sci.* 101:3476–3487. <https://doi.org/10.3168/jds.2017-13234>.
- Duffield, T. F., D. F. Kelton, K. E. Leslie, K. D. Lissimore, and J. H. Lumsden. 1997. Use of test day milk fat and milk protein to detect subclinical ketosis in dairy cattle in Ontario. *Can. Vet. J.* 38:713–718.
- Duffield, T. F., K. D. Lissimore, B. W. McBride, and K. E. Leslie. 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. *J. Dairy Sci.* 92:571–580. <https://doi.org/10.3168/jds.2008-1507>.
- Edmonson, A. J., I. J. Lean, L. D. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. *J. Dairy Sci.* 72:68–78. [https://doi.org/10.3168/jds.S0022-0302\(89\)79081-0](https://doi.org/10.3168/jds.S0022-0302(89)79081-0).
- Eskes, R., B. Antonsson, A. Osen-Sand, S. Montessuit, C. Richter, R. Sadoul, G. Mazzei, A. Nichols, and J. C. Martinou. 1998. Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg^{2+} ions. *J. Cell Biol.* 143:217–224. <https://doi.org/10.1083/jcb.143.1.217>.
- Eslami, H., R. A. Batavani, S. Asr I-Rezaei, and R. Hobbenaghi. 2015. Changes of stress oxidative enzymes in rat mammary tissue, blood and milk after experimental mastitis induced by *E. coli* lipopolysaccharide. *Vet. Res. Forum* 6:131–136.
- Esposito, G., P. C. Irons, E. C. Webb, and A. Chapwanya. 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. *Anim. Reprod. Sci.* 144:60–71. <https://doi.org/10.1016/j.anireprosci.2013.11.007>.
- Fan, C., Q. Li, D. Ross, and J. F. Engelhardt. 2003. Tyrosine phosphorylation of $I\kappa B\alpha$ activates NF- κB through a redox-regulated and c-Src-dependent mechanism following hypoxia/reoxygenation. *J. Biol. Chem.* 278:2072–2080. <https://doi.org/10.1074/jbc.M206718200>.
- Farr, V. C., K. Stelwagen, L. R. Cate, A. J. Molenaar, T. B. McFadden, and S. R. Davis. 1996. An improved method for the routine biopsy of bovine mammary tissue. *J. Dairy Sci.* 79:543–549. [https://doi.org/10.3168/jds.S0022-0302\(96\)76398-1](https://doi.org/10.3168/jds.S0022-0302(96)76398-1).
- Garavaglia, L., S. Galletti, and D. Tedesco. 2015. Silymarin and lycopene administration in periparturient dairy cows: Effects on milk production and oxidative status. *N. Z. Vet. J.* 63:313–318. <https://doi.org/10.1080/00480169.2015.1047911>.
- Gross, J. J., and R. M. Bruckmaier. 2019. Review: Metabolic challenges in lactating dairy cows and their assessment via established and novel indicators in milk. *Animal* 13(Suppl. 1):s75–s81. <https://doi.org/10.1017/S175173111800349X>.
- Gross, J. J., F. J. Schwarz, K. Eder, H. A. Van Dorland, and R. M. Bruckmaier. 2013. Liver fat content and lipid metabolism in dairy cows during early lactation and during a mid-lactation feed restriction. *J. Dairy Sci.* 96:5008–5017. <https://doi.org/10.3168/jds.2012-6245>.
- Hadsell, D., J. George, and D. Torres. 2007. The declining phase of lactation: Peripheral or central, programmed or pathological? *J. Mammary Gland Biol. Neoplasia* 12:59–70. <https://doi.org/10.1007/s10911-007-9038-4>.
- Heiko, V., and W. W. Christopher. 2011. Accessing the transcriptome: How to normalize mRNA pools. *Methods Mol. Biol.* 772:105–128.
- Itle, A. J., J. M. Huzzey, D. M. Weary, and M. A. von Keyserlingk. 2015. Clinical ketosis and standing behavior in transition cows. *J. Dairy Sci.* 98:128–134. <https://doi.org/10.3168/jds.2014-7932>.
- Kruk, J., and E. Duchnik. 2014. Oxidative stress and skin diseases: Possible role of physical activity. *Asian Pac. J. Cancer Prev.* 15:561–568. <https://doi.org/10.7314/APJCP.2014.15.2.561>.
- Lecoeur, H., E. Prina, T. Rosazza, K. Kokou, P. N'Diaye, N. Aulner, H. Varet, G. Bussotti, Y. Xing, G. Milon, R. Weil, G. Meng, and G. F. Späth. 2020. Targeting macrophage histone H3 modification as a leishmania strategy to dampen the NF- κB /NLRP3-mediated inflammatory response. *Cell Rep.* 30:1870–1882. <https://doi.org/10.1016/j.celrep.2020.01.030>.
- Lee, J. Y., K. H. Sohn, S. H. Rhee, and D. Hwang. 2001. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through toll-like receptor 4. *J. Biol. Chem.* 276:16683–16689. <https://doi.org/10.1074/jbc.M011695200>.
- Li, D., S. Ni, K. S. Miao, and C. Zhuang. 2019a. PI3K/Akt and caspase pathways mediate oxidative stress-induced chondrocyte apoptosis. *Cell Stress Chaperones* 24:195–202. <https://doi.org/10.1007/s12192-018-0956-4>.
- Li, P., L. Li, C. Zhang, X. Cheng, Y. Zhang, Y. Guo, M. Long, S. Yang, and J. He. 2019b. Palmitic acid and β -hydroxybutyrate induce inflammatory responses in bovine endometrial cells by activating oxidative stress-mediated NF- κB signaling. *Molecules* 24:2421. <https://doi.org/10.3390/molecules24132421>.
- Li, X., W. Huang, J. Gu, X. Du, L. Lei, X. Yuan, G. Sun, Z. Wang, X. Li, and G. Liu. 2015. SREBP-1c overactivates ROS-mediated hepatic NF- κB inflammatory pathway in dairy cows with fatty liver. *Cell. Signal.* 27:2099–2109. <https://doi.org/10.1016/j.cellsig.2015.07.011>.
- Li, Y., H. Y. Ding, X. C. Wang, S. B. Feng, X. B. Li, Z. Wang, G. W. Liu, and X. W. Li. 2016. An association between the level of oxidative stress and the concentrations of NEFA and BHBA in the plasma of ketotic dairy cows. *J. Anim. Physiol. Anim. Nutr. (Berl.)* 100:844–851. <https://doi.org/10.1111/jpn.12454>.
- Loor, J. J., R. E. Everts, M. Bionaz, H. M. Dann, D. E. Morin, R. Oliveira, S. L. Rodriguez-Zas, J. K. Drackley, and H. A. Lewin. 2007. Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. *Physiol. Genomics* 32:105–116. <https://doi.org/10.1152/physiolgenomics.00188.2007>.
- Miao, J. F., Y. M. Zhu, B. B. Gu, X. B. Wang, S. X. Zou, and Y. E. Deng. 2007. Evaluation of the changes of immune cells during lipopolysaccharide-induced mastitis in rats. *Cytokine* 40:135–143. <https://doi.org/10.1016/j.cyto.2007.08.012>.
- Miranda, S. G., N. G. Purdie, V. R. Osborne, B. L. Coomber, and J. P. Cant. 2011. Selenomethionine increases proliferation and reduces apoptosis in bovine mammary epithelial cells under oxidative stress. *J. Dairy Sci.* 94:165–173. <https://doi.org/10.3168/jds.2010-3366>.
- Moyes, K. M., J. K. Drackley, D. E. Morin, S. L. Rodriguez-Zas, R. E. Everts, H. A. Lewin, and J. J. Loor. 2011. Predisposition of cows to mastitis in non-infected mammary glands: Effects of dietary-induced negative energy balance during mid-lactation on immune-related genes. *Funct. Integr. Genomics* 11:151–156. <https://doi.org/10.1007/s10142-010-0186-z>.
- Nakajima, S., and M. Kitamura. 2013. Bidirectional regulation of NF- κB by reactive oxygen species: A role of unfolded protein response. *Free Radic. Biol. Med.* 65:162–174. <https://doi.org/10.1016/j.freeradbiomed.2013.06.020>.
- Pi, J., L. Leung, P. Xue, W. Wang, Y. Hou, D. Liu, E. Yehuda-Shnaidman, C. Lee, J. Lau, T. W. Kurtz, and J. Y. Chan. 2010. Deficiency in the nuclear factor E2-related factor-2 transcription factor results in impaired adipogenesis and protects against diet-induced obesity. *J. Biol. Chem.* 285:9292–9300. <https://doi.org/10.1074/jbc.M109.093955>.
- Putman, A. K., J. L. Brown, J. C. Gandy, L. Wisnieski, and L. M. Sordillo. 2018. Changes in biomarkers of nutrient metabolism, inflammation, and oxidative stress in dairy cows during the transition into the early dry period. *J. Dairy Sci.* 101:9350–9359. <https://doi.org/10.3168/jds.2018-14591>.
- Rubartelli, A. 2012. Redox control of NLRP3 inflammasome activation in health and disease. *J. Leukoc. Biol.* 92:951–958. <https://doi.org/10.1189/jlb.0512265>.
- Sahoo, S. S., R. C. Patra, P. C. Behera, and D. Swarup. 2009. Oxidative stress indices in the erythrocytes from lactating cows after treatment for subclinical ketosis with antioxidant incorporated in

- the therapeutic regime. *Vet. Res. Commun.* 33:281–290. <https://doi.org/10.1007/s11259-008-9176-1>.
- Sakemi, Y., Y. Tamura, and K. Hagiwara. 2011. Interleukin-6 in quarter milk as a further prediction marker for bovine subclinical mastitis. *J. Dairy Res.* 78:118–121. <https://doi.org/10.1017/S0022029910000828>.
- Seegers, H., C. Fourichon, and F. Beaudeau. 2003. Production effects related to mastitis and mastitis economics in dairy cattle herds. *Vet. Res.* 34:475–491. <https://doi.org/10.1051/vetres:2003027>.
- Shen, T., X. Li, J. J. Loor, Y. Zhu, X. Du, X. Wang, D. Xing, Z. Shi, Z. Fang, X. Li, and G. Liu. 2019. Hepatic nuclear factor κ B signaling pathway and NLR family pyrin domain containing 3 inflammasome is over-activated in ketotic dairy cows. *J. Dairy Sci.* 102:10554–10563.
- Shu, X., Z. Fang, Y. Guan, X. Chen, J. J. Loor, H. Jia, J. Dong, Y. Wang, R. Zuo, G. Liu, X. Li, and X. Li. 2020. High levels of fatty acids inhibit β -casein synthesis through suppression of the JAK2/STAT5 and mTOR signaling pathways in mammary epithelial cells of cows with clinical ketosis. *J. Dairy Res.* 87:212–219. <https://doi.org/10.1017/S0022029920000175>.
- Song, Y., N. Li, J. Gu, S. Fu, Z. Peng, C. Zhao, Y. Zhang, X. Li, Z. Wang, X. Li, and G. Liu. 2016. β -Hydroxybutyrate induces bovine hepatocyte apoptosis via an ROS-p38 signaling pathway. *J. Dairy Sci.* 99:9184–9198. <https://doi.org/10.3168/jds.2016-11219>.
- Song, Y., X. Li, Y. Li, N. Li, X. Shi, H. Ding, Y. Zhang, X. Li, G. Liu, and Z. Wang. 2014. Non-esterified fatty acids activate the ROS-p38-p53/Nrf2 signaling pathway to induce bovine hepatocyte apoptosis in vitro. *Apoptosis* 19:984–997. <https://doi.org/10.1007/s10495-014-0982-3>.
- Sun, X., H. Jia, Q. Xu, C. Zhao, and C. Xu. 2019a. Lycopene alleviates H_2O_2 -induced oxidative stress, inflammation and apoptosis in bovine mammary epithelial cells via the NFE2L2 signaling pathway. *Food Funct.* 10:6276–6285. <https://doi.org/10.1039/C9FO01922G>.
- Sun, X., Y. Wang, J. J. Loor, R. Bucktrout, X. Shu, H. Jia, J. Dong, R. Zuo, G. Liu, X. Li, and X. Li. 2019b. High expression of cell death-inducing DFFA-like effector a (CIDEA) promotes milk fat content in dairy cows with clinical ketosis. *J. Dairy Sci.* 102:1682–1692. <https://doi.org/10.3168/jds.2018-15439>.
- Tschopp, J., and K. Schroder. 2010. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nat. Rev. Immunol.* 10:210–215. <https://doi.org/10.1038/nri2725>.
- Vanholder, T., J. Papen, R. Bemers, G. Vertenten, and A. C. Berge. 2015. Risk factors for subclinical and clinical ketosis and association with production parameters in dairy cows in the Netherlands. *J. Dairy Sci.* 98:880–888. <https://doi.org/10.3168/jds.2014-8362>.
- Wen, H., D. Gris, Y. Lei, S. Jha, L. Zhang, M. T. Huang, W. J. Brickey, and J. P. Ting. 2011. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nat. Immunol.* 12:408–415. <https://doi.org/10.1038/ni.2022>.
- Youssef, M., and M. El-Ashker. 2017. Significance of insulin resistance and oxidative stress in dairy cattle with subclinical ketosis during the transition period. *Trop. Anim. Health Prod.* 49:239–244. <https://doi.org/10.1007/s11250-016-1211-6>.
- Zandi, E., D. M. Rothwarf, M. Delhase, M. Hayakawa, and M. Karin. 1997. The $I\kappa$ B kinase complex (IKK) contains two kinase subunits, IKK α and IKK β , necessary for $I\kappa$ B phosphorylation and NF- κ B activation. *Cell* 91:243–252. [https://doi.org/10.1016/S0092-8674\(00\)80406-7](https://doi.org/10.1016/S0092-8674(00)80406-7).
- Zhang, J., X. Wang, V. Vikash, Q. Ye, D. Wu, Y. Liu, and W. Dong. 2016. ROS and ROS-mediated cellular signaling. *Oxid. Med. Cell. Longev.* 2016:4350965. <https://doi.org/10.1155/2016/4350965>.
- Zhang, M., S. Zhang, Q. Hui, L. Lei, X. Du, W. Gao, R. Zhang, G. Liu, X. Li, and X. Li. 2015. β -Hydroxybutyrate facilitates fatty acids synthesis mediated by sterol regulatory element-binding protein1 in bovine mammary epithelial cells. *Cell. Physiol. Biochem.* 37:2115–2124. <https://doi.org/10.1159/000438569>.
- Zhao, X. J., Y. Z. Yang, Y. J. Zheng, S. C. Wang, H. M. Gu, Y. Pan, S. J. Wang, H. J. Xu, and L. D. Kong. 2017. Magnesium isoglycyrrhizinate blocks fructose-induced hepatic NF- κ B/NLRP3 inflammasome activation and lipid metabolism disorder. *Eur. J. Pharmacol.* 809:141–150. <https://doi.org/10.1016/j.ejphar.2017.05.032>.
- Zhou, Z., O. Bulgari, M. Vailati-Riboni, E. Trevisi, M. A. Ballou, F. C. Cardoso, D. N. Luchini, and J. J. Loor. 2016. Rumen-protected methionine compared with rumen-protected choline improves immunometabolic status in dairy cows during the peripartur period. *J. Dairy Sci.* 99:8956–8969. <https://doi.org/10.3168/jds.2016-10986>.
- Zhu, Y., G. Liu, X. Du, Z. Shi, M. Jin, X. Sha, X. Li, Z. Wang, and X. Li. 2019. Expression patterns of hepatic genes involved in lipid metabolism in cows with subclinical or clinical ketosis. *J. Dairy Sci.* 102:1725–1735. <https://doi.org/10.3168/jds.2018-14965>.