ABSTRACT

Ketosis is a common metabolic disorder in high-producing dairy cows during the peripartal period. Negative energy balance leads to increased circulating levels of nonesterified fatty acids (NEFA) and β-hydroxybutyrate (BHB), consequently increasing the risk of ketosis. It is well-known that NEFA and BHB can induce lipotoxicity and oxidative stress in bovine tissues/organs including the liver and adipose tissue. Although the mammary gland is one important site for NEFA and BHB metabolism, whether an overload in their concentrations within mammary cells causes oxidative stress during ketosis remains unclear. Thus, the present study compared oxidative stress status and mitochondrial function in mammary tissues harvested by biopsy from healthy (n = 15) and clinically ketotic (n = 15) dairy cows within 2 to 3 wk postpartum. Compared with healthy cows, ketotic cows had depressed daily milk yield (median: 28.92 vs. 21.56 kg) and dry matter intake (median: 22.36 vs. 19.92 kg/d), accompanied by elevated plasma NEFA (median: 0.32 vs. 1.26 mM), BHB (median: 0.52 vs. 3.69 mM), and lower plasma glucose (median: 4.55 vs. 2.13 mM). As detected by a commercial kit, a greater level of reactive oxygen species in mammary epithelial cells of ketotic cows, and greater oxidant indices including hydrogen peroxide and malondialdehyde coupled with lower antioxidant indices including glutathione peroxidase, catalase, and superoxide dismutase activities as detected by the respective biochemical kits in the homogenate of mammary tissue of ketotic cows indicated increased oxidative stress status. Lower citrate synthase activity and ATP production as detected by the respective commercial kits coupled with lower mRNA and protein abundance of mitochondrial respiratory chain oxidative phosphorylation complexes I–V (CO I–V) in ketotic cows suggested an impairment of mitochondrial function. This was supported by lower mRNA and protein abundance of nucleus-derived mitochondrial function regulators including peroxisome proliferator activated receptor gamma coactivator 1 α, mitofusin 2, nuclear respiratory factor 1, and mitochondrial transcription factor A. Lower mitochondrial membrane potential evaluated via the tetraethylbenzimidazolylcarbocyanine iodide (JC-1) labeling method and swollen mitochondria in mammary epithelial cells of ketotic cows suggested the existence of mitochondrial damage. Overall, the present study revealed extensive mitochondrial dysfunction and oxidative stress in the mammary gland of clinically ketotic cows. As such, data suggest that reduced milk yield in cows with ketosis is partly due to enhanced oxidative stress along with mitochondrial dysregulation in the mammary gland.

Key words: dairy cow, ketosis, mammary gland, mitochondria, oxidative stress

INTRODUCTION

Ketosis, a major metabolic disease in dairy cows, is primarily caused by excessive nonesterified fatty acids (NEFA) and BHB generation due to negative energy balance (NEB; McArt et al., 2012; White, 2015; Suriyasathaporn et al., 2000); furthermore, ketosis leads to decreased milk yield (Hillreiner et al., 2016). Excessive plasma NEFA and BHB concentrations in dairy cows can be lipotoxic and result in oxidative stress, inflammation, or apoptosis in several organs/tissues (e.g., liver and adipose tissue) through overactivation of mitogen-activated protein kinase or nuclear factor kappa B signaling pathways (Song et al., 2014, 2016; Shi et al., 2018). Whether these mechanisms impair mammary gland function during ketosis is not well understood.

Mitochondrial reactive oxygen species (ROS) can lead to development of oxidative stress (Rolo et al., 2012).
Mitochondrial homeostasis plays central roles in both ROS disposal and ATP production, maintaining normal function of tissues/organs, an imbalance of which may lead to enhanced mammary oxidative stress and impaired milk yield (Cheng and Ristow, 2013). Maintenance of mitochondrial function requires not only mitochondrial respiratory chain oxidative phosphorylation (OXPHOS) complexes (CO I–V), but also nucleus-derived regulators including peroxisome proliferator activated receptor-γ coactivator-1α (PGC-1α) and mitofusin-2 (MFN2), nuclear respiratory factor 1 (NRF-1), and transcription factor A, mitochondrial (TFAM) (Bongard et al., 2015; Kang and Ji, 2016).

The objective of this study was to explore the mitochondrial function and oxidative stress status in bovine mammary gland during clinical ketosis, which might provide some insights into the pathologic tissue-specific mechanisms of depressed milk synthesis in cows with ketosis. Our general hypothesis was that ketosis leads to depressed milk yield via impairing mammary mitochondria homeostasis and redox balance.

**MATERIALS AND METHODS**

**Animals**

The experimental protocol (No. SY202012019) was approved by the Ethics Committee on the Use and Care of Animals at Jilin University (Changchun, China). Animals used in the current study received humane care according to the principles and guidelines of the Guidelines for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). To guarantee absence of other comorbidities, all cows (within 2–3 wk postpartum) received a routine physical examination. Lactating Holstein cows with similar parity (median = 3, range = 2–4) and DIM (median = 6 d, range = 3–10 d) were preselected from a 10,000-cow dairy farm located in Changchun City (Jilin Province, China). Veterinarians classified cows as suspected clinically ketotic if a nitroprusside test for ketone bodies in milk was positive. Accordingly, 40 suspected ketotic postpartum and 40 healthy postpartum cows were preselected. Serum BHB concentrations in the preselected cows were analyzed. According to clinical symptoms and serum BHB concentrations (Itle et al., 2015; Sun et al., 2019), the final cohort of cows included 15 clinically ketotic (serum BHB concentrations >3 mmol/L) and 15 control cows (serum BHB concentrations <0.6 mmol/L) with similar BW. Cows had ad libitum access to the same diet (Sun et al., 2019) that was fed twice daily (0830 and 1600 h), and fresh water was supplied continuously. During 10 d of experimental work, selected cows were housed in a climate-controlled barn with individual tie-stalls to reduce environmental interference.

The first day after the determination of cohorts of healthy and clinical ketosis cows was regarded as d 1. The biopsy was conducted on d 4. The tissue biopsy procedure was shown to reduce milk yield in the first 3 milkings postbiopsy (Farr et al., 1996). To remove the effect of tissue biopsy on milk production results, data from both the day of biopsy and the first-day postbiopsy were disregarded. Thus, cows were milked twice daily at 0800 and 1530 h, and daily milk production per cow was calculated based on data during d 1 to 3 before and d 6 to 10 after mammary biopsy. Blood samples were collected from the jugular vein at 0730 h on d 1, 3, 5, and 7 before feeding and centrifuged at 3,000 × g for 15 min at 4°C to obtain serum within 30 min of drawing the blood. Once the serum was obtained, it was stored at −20°C until it was used for detection of biochemical indexes within 2 d. Serum glucose (GLU), BHB, and NEFA were detected using commercially available kits (GLU, cat. no. GL3815; BHB, cat. no. RB1008; and NEFA, cat. no. FA115; Randox Laboratories) by a Hitachi 7170 autoanalyzer (Hitachi). The average BW of each cow was detected via weigh-bridge at 0750 h on d 1, 3, 5, and 7. The DMI of each cow was calculated based on feed fed and feed refused on d 1, 3, 5, and 7. Characteristics, including BW, DMI, milk production, and serum GLU, BHB, and NEFA concentrations of the selected control and clinically ketotic cows are in Table 1.

Mammary gland tissue was collected by percutaneous biopsies from the 15 cows in each group. The biopsy was conducted on d 4 from the right or left rear quarter of the mammary gland according to a published protocol (Bionaz and Loor, 2008). Briefly, after sterilization and skin incision, blunt dissection of the mammary capsule was performed to ensure that a sample of mammary parenchyma was obtained. For each cow, 3 biopsies were taken located 2 to 3 inches from each other at the right or left rear quarter of the mammary gland. In total, 2.5 to 3 g of tissue was collected from each cow. The bleeding was stopped immediately by pressing the wound after removal of the biopsy instrument from the capsule. The skin incision was closed with 4 or 5 Michel clips (11 mm; Henry Schein) and sprayed with a topical antiseptic (povidone-iodine ointment, 10%; Taro Pharmaceuticals). The health of cows postbiopsy was monitored by recording rectal temperature, daily milk yield, and daily DMI for 7 d. No incidence of mastitis was disregarded. Thus, cows were milked twice daily at 0800 and 1530 h, and daily milk production per cow was calculated based on data during d 1 to 3 before and d 6 to 10 after mammary biopsy. Blood samples were collected from the jugular vein at 0730 h on d 1, 3, 5, and 7 before feeding and centrifuged at 3,000 × g for 15 min at 4°C to obtain serum within 30 min of drawing the blood. Once the serum was obtained, it was stored at −20°C until it was used for detection of biochemical indexes within 2 d. Serum glucose (GLU), BHB, and NEFA were detected using commercially available kits (GLU, cat. no. GL3815; BHB, cat. no. RB1008; and NEFA, cat. no. FA115; Randox Laboratories) by a Hitachi 7170 autoanalyzer (Hitachi). The average BW of each cow was detected via weigh-bridge at 0750 h on d 1, 3, 5, and 7. The DMI of each cow was calculated based on feed fed and feed refused on d 1, 3, 5, and 7. Characteristics, including BW, DMI, milk production, and serum GLU, BHB, and NEFA concentrations of the selected control and clinically ketotic cows are in Table 1.
real-time PCR were washed with cold PBS for 3 × 3 min before freezing in liquid nitrogen and stored at −80°C until further analysis. Samples for use in other experiments in the present study were stored in tissue storage solution after washing with PBS (GenSpark Biotechnology) at 4°C.

**Detection of Oxidative and Antioxidant Indices in Mammary Gland Homogenates**

Pre-washed sterile mammary gland tissue (~150 mg) from each cow was placed into 1 mL of ice-cold RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology). Samples were then homogenized with a Polytron homogenizer (Janke and Kunkel, IKA) followed by centrifugation at 1,200 × g for 15 min at 4°C. The supernatant was collected for measurement of superoxide dismutase (SOD; cat. no. A001-3-2; Jiacheng Bioengineering Institute), malondialdehyde (MDA; cat. no. A003-1-2; Jiacheng Bioengineering Institute), glutathione peroxidase (GSH-Px; cat. no. A005-1-2; Jiacheng Bioengineering Institute), catalase (CAT; cat. no. A007-1-1; Jiacheng Bioengineering Institute; and H2O2; cat. no. S0038; Beyotime) using commercial kits according to the instructions of the manufacturers. The activities of these oxidative and antioxidant indices were detected as previously described (Song et al., 2016; Sun et al., 2020). In general, SOD activity was determined spectrophotometrically at 550 nm by xanthine and xanthine oxidase systems and expressed in units per milligram of protein. The MDA measurement was based on the reaction with thiobarbituric acid in an acidic medium at 95°C at 533 nm. The MDA concentration was calculated from the standard curve and expressed as millimoles per gram of protein. Catalase was detected with a spectrometer at a wavelength of 405 nm. One unit of CAT activity was defined as the amount of protein that decomposes 1 μmol of H2O2 per second; thus, its content was reported as units per milligram protein. Hydrogen peroxide was determined with a spectrometer at a wavelength of 560 nm, and its content reported as millimoles per liter.

**Mammary Gland Protein Extraction and Western Blotting**

Total protein from mammary gland tissue was extracted using a protein extraction kit (cat. no. C600589; Sangon Biotech Ltd.) according to the manufacturer’s protocols. The protein concentration was determined by the bicinchoninic acid protein assay (BCA protein assay kit, Pierce). A total of 300 to 500 mg of tissue from each cow was used for protein extraction; 20 μg of protein from each sample were separated by polyacrylamide gel (10%) electrophoresis and electrotransferred onto polyvinylidene fluoride (PVDF) membranes. Then, the nonspecific binding sites on PVDF membranes were blocked with 3% BSA. The PVDF membranes were then hybridized with primary antibodies against PGC-1α (1:1,000, cat. no. ab54481, Abcam), MFN2 (1:1000, cat. no. ab56889, Abcam), CO I (1:1,000, cat. no. sc-19998, Santa Cruz Biotechnology), CO II (1:1,000, cat. no. sc-514489, Santa Cruz Biotechnology), CO III (1:1,000, cat. no. ab14745, Abcam), CO IV (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), CO V (1:1,000, cat. no. ab14730, Abcam), and TFAM (1:1,000, cat. no. sc166965, Santa Cruz Biotechnology), respectively. The PVDF membranes were then washed for 3 × 10 min with PBS and each incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:5000, Boster) at room temperature for 45 min. After washing the PVDF membranes for 3 × 10 min with PBS, immunoreactive bands were enhanced with chemiluminescence solution (Pierce Biotechnology Inc.). Last, bands were imaged using a Protein Simple Imager (ProteinSimple). The gray value of bands was analyzed using the Image-Pro

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Table 1. Characteristics and blood variable of selected peripartal dairy cows

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Clinical ketosis</th>
<th>P-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (kg)</td>
<td>621.46</td>
<td>634.24</td>
<td>0.6974</td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>21.96</td>
<td>19.87</td>
<td>0.0476</td>
</tr>
<tr>
<td>Milk production (kg/cow per day)</td>
<td>28.57</td>
<td>21.49</td>
<td>0.0235</td>
</tr>
<tr>
<td>Serum GLU (mM)</td>
<td>4.68</td>
<td>2.64</td>
<td>0.0051</td>
</tr>
<tr>
<td>Serum BHB (mM)</td>
<td>0.36</td>
<td>3.73</td>
<td>0.0053</td>
</tr>
<tr>
<td>Serum NEFA (mM)</td>
<td>0.29</td>
<td>1.25</td>
<td>0.0046</td>
</tr>
</tbody>
</table>

1BW, DMI, milk production, concentrations of serum glucose (GLU), BHB, and nonesterified fatty acid (NEFA) in healthy (Control; n = 15) and clinical ketotic (Ketosis; n = 15) cows (within 2–3 wk postpartum) were shown as median (interquartile range).

²P < 0.05 was considered significant.
Plus (Media Cybernetics). β-Actin was used as a reference protein, and each band was compared against β-actin in each sample.

Quantitative Reverse Transcription PCR Assay

Quantitative real-time PCR was performed as described previously (Sun et al., 2017). Total RNA from mammary gland tissue was extracted using Trizol reagent (cat. no. 9108; TaKaRa Biotechnology Co. Ltd.) according to the supplier’s protocol. Samples of 150 to 300 mg of tissue from each cow were used for RNA extraction. The RNA concentration and quality were measured using a K5500 MicroSpectrophotometer (Beijing Kaiao Technology Development Ltd.) and electrophoresis. A total of 3 μg of RNA were transcribed into cDNA using a PrimeScript Reverse Transcriptase Kit (cat. no. 6110B; TaKaRa Biotechnology Co. Ltd.). We evaluated the mRNA expression levels using the SYBR green plus reagent kit (cat. no. DRR041A; TaKaRa Biotechnology Co. Ltd.) with a 7500 Real-Time PCR System (Applied Biosystems). Each sample was run in triplicate (n = 15/group). Relative transcription of target genes was normalized to that of \textit{ACTB} and determined by the 2$^{−\Delta\Delta C_{T}}$ method. Primers were designed with the Primer Express software (Applied Biosystems) as shown in Table 2.

Determination of ATP Concentration and Citrate Synthase Activity in Mammary Gland Homogenates

The ATP concentration was measured with an ATP analysis kit (cat. no. A095-1-1; Jiancheng Biotechnology Institute). According to the manufacturer’s instruction, mammary tissue (~20 mg) was homogenized in cold PBS by repeated freeze-thaw cycles. The homogenate was incubated in boiling water for 10 min, and the supernatant was collected by centrifugation at 13,000 $\times$ g for 10 min at room temperature, followed by mixing with substrate solution and accelerant. The mixture was incubated for 30 min at 37°C followed by mixing with precipitant and centrifuged for 5 min at 1,500 $\times$ g. Color-developing solution and stop solution were subsequently used before measuring with a spectrometer at a wavelength of 636 nm. Adenosine triphosphate content was related to protein content.

Citrate synthase activity was measured with a Citrate Synthase Activity assay kit (cat. no. ab119692; Abcom). According to the instructions of the manufacturer, mammary gland tissue (~20 mg) was homogenized and lysed in lysis buffer containing 25 mM Tris-HCl (pH 7.5) and 0.5% β-dodecylmaltoside. The assay was initiated by adding acetyl-CoA to the homogenate, 1 mM Ellman’s reagent [5,5′-dithiobis(2-nitrobenzoic acid)], and 1 mM oxaloacetate, followed by measuring absorbance at 412 nm.

Transmission Electron Microscopy of Primary Mammary Epithelial Cells

Washed sterile mammary gland tissue (~200 mg) from each cow was sliced and digested with 0.5% collagenase III (cat. no. C8490; Solarbio) for 2 h at 37°C. The solution was filtered through a 74-μm strainer and centrifuged at 150 $\times$ g for 10 min at 4°C to collect cell agglomerates. Ultrastructural characteristics of intracellular mitochondria were visualized by transmission electron microscopy. Mammary epithelial cells were fixed with 2.5% glutaraldehyde and 2% paraformalde-

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Table 2. The primer sequences used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>RefSeq</th>
<th>Forward/reverse primer sequences (5′-3′)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARGC1A (PGC-1α)</td>
<td>NM_177945.3</td>
<td>FOR: CCGGTGCTACCTGAGAGAGAGAGAGAGA&lt;br&gt;REV: CTTGACTGGGATGACCGAAG</td>
<td>125</td>
</tr>
<tr>
<td>MFN2</td>
<td>NM_001190270.1</td>
<td>FOR: TGGCGCAAGACTA&lt;br&gt;REV: TCGTCCACCAACACAGAGAGAG</td>
<td>109</td>
</tr>
<tr>
<td>NRF-1</td>
<td>NM_001098002.2</td>
<td>FOR: CAGGGGTGGGCAATAAGA&lt;br&gt;REV: TTAGCAGGAAGTCA&lt;br&gt;REV: TTAGCAGGAAGTCA</td>
<td>214</td>
</tr>
<tr>
<td>TFAM</td>
<td>NM_001034016.2</td>
<td>FOR: TTCACCATCGACAGTAGGAA&lt;br&gt;REV: CTCCTCATGCAGTAGTAGGAG</td>
<td>134</td>
</tr>
<tr>
<td>NDUFA9 (CO I)</td>
<td>NM_205817.1</td>
<td>FOR: TATGGGATGGAAACCGGAGAGAGAGAG</td>
<td>162</td>
</tr>
<tr>
<td>SDHA (CO II)</td>
<td>NM_174178.2</td>
<td>FOR: GCTCTTTTTGGGGAACCTTGGG&lt;br&gt;REV: CAGAACCTGGAAGGAGAG</td>
<td>106</td>
</tr>
<tr>
<td>UQCRCC2 (CO III)</td>
<td>NM_174650.2</td>
<td>FOR: TCTCATTGCTGGTGGG&lt;br&gt;REV: TTGCTGTGGG&lt;br&gt;REV: TTGCTGTGGGAC&lt;br&gt;REV: TTGCTGTGGGAC</td>
<td>197</td>
</tr>
<tr>
<td>COX6A1 (CO IV)</td>
<td>NM_001077831.2</td>
<td>FOR: ATCTGCGGTGGTTTTGGGTTGC&lt;br&gt;REV: GGGGTGGGTCGCCAGT&lt;br&gt;REV: TCCACGCCTCAGAGGAC&lt;br&gt;REV: TCCACGCCTCAGAGGAC</td>
<td>334</td>
</tr>
<tr>
<td>ATP5F1B (CO V)</td>
<td>NM_175796.3</td>
<td>FOR: CTACAGTCCGGCTAGAGC&lt;br&gt;REV: GTCATCACCATCGGCAATGAG</td>
<td>148</td>
</tr>
<tr>
<td>ACTB (β-Actin)</td>
<td>NM_001101.4</td>
<td>FOR: CTAACAGTCCGCCTAGAGC&lt;br&gt;REV: GTCATCACCATCGGCAATGAG</td>
<td>403</td>
</tr>
</tbody>
</table>
Mitochondrial Membrane Potential and ROS Levels in Primary Mammary Epithelial Cells

Pre-washed sterile mammary gland tissue (300–500 mg) was sliced and digested with 0.5% collagenase III (cat. no. C8490, Solarbio) for 2 h at 37°C. The solution was filtered through a 74-μm strainer and centrifuged at 150 × g for 10 min at 4°C to collect cells. For each sample, 1 × 10^6 cells were seeded in a 6-well plate recovered in Dulbecco’s modified Eagle medium (DMEM/F-12, supplemented with 10% fetal bovine serum) for 24 h at 37°C. The cells were then washed in PBS, followed by the addition of JC solution and incubation with suspension cells for 30 min at 37°C followed by washing steps with the dilution buffer. At low concentrations (due to low MMP), JC-1 predominantly forms a monomer in the cytosol that yields green fluorescence with the emission of ~530 nm. At high concentrations (due to high MMP), the dye aggregates, yielding a red-colored emission (~590 nm) inside the mitochondria. The fluorescence was measured by flow cytometry (CytoFLEX, Beckman Coulter).

Intracellular ROS concentrations were measured using a peroxide-sensitive fluorescent probe 2’,7’-dichlorofluorescein diacetate (cat. no. S0033; Beyotime Biotechnology Inc.). Cells were exposed to a serum-free medium containing 10 μmol/L dichlorofluorescein diacetate and propidium iodide and protected from light for 30 min followed by washing 3 times with cold PBS. The fluorescence was measured by flow cytometry (CytoFLEX, Beckman Coulter).

Statistical Analysis

All experiments were repeated at least 3 times. Data are expressed as means ± standard error (unless otherwise specified). Statistical analyses were performed using GraphPad Prism 5 (GraphPad InStat Software, https://www.graphpad.com/scientific-software/instat/). Normally distributed data were analyzed with Student t-tests; MMP data were analyzed with paired t-tests; other nonnormally distributed data were analyzed with Wilcoxon signed-rank test. We considered \( P < 0.05 \) statistically significant.

RESULTS

Characteristics and Blood Variables of Selected Peripartal Dairy Cows

Milk yield and DMI of clinical ketotic cows were lower (\( P < 0.05 \)) compared with the control of healthy cows (Table 1). Additionally, ketotic cows had lower (\( P < 0.01 \)) GLU concentrations coupled with greater (\( P < 0.01 \)) BHB and NEFA concentrations in serum (Table 1).

Clinical Ketosis Promoted Oxidative Stress of the Mammary Tissue

As shown in Figure 1A and B, ketotic cows had greater (\( P < 0.01 \)) \( \text{H}_2\text{O}_2 \) and MDA concentrations in the mammary gland. Compared with healthy cows, ketotic cows had lower activities of antioxidant enzymes [e.g., GSH-Px (\( P < 0.01 \)), CAT (\( P < 0.01 \)), and SOD (\( P < 0.05 \)); Figure 1C-E]. Concentrations of ROS in primary mammary epithelial cells was greater (\( P < 0.01 \)) in ketotic compared with healthy cows (Figure 1F and C).

Clinical Ketosis Impaired Mitochondrial Integrity and Function in the Mammary Tissue

As shown in Figure 2, compared with healthy cows, mRNA and protein abundance of OXPHOS (CO I–V) were lower (\( P < 0.05 \)) in ketotic cows. Ketotic cows also had downregulated (\( P < 0.05 \)) nucleus-derived function regulators of mitochondria—PGC1-α, MFN2, NRF-1, and TFAM—at both transcription and translation levels (Figure 3A–C). Compared with healthy cows, citrate synthase activity, both a key enzyme of the TCA cycle and a quantitative maker of intact mitochondria, was lower (\( P < 0.01 \)) in the mammary tissue of ketotic cows (Figure 3D). Clinical ketosis led to lower (\( P < 0.01 \)) ATP content in mammary tissue (Figure 3E).

Clinical Ketosis Results in Swollen Mitochondria and Impaired Mitochondrial Membrane Potential in Mammary Epithelial Cells

As shown in Figure 4A, compared with healthy cows, mammary epithelial cell mitochondria in ketotic cows...
had a swollen inner space (lower electron density) and cristae (increased electron density). In addition, epithelial mitochondria number quantified by transmission electron microscopy was lower \( (P < 0.01) \) in ketotic cows (Figure 4B). Compared with healthy cows, there were more \( (P < 0.01) \) JC-1 monomers in mammary epithelial cells of cows with clinical ketosis (Figure 4C-D).

**DISCUSSION**

High levels of blood NEFA and BHB are hematological features of ketosis, both of which can be loaded and used in the mammary gland (Bauman and Griinari, 2000; van Knegsel et al., 2005) especially during the time cows experience NEB. Not only is BHB a precursor for milk fat but it is also a primary mitochondrial fuel (Henderson, 2008). However, excessive plasma NEFA and BHB contribute to the development of ketosis in dairy cows (White, 2015). Additionally, excessive NEFA in peripartal dairy cows enhances ROS production during \( \beta \)-oxidation when used as an energy substrate in peripheral tissues (Abuelo et al., 2015).

Oxidative stress enhances lipolysis, thereby contributing to higher blood NEFA concentrations, entering into a vicious cycle (Abuelo et al., 2015). Elevated NEFA and BHB concentrations trigger mitogen-activated protein kinase and nuclear factor-κB signaling pathways involved in ROS-induced apoptosis and inflammation in primary hepatocytes from dairy cows (Song et al., 2014, 2016; Shi et al., 2018). Excessive ketone bodies including BHB and acetoacetate were also shown to enhance oxidative stress in the liver of dairy cows (Du et al., 2017).

In the present study, elevated \( \text{H}_2\text{O}_2 \), MDA, and ROS levels coupled with reduced GSH-Px, CAT, and SOD activities all contributed to the exacerbation of mammary oxidative stress status during ketosis. High levels of cellular oxidative stress can induce peroxidation of lipid, change in protein structure, and strand break in nucleic acids, leading to damaged cells and organelles by altering cell membrane integrity and function in many species (Schieber and Chandel, 2014). In dairy cows, exacerbated oxidative stress has been shown to impair organ function including the liver and mam-
The imbalance of antioxidants and free radicals is closely related to mitochondrial dysfunction. The mitochondrial electron transport chain mediated by OXPHOS complexes (CO I–V) is the main source of cellular ROS (Rolo et al., 2012). Reduced protein expression of OXPHOS complexes could induce a "leaky" transfer of electrons to molecular oxygen and increase the generation of ROS (Nolfi-Donegan et al., 2020). Our results showed that both mRNA and protein levels of CO I–V in the mammary gland of ketosis cows were lower than those of healthy cows. In addition, elevated cellular ROS can in turn damage mitochondrial structure (Shokolenko et al., 2009), as also revealed by our electron microscopy results showing that the mitochondria of ketotic cows had swollen inner space and cristae. Although mammary epithelial cells of ketotic cows had lower mitochondrial numbers, the oxidative stress level was still greater compared with healthy cows, partly

Figure 2. Decreased mRNA and protein abundance of oxidative phosphorylation (OXPHOS) subunits in the mammary gland of healthy (control) and clinical ketotic (ketosis) dairy cows (n = 15 per group). (A, B) Protein expression of 5 representative subunits of OXPHOS complexes (CO I to V) in the mitochondrial respiratory chain. Three representative western blots in both groups (3 representative wells of each group represent 3 samples from different cows, respectively) are shown in A, and total data are quantified in B. (C) mRNA abundance of OXPHOS complex subunits by quantitative real-time PCR; β-actin (ACTB) was used to normalize the protein and mRNA levels of targeted genes. All data are expressed as the mean ± SE; *P < 0.05 compared with control group; **P < 0.01 compared with control group.

Figure 3. Impaired mitochondrial function in the mammary gland of healthy (control) and clinical ketotic (ketosis) dairy cows (n = 15 per group). (A, B) Protein expression analysis of mitochondrial function/biogenesis regulators, including PPAR-γ coactivator 1 α (PGC1-α), mitofusin2 (MFN2), nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (TFAM). Three representative western blots in both groups (3 representative wells of each group represent 3 samples from different cows, respectively) are shown in A, and the total data are quantified in B. (C) mRNA abundance of mitochondrial function regulators by quantitative real-time PCR; β-actin (ACTB) was used to normalize the protein or mRNA levels of targeted genes. (D) Citrate synthase activity. (E) Relative ATP content (%). All data are expressed as the mean ± SE; *P < 0.05 compared with control group; **P < 0.01 compared with control group.
due to dysfunction of the remaining mitochondria resulting from the lipotoxicity induced by excessive blood NEFA and BHB.

Several nucleus-derived mitochondrial function regulators are tightly involved in mitochondrial function. For instance, PGC1-α, a transcription coactivator, is responsible for the regulation of mitochondrial function (Kelly and Scarpulla, 2004); MFN2 is an outer mitochondrial membrane protein involved in mitochondrial dynamics (Misko et al., 2010); NRF-1 is a transcription factor that activates nuclear genes coding for mitochondrial import machinery and components of the respiratory chain; TFAM, a member of the high mobility group proteins, is essential for maintenance and activation of mitochondrial DNA (Kanki et al., 2004). In the present study, mRNA and protein abundance of these nucleus-derived mitochondrial function regulators were lower in ketotic dairy cows, which along with reduced ATP production and citrate synthase activity underscored the reduction in mitochondrial function.

The marked reduction of MMP, the basis of maintaining mitochondrial energy conversion (Kuhlbrandt, 2015), in mammary tissue of dairy cows with ketosis also was related to lower expression of the mitochondrial regulatory factors studied. For instance, overexpression of PGC-1α not only causes elevated CO IV protein expression, a downstream target of PGC-1α, but also results in increased MMP level (Valle et al., 2005); in mice, mutations in MFN2 lead to defective mitochondrial bioenergetics, with reduced MMP level and increased oxidative stress (Loiseau et al., 2007); and NRF-1 and TFAM have positive effects on MMP (Filadi et al., 2018). Thus, reduced mRNA and protein abundance of mitochondrialOXPHOS complexes and nucleus-derived mitochondrial function regulators suggested mitochondrial dysfunction in the ketotic mammary gland. Those data are similar to a previous study in which high plasma NEFA and BHB concentrations and clinical ketosis led to hepatic mitochondrial dysfunction in dairy cows (Gao et al., 2018). Further research is warranted to verify whether NEFA and BHB are the primary pathogenic factors of mammary mitochondrial dysfunction and oxidative stress in ketotic cows.

A large amount of evidence suggests that oxidative stress and mitochondrial dysfunction of the mammary gland are closely related to lactation. For example, oxidative stress can lead to subclinical inflammation of the breast, which will increase the incidence rate of mastitis while reducing milk production and milk quality (Jóźwik et al., 2004, 2012; Strzalkowska et al., 2009). In addition, a previous study proposed that ROS levels regulate mammary epithelial cell differentiation, which was directly correlated with the loss of milk-producing capacity in the mammary gland of dairy cows (Baratta et al., 2019). Importantly, the plasma concentration

**Figure 4.** Damaged mitochondria integrity and reduced mitochondrial number in the mammary gland of healthy (control) and clinical ketotic (ketosis) dairy cows (n = 15 per group). (A) Representative (from at least 5 random complete views of cells) transmission electron microscope (TEM) images of mammary epithelial cells (insets in top images correspond to lower images at larger magnification (3,000×); M = mitochondria; N = nucleus; arrows = swollen mitochondrial inner space and cristae. (B) Quantification of recognized mitochondria number per epithelial cell (counted from images of A). At least 50 cells of each group were counted for the quantification. (C) Mitochondrial membrane potential (MMP) of primary mammary epithelial cells. Tetraethylbenzimidazolylcarbocyanine iodide (JC-1) monomers yielding green fluorescence were detected through the fluorescein isothiocyanate (FITC) channel of flow cytometry; JC-1 aggregates yielding green fluorescence were detected through the phycoerythrin (PE) channel. (D) Ratio of monomers to aggregates mean fluorescence intensity (MFI) in control and ketosis groups. Data are expressed as the mean ± SE; *P < 0.05 compared with control group; **P < 0.01 compared with control group.
of antioxidative factor ascorbic acid correlated positively with milk yield and milk lactose percentage and negatively with milk protein percentage, which further confirmed a potential relationship between system oxidative stress and milking (Tanaka et al., 2008). On the other hand, in dairy cows, complete mitochondrial structure and function are not only the main source of ATP supply but also participate in the signal regulation of milk biosynthesis and proliferation through relative signaling proteins on mitochondrial membrane of mammary epithelial cells (Chen et al., 2018). Moreover, previous studies in nonruminants have demonstrated that mitochondrial function associated with MMP is an important index of apoptosis (Wang et al., 2014; Saramlamma et al., 2017), and increased mammary apoptosis was shown to be associated with reduced milk yield in cows experiencing NEB (Capuco et al., 2001; Stefanon et al., 2002). Overall, mitochondrial dysfunction and aggravated oxidative stress of the mammary gland caused by ketosis may be at least partly responsible for milk yield depression in perinatal dairy cows.

In conclusion, mitochondrial dysfunction and aggravated oxidative stress status were observed in the mammary glands of ketotic cows and might partly explain the reduced milk synthesis capacity. These findings provide new avenues for research and development of new treatment methods for ketosis in dairy cows, for example, drugs to improve mitochondrial function and antioxidant capacity.

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