When ketosis occurs, supraphysiological levels of free fatty acids (FFA) can cause oxidative injury to the mammary gland and autophagy can regulate the cellular oxidative status. The aim of this study was to investigate the autophagy status of mammary tissue and its associations with oxidative stress in healthy and clinically ketotic dairy cows. Mammary tissue and blood samples were collected from healthy cows \( n = 15 \), \( \beta \)-hydroxybutyrate (BHB) \(<0.6 \text{mM} \) and clinically ketotic cows \( n = 15 \), BHB \( >3.0 \text{mM} \) at 3 to 15 (average = 7) days in milk. For in vitro study, bovine mammary epithelial cells (BMEC) isolated from healthy cows were treated with 0, 0.3, 0.6, or 1.2 \( \text{mM} \) FFA for 24 h. Furthermore, BMEC were pretreated with 100 \( \text{nM} \) rapamycin, an autophagy activator, for 4 h or 50 \( \text{mM} \) 3-methyladenine (3-MA), an autophagy inhibitor, for 1 h, followed by treatment with or without FFA (1.2 \( \text{mM} \)) for another 24 h. Oxidation indicators and autophagy-related protein abundance were measured. Compared with healthy cows, serum concentrations of FFA, BHB, and malondialdehyde were greater in clinically ketotic cows, but milk production (kg/d), milk protein (kg/d), activities of superoxide dismutase, catalase, and glutathione peroxidase were lower. Abundances of mRNA and protein of autophagy-related gene 5 (ATG5) and 7 (ATG7) were lower, but sequestosome-1 (SQSTM1, also called p62) greater in mammary tissue of clinically ketotic cows. In vitro, exogenous FFA increased the content of malondialdehyde and reactive oxygen species, but decreased the activities of superoxide dismutase, catalase, and plasma glutathione peroxidase. Compared with the 0 \( \text{mM} \) FFA group, abundance of ATG5, ATG7, LC3-II was greater, but p62 was lower in the 0.6 \( \text{mM} \) FFA-treated cells. Similarly, abundance of ATG5, ATG7, and LC3-II was lower, but p62 greater in the 1.2 \( \text{mM} \) FFA-treated cells relative to 0 \( \text{mM} \) FFA group. Culture with rapamycin alleviated oxidative stress induced by 1.2 \( \text{mM} \) FFA, whereas 3-MA aggravated it. Overall, results indicated that a low concentration (0.6 \( \text{mM} \)) of FFA can induce oxidative stress and activate autophagy in BMEC. At higher concentrations of FFA (1.2 \( \text{mM} \)), autophagy is impaired and oxidative stress is aggravated. Autophagy is a mechanism for BMEC to counteract FFA-induced stress. As such, it could serve as a potential target for further development of novel strategies against oxidative stress.

**Key words:** ketosis, FFA, mammary gland, oxidative stress, autophagy

**INTRODUCTION**

Ketosis is a common metabolic disease in high-yielding cows with a high prevalence and poses a severe economic loss (Brunner et al., 2018). In the peripartal period, fetal development and lactation require a significant incremental amount of energy, but the typical decrease in DMI leads to negative energy balance (NEB). The NEB initiates fat mobilization followed by a significant increase in blood concentrations of free fatty acids (FFA), which are the hallmark of NEB status (Duffield et al., 1998; Itle et al., 2015). Because an overload of hepatocytes with FFA is beyond the processing capacity of the liver, a large number of ketones is synthesized, among which the dominant is BHB, leading to the onset of ketosis (Peng et al., 2019).

Ketosis is usually accompanied by oxidative stress, in part due to the marked increase in oxidative metabolism of the liver that generates free radicals, such as reactive oxygen species (ROS; Graugnard et al.,...
In pathological conditions, free radicals derived from increased metabolism of FFA accumulate and, depending on the antioxidant status, could cause oxidative stress (Schönfeld and Wojtczak, 2008; Contreras and Sordillo, 2011). In clinical ketotic cows (i.e., blood BHB >3.0 mM), milk synthesis capacity is diminished and the mammary gland is also subject to metabolic stress arising from the increased oxidative metabolism of FFA, often contributing to mammary cell death (Bae et al., 2017; Zahrazadeh et al., 2018). The underlying mechanisms of FFA-induced oxidative stress in the mammary gland of clinically ketotic cows is not well characterized.

Autophagy refers to a catabolic process aimed at recycling cellular components and damaged organelles in response to diverse stress conditions. Autophagic activity is mediated by a complex molecular mechanism including more than 30 autophagy-related genes and their expression products (ATG), among which ATG5 and ATG7 are involved as core proteins in the formation of autophagosomes (Kang et al., 2011; Khambu et al., 2018). During assembly and maturation, microtubule-associated protein 1 light chain 3 (LC3) and sequestosome-1 (SQSTM1, also called p62) serve as 2 protein markers for autophagosome formation and autophagic flux (Mizushima and Klionsky, 2007). In the context of oxidative stress, autophagy contributes to clearing cells of all irreversibly oxidized biomolecules such as DNA, proteins, lipids, and so on, and damaged mitochondria (Filomeni et al., 2015). It has been reported that p62 can bridge autophagy-targeted mitochondria to LC3 located on the autophagosomes surface, thereby driving their degradation to remove damaged mitochondria (Vadlamudi et al., 1996; Bjorkoy et al., 2005).

Although previous studies illustrated some of the roles of autophagy in oxidative stress, the mechanisms whereby autophagy participates in FFA-induced oxidative stress in the mammary gland of clinically ketotic cows are unknown. Thus, the aim of this study was to investigate (1) oxidative stress and autophagy status in mammary gland tissue from clinically ketotic cows and (2) the role of autophagy in FFA-induced oxidative stress in mammary epithelial cells.

Our experiment took a total of 4 mo and a total of 103 lactating dairy cows (numbers of parities: median = 3, range = 2 to 4; DIM: median = 7 d, range = 3 to 15 d) were selected from a 5,000-cow dairy farm located in Changchun City, Jilin Province, China. Selected cows received a routine physical examination to ensure absence of other peripartal diseases. Cows were classified as suspected ketosis if results for a nitroprusside-based milk ketone test were positive in the presence of clinical symptoms, such as reduction of feed intake, milk yield, or both. Then, we preselected 68 healthy cows and 35 cows with suspected ketosis.

To diagnose clinical ketosis, the blood concentration of BHB in these cows was measured. According to plasma parameters and clinical signs (Oetzel, 2004; Itle et al., 2015), 15 control cows (BHB <0.6 mM) were selected randomly among 68 healthy cows, and 15 clinically ketotic cows (BHB >3.0 mM) were selected randomly among 35 cows with suspected ketosis. All selected cows were housed in a climate-controlled barn (daily air temperature 10–20°C) with individual tiestalls to reduce environmental interference. Cows had ad libitum access to the same diet (Supplemental Table S1, https://figshare.com/articles/dataset/Basal_diet_formulation_/19431380) and were offered this diet twice a day, at 0830 and 1600 h.

Screened cows were milked twice daily at 0800 and 1530 h. Milk yield was electronically recorded, and milk samples were collected at each milking for 5 d and stored at 4°C with a preservative (1 mg/mL of potassium dichromate). Considering daily variation in milk yield, as well as the effect of tissue biopsy on milk production, data from the 3 d before biopsy were averaged with data from 2 d postbiopsy, without regard to production data from both the day of biopsy and the first day postbiopsy. This was considered necessary because the biopsy procedure typically results in reduced milk yield for the first 2 to 3 milkings (Farr et al., 1996). The percentage of milk fat, protein, and lactose was measured by infrared analysis with a 4-channel spectrophotometer (Multispec, Foss Food Technology), then the amount (kg/d) of each was calculated from milk yield and composition. Dry matter intake for each group (kg/d) was calculated as the difference between feed offered and feed refused every day for the last 3 d before biopsy. The productive and main physiological parameters of cows are reported in Table 1.

Before morning feeding, blood samples were collected without anticoagulant between 0730 and 0830 h by jugular venipuncture for 3 consecutive days. Subsequently, blood samples were (1) centrifuged at 3,000 × g for 15 min at 4°C to obtain serum, and serum was harvested and stored at −80°C until further analysis and (2) kept at room temperature until transported to

**MATERIALS AND METHODS**

**Animals and Sampling**

The study protocol was approved by the Ethics Committee for the Use and Care of Animals, Jilin University (Changchun, China). Animal studies were performed in accordance with the Guiding Principles of Animals adopted by the Chinese Association for Laboratory Animal Sciences.
the laboratory (within 30 min after collection). Concentrations of glucose, BHB, and FFA in serum were determined using a Hitachi 7170 autoanalyzer (Hitachi) with commercially available kits (BHB: catalog no. RB1008; FFA: catalog no. FA115; glucose: catalog no. GL3815; Randox Laboratories). Malondialdehyde (MDA) was analyzed by the lipid peroxidation MDA assay kit (S0131S, Beyotime) according to the manufacturer’s protocols (https://www.beyotime.com/product/S0131S.htm). Superoxide dismutase (SOD) was analyzed by the total SOD assay kit with NBT (S0109, Beyotime) according to the manufacturer’s protocols (https://www.beyotime.com/product/S0109.htm). Catalase (CAT) was analyzed by the CAT assay kit (S0051, Beyotime) according to the manufacturer’s protocols (https://www.beyotime.com/product/S0051.htm). Glutathione peroxidase (GSH-Px) was analyzed by total GSH-Px assay kit with NADPH (S0058, Beyotime) according to the manufacturer’s protocols (https://www.beyotime.com/product/S0058.htm).

Percutaneous biopsies from cows were obtained from the right or left rear quarter of the mammary gland according to published procedures (Bae et al., 2017). Biopsies were conducted at 0800 h (after morning milking). Briefly, after making the skin incision, we performed blunt dissection of the mammary capsule to ensure tissue obtained during the biopsy was mammary parenchyma. Immediately after the biopsy, the instrument was removed from the capsule and pressure was applied to the wound until visual signs of bleeding were absent. The skin incision was closed with 4 or 5 Michel 11-mm clips (Henry Schein). The incision site was sprayed with topical antiseptic (povidone iodine ointment, 10%; Taro Pharmaceuticals). Health after surgery was monitored by recording rectal temperature, milk yield, and daily feed intake for 7 d. Surgical clips were removed 7 d after biopsy. No incidence of mastitis was detected for any cow during the 7 d after surgery. Tissue samples for quantitation of protein and RNA were frozen immediately in liquid nitrogen and stored at −80°C until analysis.

**Primary Cell Culture**

Primary mammary epithelial cells from 3 healthy lactating dairy cows (DIM = 100 ± 5 d) were harvested as previously described (Wang et al., 2014, 2017). Immediately after exsanguination, parenchyma samples were aseptically removed from the core of the mammary gland. Tissue was sliced into pieces and digested with 0.5% collagenase III (C8490, Solarbio) for 3 h at 38°C. The solution was sequentially filtered with a 74-μm strainer and centrifuged at 150 × g for 10 min at 4°C. Cells were cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, HyClone) supplemented with 10% fetal bovine serum, 500 ng/mL of hydrocortisone (Sigma-Aldrich), 1 μg/L of prolactin (Sigma-Aldrich), 10 ng/mL of epidermal growth factor (PeproTech), and 100 IU/mL of penicillin/streptomycin (Baoman Biotechnology) for 48 h at 37°C under an atmosphere containing 5% CO2. Cells were then digested with 100 μM trypsin (a 3-min digestion followed by a 5-min digestion) to purify epithelial cells. After 3 passages, cells were seeded into a 6-well tissue culture plate (2 mL/well) with DMEM/F-12 supplemented with 10% fetal bovine serum and 100 IU/mL penicillin/streptomycin. Medium was changed once every 24 h.

**Cell Treatment**

The FFA mixture was diluted in 0.1 M KOH at 60°C, and the pH of the solution was adjusted to 7.4 with hydrochloric acid (1 M). The FFA stock (52.7 mM) solution included oleic acid (22.9 mM), linoleic acid (2.6 mM), palmitic acid (16.8 mM), stearic acid (7.6 mM), and palmitoleic acid (2.8 mM). Mammary epithelial cells were cultured with 0, 0.3, 0.6, or 1.2

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**Table 1.** Means for BW, DMI, production characteristics, and blood parameters in healthy and clinically ketotic cows

<table>
<thead>
<tr>
<th>Item</th>
<th>Control (n = 15)</th>
<th>Clinical ketosis (n = 15)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (kg)</td>
<td>621.27 ± 8.56</td>
<td>654.46 ± 5.92</td>
<td>0.015</td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>22.18 ± 0.55</td>
<td>20.36 ± 0.47</td>
<td>0.001</td>
</tr>
<tr>
<td>Milk production (kg of milk/cow per day)</td>
<td>28.96 ± 0.75</td>
<td>25.89 ± 0.68</td>
<td>0.005</td>
</tr>
<tr>
<td>Milk fat (kg/d)</td>
<td>1.06 ± 0.09</td>
<td>1.27 ± 0.11</td>
<td>0.001</td>
</tr>
<tr>
<td>Milk protein (kg/d)</td>
<td>0.99 ± 0.17</td>
<td>0.70 ± 0.16</td>
<td>0.001</td>
</tr>
<tr>
<td>Milk lactose (kg/d)</td>
<td>1.29 ± 0.11</td>
<td>1.03 ± 0.09</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum glucose (mM)</td>
<td>3.73 ± 0.32</td>
<td>2.93 ± 0.25</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum free fatty acids (mM)</td>
<td>0.29 ± 0.07</td>
<td>1.08 ± 0.35</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum BHB (mM)</td>
<td>0.51 ± 0.12</td>
<td>3.69 ± 0.58</td>
<td>0.001</td>
</tr>
</tbody>
</table>

1Values are means ± SEM.
mM FFA in medium with 2% BSA for 24 h. Mammary cells take up fatty acids from BSA-conjugated FFA (Bionaz and Loor, 2008); thus, cells were cultured with FFA in medium with 2% BSA. For experiments involving autophagy modulation, rapamycin (99045, Cell Signaling Technology) and 3-methyladenine (3-MA; M9281, Sigma-Aldrich) were used at concentrations of 100 nM and 5 mM, respectively, in 1.2 mM FFA group for 24 h.

**Determination of Intracellular ROS Concentration and Oxidative Stress Indicators**

After treatment with FFA for 24 h, the bovine mammary epithelial cells (BMEC) were incubated with dichlorofluorescein diacetate (DCFH-DA; Beyotime Biotechnology Inc.) for 30 min at 37°C. The fluorescence intensity of cells was measured by flow cytometry (Becton Dickinson). Malondialdehyde was analyzed by the lipid peroxidation MDA assay kit; SOD was analyzed by total SOD assay kit with NBT; CAT was analyzed by CAT assay kit; and GSH-Px was analyzed by total GSH-Px assay kit with NADPH according to the manufacturer’s protocols as above.

**Quantitative Reverse-Transcription PCR Assay**

Total RNA from mammary tissues and cultured mammary epithelial cells were extracted with RNAiso Plus (Tiangen Biotech Co. Ltd.) in accordance with the manufacturer’s instructions. The protein concentration was measured by the BCA method (P1511; Applygen Technologies). A total of 30 μg of protein from each sample were separated by 12% SDS-PAGE. The molecular location of the bovine target protein on gel was consistent with the manufacturer’s statement of antibody (molecular weight of the target protein) by referring the molecular weight marker (26616; Thermo Fisher Scientific). The target protein on gel was electrophoretically transferred to a polyvinylidene difluoride membrane (PVDF). The membranes were blocked in 3% BSA/Tris-buffered saline/Tween (TBS-T) buffer for 4 h, and then incubated overnight at 4°C with primary antibodies against ATG5 (1:500; NB110; Novus Biologicals), ATG7 (1:1,000; D12B11; Cell Signaling Technology), p62 (1:2,000; ab101266; Abcam), LC3 (1:1,000; ab48394; Abcam), and β-actin (1:2,000; ab8226; Abcam), respectively. The antibodies of ATG5, ATG7, p62, LC3 and β-actin can react with bovine target proteins according to the manufacturer’s statement. Subsequently, the PVDF membranes were washed with TBS-T 3 times and incubated with horseradish peroxidase-conjugated anti-rabbit or anti mouse immunoglobulin for 45 min at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence solution (Millipore). β-Actin was used as a reference protein. Finally, all western blot bands were analyzed using Image-pro Plus (Media Cybernetics).

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 5 (GraphPad InStat Software) or SPSS 23.0 software (IBM Corp.). For in vivo results with a normal distribution, parametric statistical analysis was performed using independent samples t-tests. For in vitro results, linear and quadratic contrasts were conducted.
to evaluate dose-dependent effects. One-way or 2-way ANOVA was performed for multiple comparisons with Bonferroni correction for data meeting homogeneity of variance. Data are presented as the mean ± SEM. A P-value <0.05 was considered statistically significant, and P < 0.01 was considered extremely significant.

RESULTS

Baseline Characteristics and Blood Parameters

Compared with healthy cows, cows with clinical ketosis had lower milk production (P = 0.005) and DMI (P = 0.001). Cows with clinical ketosis had lower content of milk protein (P = 0.001), but greater milk fat (P = 0.001). Cows with clinical ketosis had greater concentrations of FFA (P = 0.001) and BHB (P = 0.001). In contrast, cows with clinical ketosis had a lower concentration of glucose (P = 0.001).

Changes in Oxidative and Antioxidant Status in Ketotic and Healthy Cows

Compared with healthy cows, cows with clinical ketosis had greater plasma concentration of MDA (Figure 1A, P = 0.001), but lower plasma activities of SOD (Figure 1B, P = 0.007), CAT (Figure 1C, P = 0.001), and GSH-Px (Figure 1D, P = 0.001).

Autophagy-Related mRNA and Protein Abundance in Mammary Tissue

Compared with mammary tissue of healthy cows, mammary tissue of clinically ketotic cows had lower
protein abundance of ATG5 (Figure 2B, \(P = 0.002\)), ATG7 (Figure 2C, \(P = 0.006\)), LC3-II (Figure 2E, \(P = 0.005\)), but greater protein abundance of p62 (Figure 2D, \(P = 0.004\)). Similarly, mammary tissue of clinically ketotic cows had lower mRNA abundance of ATG5 (Figure 2F, \(P = 0.009\)), ATG7 (Figure 2G, \(P = 0.011\)), MAP1LC3 (Figure 2I, \(P = 0.002\)), but greater mRNA abundance of SQSTM1 (Figure 2H, \(P = 0.011\)).

**Effects of Autophagy on the Oxidant and Antioxidant Status in BMEC**

Rapamycin relieved, but 3-MA aggravated the FFA (1.2 mM) induced increase in the content of MDA (Figure 4A, \(P = 0.024\), \(P = 0.018\), respectively), ROS (Figure 4E, \(P = 0.039\), \(P = 0.028\), respectively), and the decrease in activities of SOD (Figure 4B, \(P = 0.049\), \(P = 0.023\), respectively), CAT (Figure 4C, \(P = 0.036\), \(P = 0.034\), respectively), and GSH-Px (Figure 4D, \(P = 0.003\), \(P = 0.005\), respectively).

**Oxidant and Antioxidant Status and Autophagy-Related Proteins Are Altered by FFA in BMEC**

Compared with the 0 mM FFA group, treatment with increasing concentrations of FFA, especially 0.6 and 1.2 mM FFA, resulted in a dose-dependent increase in the content of MDA (linear, \(P < 0.01\); Figure 3A, \(P = 0.049\), \(P = 0.001\), respectively), ROS (linear, \(P < 0.01\); Figure 3E, \(P = 0.012\), \(P = 0.001\), respectively), and a decrease in the activities of SOD (linear, \(P < 0.01\); Figure 3B, \(P = 0.014\), \(P = 0.001\), respectively), CAT (linear, \(P < 0.01\); Figure 3C, \(P = 0.038\), \(P = 0.001\), respectively), GSH-Px (linear, \(P < 0.01\); Figure 3D, \(P = 0.012\), \(P = 0.004\), respectively). Similarly, compared with the 0 mM FFA group, 0.6 mM FFA group had greater but 1.2 mM FFA group had lower protein abundance of ATG5 (linear and quadratic, \(P < 0.01\); Figure 3G, \(P = 0.042\), \(P = 0.012\), respectively), ATG7 (linear and quadratic, \(P < 0.01\); Figure 3H, \(P = 0.027\), \(P = 0.029\), respectively), LC3-II (linear and quadratic, \(P < 0.01\); Figure 3J, \(P = 0.044\), \(P = 0.002\), respectively). The 0.6 mM FFA group had lower but the 1.2 mM FFA group had greater protein abundance of p62 (linear and quadratic, \(P < 0.01\); Figure 3I, \(P = 0.013\), \(P = 0.004\), respectively).

**DISCUSSION**

Oxidative stress is a common pathological foundation of ketosis, which results partly from a deficit in antioxidant capacity (Miller et al., 1993; Du et al., 2017). In addition to the typical reductions in milk production during ketosis, previous studies indicated that persistent oxidative stress affects lactation quality and reduces performance in dairy cows (Miller et al., 1993; Zheng et al., 2021). When ketosis occurs, the oxidative stress induced by excessive FFA accumulation causes mammary gland injury. For instance, in bovine hepatocytes, FFA can induce cellular oxidative stress and injury (Shi et al., 2015). Data from BMEC indicated that oxidative stress increases the number of apoptotic cells, decreases cell viability, and alters cell morphology (Miranda et al., 2011). Together, available data led us to investigate in greater detail regulatory mechanisms whereby oxidative stress in mammary tissue causes damage, particularly during periods of NEB, which leads to overproduction of prooxidant molecules such as FFA.
Autophagy is a highly conserved and strictly regulated cellular self-degradation process. It involves degradation of long-lived proteins, entire organelles, oligomers, and protein aggregates, which are sequestered by their autophagosomal double-membrane vesicles. These vesicles are transported to lysosomes for fusion or to endosomes for their eventual degradation before fusion with lysosomes, followed by recy-
clinging of hydrolysis products (Mizushima, 2007; Ravikumar et al., 2009; Rubinsztein et al., 2012). When autophagy is activated, cytosolic LC3-I is converted into membrane-bound LC3-II, and the conversion between LC3-I and LC3-II promotes the formation and lengthening of the autophagosome; p62 can interact with LC3 and facilitate degradation of ubiquitinated protein aggregates by autophagy (Feng et al., 2016; Hwang et al., 2017). Thus, p62 can serve as a selective substrate of autophagy. In this process, LC3-II and p62 are markers for the evaluation of autophagic flux (Kabeya et al., 2000; Mizunoe et al., 2018).

A growing number of studies in nonruminant models have demonstrated that autophagy not only plays a significant role in maintaining stability of the intracellular environment, but also can play a role in responding to diverse conditions of stress such as selectively clearing irreversibly oxidized biomolecules (Filomeni et al., 2015; Liao et al., 2019; Xu et al., 2021). Autophagic activity in the mammary gland of cows with subclinical ketosis was significantly increased and was associated with a greater ability of mammary epithelial cells to control metabolic stress (Li et al., 2020). However, cows with clinical ketosis in the present study had greater abundance of p62 and lower abundance of ATG5, ATG7, and LC3-II, suggesting an overall decrease of autophagy activity. One possible explanation for these effects is that cows with clinical ketosis failed to control oxidative stress because of the decrease in autophagy, or that the degree of oxidative stress was beyond the clearance capacity of autophagy. Alternatively, it is possible that the excessive concentrations of FFA had a negative effect on mechanisms related to autophagy activity.

Figure 4. Effects of autophagy on the oxidation indicators and autophagy relative proteins in bovine mammary epithelial cells (BMEC). The BMEC were pretreated with 100 nM rapamycin for 4 h or 50 mM 3-methyladenine (3-MA) for 1 h, respectively. Then they were treated with or without 1.2 mM free fatty acids (FFA) for another 24 h. (A–E) Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and reactive oxygen species (ROS) contents in BMEC. (F) Western blot analysis of sequestosome-1 (SQSTM1, also called p62) and microtubule-associated protein 1 light chain 3 (LC3) in BMEC. (G, H) Protein abundance of p62 and microtubule-associated protein 1 light chain 3-II (LC3-II) in BMEC. Data were analyzed using 2-way ANOVA with subsequent Bonferroni correction and expressed as mean ± SEM.
The present study revealed a dose-dependent effect of FFA on autophagy activity. Our in vitro results revealed at a low concentration of FFA (0.6 mM), the increased level of ROS, along with increases in ATG5, ATG7, and LC3-II abundance, and a decrease in p62 abundance suggested that autophagy activity was enhanced. These responses support the notion that subclinical ketosis is associated with enhanced autophagy (Li et al., 2020). At a high FFA concentration, the opposite effects were observed, which is consistent with our in vivo experimental data that autophagy activity in mammary glands of clinically ketogenic cows was decreased. Furthermore, studies in Huh7 and HepG2 cell lines also showed that high concentrations of fatty acids inhibited autophagic activity (González-Rodríguez et al., 2014; Miyagawa et al., 2016). Taken together, our in vivo and in vitro data demonstrated that low concentrations of FFA enhanced BMEC autophagy and high concentrations of FFA impaired BMEC autophagy. Low concentrations of FFA-induced stress can be alleviated by activated autophagy, whereas high concentrations of FFA have a strong lipotoxicity that impairs autophagy processes and so cannot be countered. Disruption of autophagy results in inhibition of ubiquitination, accumulation of ROS, attenuated mitochondrial function, and diminished DNA repair, which in turn causes oxidative stress (Li et al., 2015; Liu et al., 2015). Hence, autophagy is a cellular mechanism to counteract stress, and impaired autophagy may be an important induction mechanism of oxidative stress in the mammary gland of dairy cows exposed to high circulating levels of FFA or with clinical ketosis.

Oxidative stress in bovine adipocytes can be ameliorated by inducing autophagy, underscoring the role of this process in modulating oxidative stress status (Xu et al., 2021). Work in nonruminants has demonstrated that the degree of autophagy activity can alleviate or exacerbate oxidative stress in many cells such as hepatocytes, breast cancer cells, cardiomyocytes, and so on (Hickson-Bick et al., 2008; Czaja, 2011; Wang et al., 2021). Thus, we sought to investigate these processes in the context of FFA-induced oxidative stress in BMEC. The fact that activation of autophagy with rapamycin attenuated while 3-MA increased the FFA-induced oxidative stress indicated that autophagy is involved in the regulation of oxidative stress in BMEC. Thus, enhancing autophagic activity in the mammary gland may be a novel strategy against oxidative stress and decreased milk production induced by ketosis or during periods when concentrations of FFA are increased; however, the FFA threshold for impaired or activated autophagy and its potential mechanisms remain for further study.

CONCLUSIONS

Our study clarified the role of autophagy in the context of oxidative stress in the bovine mammary gland induced by high concentrations of FFA. Autophagy is a mechanism for BMEC to counteract FFA-induced stress and could serve as a potential target for development of novel strategies against oxidative stress and decreased milk production induced by ketosis.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Beijing, China; grant no. 32022084 and 32172927), the Fundamental Research Funds for the Central Universities (Changchun, China), and the Fund of Education Department of Jilin Province (Changchun, China; grant no. JJKH20221041KJ). The authors have not stated any conflicts of interest.

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