Staphylococcal enterotoxin M induced inflammation and impairment of bovine mammary epithelial cells

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ABSTRACT

Staphylococcus aureus is one of the major etiological pathogens of bovine mastitis. Its invasion into mammary epithelial cells has been proven to be a key event in the pathogenesis of mastitis. However, the specific pathogenic factors have not been clearly identified. *Staphylococcus aureus* often triggers infections by releasing virulence factor. Recent several studies reported that staphylococcal enterotoxin M was one of the most frequently found enterotoxin genes associated with bovine mastitis. Thus, the effect of staphylococcal enterotoxin M on inflammation and damage of the bovine mammary epithelial bovine mammary gland epithelial cell line (MAC-T) cells with 48 h treatment was explored in the present study. First, staphylococcal enterotoxin M protein was purified by a Ni-NTA spin column (GE Life Science, Westborough, MA). The levels of tumor necrosis factor- α , IL-6, and monocyte chemoattractant protein 1 (MCP-1) secretion were measured with the corresponding ELISA kits (R&D Systems, Abingdon, UK). Second, cell viability was assessed with a Cell Counting Kit-8 (Bioswamp, Wuhan, China) and the apoptotic percentage of cells was determined by annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI; Beyotime, Nanjing, China) staining. Third, ATP concentration, reactive oxygen species (ROS) generation and lactate dehydrogenase (LDH) release were assayed with commercial kits, then mitochondrial membrane potential ($\Delta\Psi_m$) was estimated using fluorescent probe JC-1 (Beyotime). Finally, the production intercellular cell adhesion molecule-1 (ICAM-1), microtubule-associated protein 1A/1B-light chain 3 I/II (LC3 I/II), p62 (Proteintech, Rosemont, IL), and phosphorylation of I κ B α , caspase 3, and mammalian target of rapamycin were detected by Western blot. The results showed that staphylococcal enterotoxin M

induced inflammation of epithelial cells (upregulating tumor necrosis factor- α , IL-6, MCP-1, and ICAM-1 production) and activated NF- κ B (promoting phosphorylation of I κ B α). Furthermore, staphylococcal enterotoxin M impaired MAC-T cells via cell necrosis (enhancing LDH release), apoptosis (annexin V-FITC/PI stain, exacerbating oxidative stress, decreasing $\Delta\Psi_m$ and intracellular ATP concentration, and activating caspase 3), but independent of autophagy (nonsignificantly increasing LC3-II, decreasing p62 expression, and activating mammalian target of rapamycin). Thereby, staphylococcal enterotoxin M induced the inflammatory property of bovine mammary epithelial cells by boosting cytokine, chemokine, and adhesion molecule production. Furthermore, it caused epithelial cell dysfunction via depressing cell viability and initiating cell necrosis and apoptosis. Because epithelial cells played important roles in orchestrating the inflammatory response and protecting bovine mammary tissue from mastitis, our results indicated that staphylococcal enterotoxin M may be associated with mastitis.

Key words: staphylococcal enterotoxin M, bovine mammary epithelial cell, mastitis, apoptosis

INTRODUCTION

Bovine mastitis induced by invading pathogens imposes considerable economic loss on the dairy industry worldwide through increased veterinary costs, reduced milk yield, and impaired milk quality (Viguier et al., 2009). The mammary gland is a complex organ composed of several cell types for synthesis and secretion of milk (Kobayashi and Kumura, 2011). Among the cells, a layer of mammary epithelial cells establishes the blood–milk barrier for exchange of macromolecules and solutes during milk production (Guidry et al., 1998). Upon bacterial challenge, mammary epithelial cells protect the underlying tissues from damage via eliciting proinflammatory cytokines and triggering the inflammatory cascade response in the acute phase of mastitis (Günther et al., 2011). Unfortunately, mammary epithelial cell dysfunction caused by exacerbated

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or sustained inflammation damages barrier integrity and surrounding extravascular tissues (Ryman et al., 2015). Given the important role of epithelial cells in regulating mammary gland health and disease, a better understanding of epithelial cell function during mastitis may support development of targeted therapies to protect mammary tissue.

Staphylococcus aureus is one of the major etiological pathogens of bovine mastitis (Piccinini et al., 2012). *Staphylococcus aureus* adhesion and invasion to mammary epithelial cells have been proven to be key events involved in the pathogenesis of mastitis (Kerro Dego et al., 2002). Following *S. aureus* infection, the cells recognize *S. aureus* through the plasma membrane toll-like receptor 2. The binding activates the phosphoinositide 3-kinase/Akt (**PI3K/Akt**)-mammalian target of rapamycin (**mTOR**) signaling pathway that mediates a variety of cellular responses such as survival, proliferation, apoptosis, and inflammation (Akira and Takeda, 2004), but the specific pathogenic factors in *S. aureus* leading to mastitis have not been clearly identified. *Staphylococcus aureus* often triggers infections by releasing virulence factors such as enterotoxins, a series of related proteins responsible for toxic-shock syndrome, septicemia, and food poisoning (Casman, 1965; Balaban and Rasooly, 2000). Staphylococcal enterotoxins have been traditionally subdivided into classical staphylococcal enterotoxins (from SEA to SEE) and newly identified staphylococcal enterotoxins or enterotoxin-like proteins (SEL; through SEG to SEIY; Argudín et al., 2010; Hu and Nakane, 2014). Recent several studies established the virulence gene profiles of *S. aureus* isolates collected from milk samples of cows with subclinical or clinical mastitis. These toxin genes were reported to be closely associated with the pathogenicity of the *S. aureus* causing mastitis (Wang et al., 2017). In particular, staphylococcal enterotoxin M was harbored by 19.4% *S. aureus* isolates in eastern Poland (Kot et al., 2016), by 26.9% *S. aureus* isolates in 2 major dairy production regions of China (Wang et al., 2009), by 16.0% *S. aureus* strains in 10 dairy herds located in Ningxia, China (Wang et al., 2017), by 100% *S. aureus* ST520 strains from 2014 to 2015 in the Shanghai and Zhejiang areas of China (Li et al., 2017), and by 100% methicillin-resistant *S. aureus* strains from 5 dairy farms in Gyeonggi province in Korea from 2011 to 2012 (Song et al., 2016). The frequent occurrence of the staphylococcal enterotoxin M gene associated with mastitis indicated that it might be related to mastitis. Because epithelial cells played important roles in orchestrating the inflammatory response and protecting bovine mammary tissue from mastitis, the present study investigated the possible effect of staphylococcal

enterotoxin M on inflammatory response and survival of bovine mammary epithelial cells.

MATERIALS AND METHODS

Staphylococcal Enterotoxin M Protein Preparation

Staphylococcal enterotoxin M protein was prepared as we previously described (Zhao et al., 2017). In brief, staphylococcal enterotoxin M gene was amplified from clinical *S. aureus* strain H4 associated with food poisoning. Then staphylococcal enterotoxin M gene was cloned into pET-28a plasmid (Novagen, Darmstadt, Germany) and transformed into competent *Escherichia coli* BL21 (DE3) cells (Novagen). After induction with 0.5 mmol/L isopropyl-1-thio- β -D-galactopyranoside (Sigma-Aldrich, St. Louis, MO), the cells were lysed by sonication and the tagged staphylococcal enterotoxin M recombinant protein was purified by a Ni-NTA spin column (GE Life Science, Westborough, MA). At last, the purified protein was dialyzed into 10 mmol/L PBS (pH = 7.4) and applied to 12% SDS-PAGE.

Cell Culture and Treatment

The bovine mammary gland epithelial cell line MAC-T was a kind gift from Jinfeng Miao (Nanjing Agricultural University, Nanjing, China). The MAC-T cell line was established from primary bovine mammary alveolar cells by stable transfection with SV-40 large T-antigen. The cells displayed a cobblestone shape on plastic substratum. They are widely used to investigate inflammation and proliferation assay of bovine mammary epithelial cells associated with mastitis (Jeong et al., 2017; Wang et al., 2019). In the present study, monolayer MAC-T cells were grown in high glucose Dulbecco's modified Eagle's medium (Hyclone, Fremont, CA) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA), 100 U/mL streptomycin, and 100 U/mL penicillin in the humidified atmosphere of 5% CO₂ at 37°C. For assays, in vitro cultured MAC-T cells between passages 25 and 30 were serum-starved for 24 h before treatment and then incubated in the presence of 0, 1, 100, and 10,000 μ g/L staphylococcal enterotoxin M for 48 h.

Cell Viability Assay

The effect of staphylococcal enterotoxin M on bovine mammary gland epithelial cell viability was assessed with a Cell Counting Kit-8 (CCK8, no. 1706, Bioswamp, Wuhan, China). In detail, 3×10^3 cells/well was inoculated and exposed to 0, 1, 100, and 10,000

μg/L staphylococcal enterotoxin M for 48 h as above, then 10 μL of CCK8 solution was added for 4 h and the absorbance value was calculated at 450 nm. The data were expressed as the percentage of viable cells with staphylococcal enterotoxin M treatment compared with that in the control group.

Cell Apoptosis, Reactive Oxygen Species Production, and Inflammatory Cytokine Release

After being challenged with staphylococcal enterotoxin M for 48 h, bovine mammary epithelial cells were stained with an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit according to the manufacturer's protocol (no. C1062, Beyotime, Nanjing, China). The percentage of cells at different apoptotic stages was determined by a flow cytometer (Beckman Coulter FC500, Brea, CA). At the same time, the reactive oxygen species (ROS) generation by the cells was measured with a cellular ROS detection assay kit (no. PAB180052, Bioswamp), and also analyzed by the flow cytometer. In addition, the concentrations of tumor necrosis factor-α (TNF-α, no. HM10001, Bioswamp), IL-6 (no. HM10205, Bioswamp), and monocyte chemoattractant protein 1 (MCP-1, no. HM10894, Bioswamp) secreted from bovine mammary epithelial cells were assayed using the corresponding ELISA kits (R&D Systems, Abingdon, UK).

Lactate Dehydrogenase Release

Lactate dehydrogenase (LDH) is an enzyme that is normally located in the cytoplasm and is released into the medium when the cell membrane is damaged (Loeffler et al., 2004). Thus, LDH release assay can be used to evaluate the integrity of cell membranes. After staphylococcal enterotoxin M treatment, the cell supernatants were collected for measurement of LDH release by a LDH Assay Kit (no. A020-1, Nanjing Jiancheng Chemical Industrial Co. Ltd., Nanjing, China). Then, the absorbance at 490 nm was detected.

Assessment of Mitochondrial Membrane Potential and Intracellular ATP Content

Mitochondrial membrane potential was estimated using fluorescent probe JC-1 (no. 180068, Beyotime, Nanjing, China) as described previously (Feng et al., 2017). The JC-1 monomer and aggregate signals were detected by flow cytometry analysis and the ratio of aggregate/monomer fluorescent intensity was calculated for the level of mitochondrial membrane potential. Intracellular ATP concentrations were assayed with an ATP assay kit (no. A095, Nanjing Jiancheng Chemical Industrial

Co. Ltd.). The cells were lysed and the proteins were discarded. Then, using ATP as standard, intracellular ATP contents were determined by chemiluminescence (West Pico kit, Pierce, Loughborough, UK).

Western Blot

Western blot was employed to measure phosphorylation of IκBα, caspase 3, mTOR, and the levels of intercellular cell adhesion molecule-1 (ICAM-1), microtubule-associated protein 1A/1B-light chain 3 I/II (LC3 I/II), and p62 in bovine mammary epithelial cells with staphylococcal enterotoxin M challenge. Total cellular and cytoplasmic proteins were prepared with lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich) and applied to SDS-PAGE, transferred to nitrocellulose membranes (Whatman, London, UK). Then, the membranes were blocked with 20 mmol/L Tris-HCl buffer supplemented with 0.1% Tween 20 and 5% wt/vol BSA for 1 h at room temperature. After that, the proteins on the membranes were incubated overnight at 4°C with the specific primary antibodies against NF-κB p65 (no. 10745-1-AP, 1:2,000, Proteintech, Rosemont, IL), p-IκBα (phospho Ser32, no. ab92700, 1:2,000, Abcam, Cambridge, MA), caspase 3 (no. 19677-1-AP, 1:1,000, Proteintech), p-caspase 3 (phospho S150, no. ab59425, 1:1,000, Abcam), mTOR (no. 0657-1-AP, 1:1,000, Proteintech), p-mTOR (phospho S2448, no. ab109265, 1:1,000, Abcam), ICAM-1 (no. 10020-1-AP, 1:2000, Proteintech), LC3 I/II (no. 128,025, 1:1,000, Abcam), p62 (no. 18420-1-AP, 1:2,000, Proteintech), and GAPDH (no. PAB36269, 1:1,000, Bioswamp). Membranes were washed 3 times and the corresponding horseradish peroxidase-conjugated secondary antibodies were added. Finally, specific immune blots were detected by chemiluminescence (West Pico kit, Pierce). The values from Western blot assay were expressed as the percentage of optical density with staphylococcal enterotoxin M treatment groups compared with that in the control group.

Statistical Analysis

The data were presented as average values ± standard error of the means from 3 independent experiments of cell viability assay, cell apoptosis, ROS production, inflammatory cytokine, LDH release, mitochondrial membrane potential ($\Delta\Psi_m$), intracellular ATP content, and Western blot analysis. Statistical analyses were performed with SPSS Statistics V17.0 software (SPSS Inc., Chicago, IL). These data were checked for normal distribution using Shapiro-Wilk test and the differences between 1, 100, or 10,000 μg/L staphylococcal enterotoxin M treatment groups and the control

group were made by an unpaired *t*-test with 95% confidence intervals. Statistical significance was considered at 2-tailed value of $P < 0.05$.

RESULTS

Staphylococcal Enterotoxin M Protein Preparation

The identified staphylococcal enterotoxin M gene information was submitted to GenBank (GenBank accession number: KT853047). The entire nucleotide sequence encoded a 239 amino acid polypeptide precursor. Molecular weight of the deduced protein was 27.37 kDa. Thereafter, mature staphylococcal enterotoxin M protein was expressed in *E. coli* BL21 cells and purified by Ni^{2+} -charged affinity chromatography. The purified protein with expected molecular weight (about 28 kDa) is indicated by an arrow in Figure 1.

Figure 1. Preparation of staphylococcal enterotoxin M protein. The fractions purified by Ni^{2+} -charged affinity chromatography were applied to SDS-PAGE. (1) Cellular total proteins after induction by isopropyl-1-thio- β -D-galactopyranoside; (2) fraction elution with 50 mmol/L imidazole; and (3) fraction elution with 100 mmol/L imidazole. The target protein with expected molecular weight (about 28 kDa), indicated by an arrow, was collected. M = marker.

Inflammatory Response in Mammary Epithelial Cells

Mammary epithelial cells with mastitis express proinflammatory cytokines including TNF- α and IL-6 (Zhang et al., 2016). The present study investigated whether staphylococcal enterotoxin M mediated the secretion of TNF- α and IL-6 in bovine mammary epithelial cells. Results shown that staphylococcal enterotoxin M potentially promoted TNF- α and IL-6 secretion (Figure 2A, B). Furthermore, staphylococcal enterotoxin M enhanced ICAM-1 and MCP-1 production (Figure 2C, D, E); ICAM-1 is an endothelial- and leukocyte-associated transmembrane protein facilitating leukocyte endothelial transmigration (Yang et al., 2005), whereas MCP-1 is a highly potent chemoattractant protein inducing monocytes and macrophages to sites of tissue injury and inflammatory reaction (Yadav et al., 2010). Thus, staphylococcal enterotoxin M induced proinflammatory cytokine release of bovine mammary epithelial cells, which might recruit monocytes and macrophages to the mammary gland. In addition, TNF- α and IL-6 belong to key signaling molecules of the downstream NF- κ B signaling pathway (Huang et al., 2012). To understand the molecular mechanism of staphylococcal enterotoxin M induced expression of TNF- α and IL-6, the NF- κ B was analyzed by Western blot. As shown in Figure 2D, F, G, staphylococcal enterotoxin M boosted $\text{I}\kappa\text{B}\alpha$ phosphorylation, which suggested that inflammation induced by staphylococcal enterotoxin M was probably associated with NF- κ B signaling.

Cell Viability

As described above, exacerbated inflammation would induce epithelial dysfunction (Ryman et al., 2015), so CCK8 assay was used to test the effect of staphylococcal enterotoxin M on bovine mammary epithelial cell viability. After 48 h of treatment, 1, 100, and 10,000 $\mu\text{g/L}$ staphylococcal enterotoxin M significantly inhibited cell viability ($P < 0.001$) in a dose-dependent manner as shown in Figure 3. The results indicated that staphylococcal enterotoxin M depressed epithelial cell viability, which would lead to cell death.

Integrity of Mammary Epithelial Cell Membrane

Cell death, the ultimate consequence of infection, mainly includes cell necrosis, apoptosis, and autophagy (Orrenius et al., 2011). Necrosis is characterized by loss of cell membrane integrity and release of cellular contents such as LDH. Thus, the activity of LDH in cell medium was determined by a LDH Assay Kit as shown in Figure 4. Staphylococcal enterotoxin M increased

LDH release, indicating that it impaired mammary epithelial cell membrane integrity and led to cell necrosis.

Cell Apoptosis

Cell apoptosis was investigated using annexin V-FITC/PI staining. The results in Figure 5 revealed that staphylococcal enterotoxin M significantly enhanced mammary epithelial cell apoptosis. Cell apoptosis is mainly mediated through the intrinsic and extrinsic

apoptosis pathway. Excessive ROS can induce apoptosis through both the extrinsic and intrinsic pathways (Lamkanfi and Dixit, 2010). In the present study, staphylococcal enterotoxin M promoted ROS production (Figure 6A). In addition, mitochondrial membrane potential and intracellular ATP content were assessed in Figures 6B and 6C. Staphylococcal enterotoxin M impaired mitochondrial membrane potential and further decreased intracellular ATP concentration. These data indicated staphylococcal enterotoxin M depressed

Figure 2. Inflammatory response in mammary epithelial cell cytokines. The bovine mammary gland epithelial cells were exposed for 48 h to 0, 1, 100, and 10,000 μg/L staphylococcal enterotoxin M. Then, the concentrations of tumor necrosis factor-α (TNF-α; A), IL6 (B), and monocyte chemoattractant protein 1 (MCP-1; C) secreted were measured using the corresponding ELISA kits. The level of the production intercellular cell adhesion molecule-1 (ICAM-1; E), NF-κB (F), and phosphorylation of IκBα (Ser32; p-IκBα, G) in cytoplasm was analyzed by Western blot. Data were the mean ± SEM of 3 independent experiments. ***P* < 0.01, ****P* < 0.001.

Figure 3. Viability of mammary epithelial cells. Three $\times 10^3$ cells/well was inoculated and exposed to 0, 1, 100, and 10,000 $\mu\text{g/L}$ staphylococcal enterotoxin M for 48 h as above. Then, 10 μL of Cell Counting Kit-8 (Bioswamp, Wuhan, China) solution was added for 4 h, and the absorbance value was calculated at 450 nm. The data were expressed as the percentage of viable cells with staphylococcal enterotoxin M treatment compared with that in the control group. Data were the mean $\pm$ SEM of 3 independent experiments. *** $P < 0.001$.

mitochondrial function. Furthermore, it activated caspase-3 (Figure 6D). Caspase-3 is essential for the formation of apoptotic bodies (Porter and Jänicke, 1999). Therefore, staphylococcal enterotoxin M probably depressed mammary epithelial cell viability via the cell apoptosis pathway.

Cell Autophagy

Cell death can also occur as a result of cell autophagy. During cell autophagy processes, LC3-I is conjugated to phosphatidylethanolamine to form LC3-II. Thus, detecting LC3-II/I by immunoblotting has become a reliable method for monitoring autophagy (Tanida et al., 2008). In this study, staphylococcal enterotoxin M did not significantly activate LC3-II (Figure 7A). Consistent with this, the level of the selective autophagy receptor p62 did not change (Figure 7B). Given that mTOR is a major regulator of cell autophagy, mTOR activity after staphylococcal enterotoxin M stimulation was investigated. Staphylococcal enterotoxin M did not regulate mTOR activity either (Figure 7D). Therefore, staphylococcal enterotoxin M did not obviously induce mammary epithelial cell autophagy.

DISCUSSION

Staphylococcus aureus is one of the most important pathogens inducing bovine mastitis. It has been proved

Figure 4. Lactate dehydrogenase (LDH) release from mammary epithelial cells. After 0, 1, 100, and 10,000 $\mu\text{g/L}$ staphylococcal enterotoxin M treatment for 48 h, the supernatants of mammary gland epithelial cells were collected for measurement of LDH release by a LDH Assay Kit (Nanjing Jiancheng Chemical Industrial Co. Ltd., Nanjing, China). The absorbance at 490 nm was detected. Data were the mean $\pm$ SEM of 3 independent experiments. * $P < 0.05$, *** $P < 0.001$.

that mammary epithelial cells are the main targets of *S. aureus* infection in bovine mastitis (Kerro Dego et al., 2002). However, the pathogen effectors have not been clearly elucidated. More than one report indicated the occurrence of the staphylococcal enterotoxin M gene in *S. aureus* isolate associated with mastitis (Piccinini et al., 2012; Song et al., 2016; Li et al., 2017). Thus, the distinctive role of staphylococcal enterotoxin M on mammary epithelial cells was explored in the present study.

Upon *S. aureus* challenge, mammary epithelial cells secrete chemokines and cytokines, recruiting and activating leukocytes to target sites against pathogens in mastitis (Alluwaimi, 2004). Here, staphylococcal enterotoxin M upregulated proinflammatory cytokines TNF- α and IL-6, chemokine MCP-1, and adhesion molecule ICAM-1 production. It has been reported that cytokines, such as TNF- α and IL-6, and chemokines, such as MCP-1, play important roles in the pathogenesis of mastitis (Kim et al., 2011). Elevated concentrations of chemokines induce the migration of inflammatory leukocytes to the infected area (Geng, 2001), and the cytokines amplify the inflammatory response. Moreover, activation of NF- κ B further causes the release of inflammatory cytokines and chemokines (Li et al., 2019). Then, sustained inflammatory reaction induces epithelial cell dysfunction (Ryman et al., 2015).

The blood–milk barrier formed by mammary epithelial cells is important for mammary gland resistance to bacterial infection and destruction of the blood–milk barrier aggravates the development of mastitis

Figure 5. Cell apoptosis of mammary epithelial cells. After being treated with 0, 1, 100, and 10,000 $\mu\text{g/L}$ staphylococcal enterotoxin M for 48 h, mammary epithelial cells were stained and apoptosis was analyzed with an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit (Beyotime, Nanjing, China) according to the manufacturer's protocol (<https://www.beyotime.com/product/C1062S.htm>). (A) Control, (B) 1 $\mu\text{g/L}$ staphylococcal enterotoxin M, (C) 100 $\mu\text{g/L}$ staphylococcal enterotoxin M, and (D) 10,000 $\mu\text{g/L}$ staphylococcal enterotoxin M. The cell percentage of different apoptotic stages was determined by a flow cytometer (Beckman Coulter FC500, Brea, CA; E). B1 = necrotic cells; B2 = cells at late apoptotic stage; B3 = live cells; B4 = cells at early apoptotic stage. Data were the mean $\pm$ SEM of 3 independent experiments. ** $P < 0.01$, *** $P < 0.001$.

Figure 6. Mitochondrial apoptosis pathway of mammary epithelial cells. After staphylococcal enterotoxin M administration for 48 h, the reactive oxygen species (ROS) production was measured with cellular reactive oxygen species detection assay kit, and analyzed by a flow cytometer (Beckman Coulter FC500, Brea, CA; A). Mitochondrial membrane potential was estimated using fluorescent probe JC-1 (Beyotime, Nanjing, China; B). Intracellular ATP concentrations were assayed with an ATP assay kit (Nanjing Jiancheng Chemical Industrial Co. Ltd., Nanjing, China; C). Phosphorylation of caspase 3 (p-caspase 3) was determined by Western blot (D). Data were the mean $\pm$ SEM of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Bruckmaier and Wellnitz, 2017). In the current study, staphylococcal enterotoxin M impaired the integrity of mammary epithelial cells, which may be attributed to cell necrosis, apoptosis, or both. A further important finding of this study was that staphylococcal enterotoxin M induced apoptosis of the bovine mammary epithelial cells. In our previous study, staphylococcal enterotoxin M caused intestine dysfunction via inflammatory cell invasion and loss of epithelium, which was in consistent with the present observation (Zhao and Tang, 2018). Cell apoptosis and production of ROS are closely related. Excessive ROS can cause cell apoptosis through activating Fas and the caspase cascade, which is induced through mitochondrial damage (Maiuri et al., 2007). Staphylococcal enterotoxin M enhanced ROS production and caspase-3 activation. Thus, the disruption was probably related to oxidative stress and mitochondrial damage with decreased mitochondrial membrane potential and intracellular ATP concentration. Oxidative response, cell apoptosis, autophagy, and

inflammation constitute the major defense networks in cells against pathogens (Tang et al., 2014). On the other hand, our results indicated the impairment of mammary epithelial cells by staphylococcal enterotoxin M was independent of autophagy as evidenced by no obvious variation of LC-II/I or p62.

CONCLUSIONS

Staphylococcal enterotoxin M induced inflammation of mammary epithelial cells and further impaired epithelial cell viability by ROS production, cell necrosis, and apoptosis, which was independent of cell autophagy.

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Figure 7. Autophagy of mammary epithelial cells. The mammary gland epithelial cells were exposed to 0, 1, 100, and 10,000 μg/L staphylococcal enterotoxin M for 48 h. The contents of LC3 I/II (abcam, Cambridge, MA; B), p62 (Proteintech, Rosemont, IL; C), and phosphorylation (p) of mammalian target of rapamycin (mTOR; D) were assessed using Western blot. Data were the mean ± SEM of 3 independent experiments.

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