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RESEARCH ARTICLE

ESBL-Producing *Escherichia coli* from Bovine Mastitis Induced Apoptosis of Bovine Mammary Epithelial Cells Via Alteration of ROS/MMP/bax/bcl-2 Signaling Pathway

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A B S T R A C T

Bovine mastitis is a common disease which causes important economic losses for dairy industry. *Escherichia coli* (*E. coli*) is one of major pathogens for both clinical and subclinical bovine mastitis. The persistent use and unwarranted application of antimicrobials has induced dynamic and continuous production and mutation of β-lactamases in extended-spectrum β-lactamase producing *E. coli* (ESBLEC). However, the pathogenic pathway of ESBLEC on bovine mammary epithelial cells (bMECs) is unknown. Aim of the present study was therefore to determine the apoptotic effects and mechanism of bovine ESBLEC on bMECs. Fifty-seven (14%) *E. coli* strains were isolated and identified from 400 clinical mastitic cows, and 20 (35%) strains of ESBLEC were confirmed by double-disc synergy testing and PCR. The apoptosis of bMECs infected with *E. coli* was analyzed with Annexin V/PI detection kit and TUNEL assay. Intracellular ROS generation and MMP were detected with commercial kits, respectively. The protein analysis of bax, bcl-2 and cleaved caspase-3 were determined by western blotting. Morphological changes were observed by TEM. After infection with ESBLEC, the apoptosis of bMECs increased significantly ($P<0.05$) as compared with the blank control group (bMECs were cultured without bacteria), evidenced by the decreased MMP, ROS level overproduction, upregulated expression of bax/bcl-2 ratio, cleaved caspase-3, and increased TUNEL positive cells. In addition, bMECs infected with ESBLEC manifested specific ultrastructure features like cytoplasmic cavitation, swollen mitochondria, cytomembrane disruption, and appearance of apoptotic bodies. Thus, ESBLEC has the capability to impart significant damages to the bMECs resulting in apoptosis via ROS/MMP/bax/bcl-2 pathway.

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INTRODUCTION

Mastitis is a common disease in dairy cows and is the cause of important economic losses for the dairy industry (Liang *et al.*, 2017). Mastitis causing bacteria exist everywhere, such as on the skin and in the environment of cattle, indicating that mastitis cannot be eradicated but only controlled. Bovine mastitis is caused mainly by miocrobial infecton (Ruegg, 2017). The use and misuse of antimicrobials have already contributed to the appearance of antibiotic-resistant strains (Cheng *et al.*, 2019). There is an urgent need to develop better methods to control the mastitis.

Escherichia coli (*E. coli*) is one of the leading causative agents of bovine mastitis worldwide (Blum and Leitner, 2013; Gao *et al.*, 2017). Some *E. coli* can produce extended spectrum beta-lacatamase (ESBL) encoded on a plasmid (Ali *et al.*, 2017). ESBLs can hydrolyze antibiotics such as 3rd generation cephalosporin,

monobactams, and carbapenems (Bush, 2018). Based on protein homology, ESBL-producing *E. coli* (ESBLEC) can be sorted as TEM, SHV, CTX-M, OXA, with TEM and SHV being the common types (Shahi *et al.,* 2013; Timofte *et al.,* 2014; Ali *et al.,* 2016). Increasingly, ESBL-producing *Enterobacteriaceae* are posing risks to the public health (Tansarli *et al.,* 2014), including high mortality, long hospitalization times, and high treatment costs (Maslikowska *et al.,* 2016). A recently report suggested that three quarters of healthy Thai people has been isolated ESBLEC, and transmission of ESBL genes can from animals to human via food chain (Boonyasiri *et al.,* 2014).

Previous studies indicated that *E. coli* could induce cells occur apoptosis via different pathways, such as MAPK pathway, Fas/FasL pathway and PERK-CHOP pathway (Wang *et al.,* 2011; Lu *et al.,* 2017; Zhao *et al.,* 2017). The increasing of apoptosis marker, TRPM-2, during *Streptococcus agalactiae* induced mastitis indicated that mammary gland epithelial cells died through apoptosis during bovine mastitis induced by Gram-positive pathogens (Bayles *et al.,* 1998). Apoptosis is regulated by a cascade of signaling events involving the relation of many factors. Former research has been reported that *E. coli* could induces apoptosis in mammary cells of cow (Long *et al.,* 2001). However, the pathogenic pathway of ESBLEC isolated from bovine mastitis of Chinese large dairy herds on bovine mammary epithelial cells (bMECs) is still unknown. Therefore, the patho-genesis of ESBLEC needs to be further explored. Aim of the present study was therefore to detect the apoptotic effect and mechanism of bovine ESBLEC on bMECs.

MATERIALS AND METHODS

Ethics statement: The present study was conducted in compliance with guidelines of the Beijing municipality on the Review of Welfare and Ethics of Laboratory Animals, and approved by the Beijing Municipality Administration Office of Laboratory Animals (BAOLA) and by the China Agricultural University Animal Ethics Committee (protocol CAU-AEC-2010-0603).

Clinical mastitis milk sample collection, isolation and identification of *E. coli*: A total of 400 quarter milk samples from 400 clinical mastitic cows were collected over a period of four years (2016-2019) from 21 large dairy herds (>1000 lactating cows) in 9 provinces and municipalities of China for *E. coli* isolation and identification. Milk samples were collected following NMC guidelines (Hope, 2010). Samples were collected in sterile containers (50 mL) and transported on ice to the mastitis laboratory at the China Agricultural University (Beijing, China). *E. coli* were identified with the guildeline of Ali *et al* (Ali *et al.,* 2017). The confirmed *E. coli* isolates were stored in brain heart infusion broth (BHI; Sigma-Aldrich) containing 30% glycerol at -80°C.

Polymerase chain reaction (PCR): Genomic DNA from suspected *E. coli* was isolated by the TIANamp bacteria DNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. PCR was used for the detection of partial 16S rRNA sequence (Ali *et al.,* 2017).

ESBLEC identification: *E. coli* isolates were phenotypically screened to identify ESBL-producers on Mueller-Hinton agar (MHA) containing cefotaxime (1 mg/L). The presumptive ESBLEC was further confirmed by double-disc synergy testing in accordance with recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2014).

Cultivation of bMECs: Bovine mammary epithelial cells (Shanghai Jingma Biological Technology Co., Ltd. China) were cultivated in DMEM with high glucose (HyClone, logan city, Utah state, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37°C with 5% CO_2 in cell culture plates (Corning, NY, USA).

LDH cytotoxicity analysis: LDH cytotoxicity of ESBLEC on bMECs was assessed using the LDH assay kit (Beyotime Institute of Biotechnology, Beijing, China) and following the manufacturer's instruction.

Apoptosis of bMECs infected with *E. coli* was analyzed with TUNEL assay: To detect the induction of apoptosis in bMECs by the 5 ESBLEC isolates, the bMECs were seeded in confocal petri dishes, when the confluency reached ~80%, medium was added, with MOI=1 and MOI=10 ESBLEC as treatment groups, and MOI=0 as the control group. The bMECs were then incubated for 1 h, 3 h and 6 h in the cell culture incubator (Cat: C1082, Beyotime, China). After treatment, the bMECs were treated following the manufacturer's instruction. Images were captured using a laser scanning confocal microscope (Leica, Germany).

Annexin V/PI assay: Apoptotic bMECs counting was detected using the Annexin V-FITC apoptosis detection kit (Cat: C1063, Beyotime, China) and following the manufacturer's instruction. Finally, apoptosis was detected by using a flow cytometry (BD, Becton, Dickinson and Company).

Reactive oxygen species (ROS) measurement: ROS was detected according to the manufacturer's instructions (Cat: S0033, Beyotime, China).

Detection of MMP: The MMP was investigated with JC-1 (Wang *et al.,* 2008) follow the manufacturer's instructions (Cat: C2006, Beyotime, China).

Western blotting analysis: Western blotting was performed as previously described (Kurien and Scofield, 2006).

Detection of transmission electron microscope (TEM): TEM was performed with the method of Han *et al.* (2017).

Statistical analysis: Data is represented as the mean ± standard deviations. Statistical analyses were performed using statistical software (SPSS Inc., Chicago, IL, USA). Difference between control and the treated groups were evaluated by one way – ANOVA and determined significant if $P<0.05$.

Table 1: ESBLEC identified from bovine mastitis milk samples in 21 dairy herds located in nine provinces of China

Province	No. of dairy herd	No. of milk samples	No. of *E. coli* isolates	ESBLEC from each herd
Anhui	AH1	21	4(4)	0
Beijing	BJ1, BJ2	14/20	2(1/1)	1(0/1)
Hebei	HB1, HB2, HB3	20/30	6(0/2/4)	2(0/0/2)
Henan	HN1, HN2	12/13	4(2/2)	0(0/0)
Heilongjiang	HL1, HL2, HL3	18/23/19	8(2/4/2)	2(0/2/0)
Inner Mongolia	IM1, IM2, IM3, IM4	20/23/26/17	17(4/5/5/3)	8(2/2/3/1)
Liaoning	LN1, LN2	16/20	6(2/4)	2(1/1)
Shandong	SD1, SD2, SD3	14/18/25	8(3/2/3)	4(1/2/1)
Tianjin	TJ1	14	2(2)	1(1)
Total	21	400	57(14%)	20(35%)

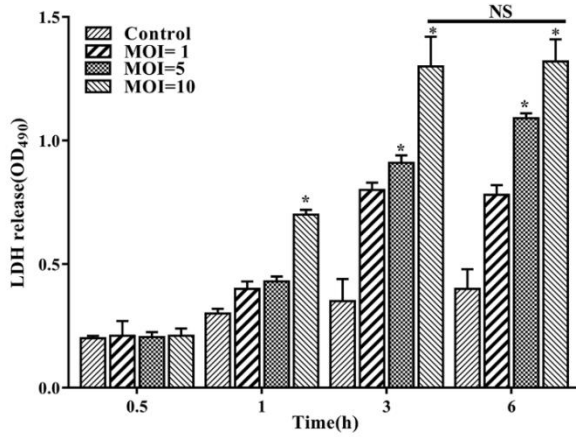

Fig. 1: LDH release of bMECs caused by 5 ESBLEC isolates. LDH level is upregulated after 1h in the MOI=10 group. Bars with a * symbol represents it has a significantly difference with control group (P<0.05); NS represents nonsignificant difference with control group.

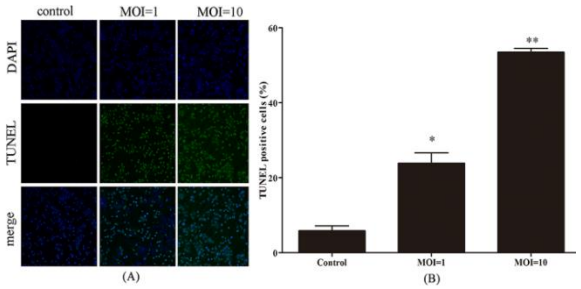

Fig. 2: Apoptosis condition detected by TUNEL method. (A) Image of confocal laser scanning microscopy（20×）at 3h. (B) The quantification of DNA fragmentation is presented as histogram. Bars with a * symbol represent significant differences with control group (P<0.05); bars with a ** symbol represent a significant difference with control group (P<0.01).

RESULTS

Isolation, identification of *E. coli* and ESBLEC: A total of 57 (14%) *E. coli* strains isolated from 400 milk samples of 120 clinical mastitic cows were collected from 21 large dairy herds (>1000 lactating cows) in nine different provinces of China included Anhui, Beijing, Hebei, Heilongjiang, Henan, Inner Mongolia, Liaoning, Shandong, Tianjin. The detail distribution of *E. coli* strains from different herds of the nine provinces are shown in Table 1. The inhibition zone of cefotaxime was ≤27 mm, while ceftriaxone ≤25 mm, ceftazidime ≤22 mm, and aztreonam ≤27 mm, indicating ESBLEC. Furthermore, the double paper adjacent test demonstrated an inflated inhibition zone, characteristic for ESBLEC. Finally, 20 (35%) isolates were found positive for ESBLs by phenotypic confirmatory tests. Eight and four ESBLEC phenotypes were isolated from dairy herds of Inner Mongolia and Shandong provinces, respectively. The rest of ESBL phenotypes were relatively identified from the herds of Hebei (*n*=2), Heilongjiang (*n*=2), Liaoning (*n*=2), Beijing (*n*=1) and Tianjin (*n*=1) provinces (Table 1). 5 of 20 recently randomly selected ESBLEC isolates have been separated for the next steps.

Evaluation of LDH cytotoxicity of bMECs infected with ESBLEC: LDH levels were up-regulated in all 5 ESBL *E. coli* isolates MOI=10 group (Fig. 1) after 1 h compared with the control group, with the level of LDH increasing over time.

Analysis of ESBLEC infected apoptosis of bMECs: The apoptosis status in bMECs was evaluated by TUNEL staining using the confocal laser scanning microscope. As showed in Fig. 2A, the ratio of DNA fragmentation increased and appeared to be concentration-dependent after bMECs were exposed to the 5 ESBLEC. There were more apoptosis cells in the MOI=10 group than the MOI=1 group (Fig. 2B). The MOI=10 group had a higher positive apoptotic ratio than the MOI=1.

The percentage of apoptotic cells was significantly higher than the control group (P<0.05) after exposure with all 5 *E. coli* isolates (Fig. 3A-C). Meanwhile, the percentage of apoptotic cells in MOI=10 group was higher than the MOI=1 group. Furthermore, the ROS level was significantly increased when cells were exposed to the ESBLEC isolates (P<0.05), and this phenomenon became more pronounced with increasing numbers of bacteria. Apoptosis in bMECs showed a dose-dependent relationship (Fig. 3D-E). In addition, changes of mitochondrial membrane potential (MMP) was shown (Fig. 4A-C) to confirm the apoptosis in bMECs exposed to the ESBLEC isolates. The MMP in the ESBLEC exposed bMECs were significantly lower than the control group in a dose dependent manner with the MOI=10 group lower than the MOI=1 group.

Detection of apoptosis proteins in bMECs infected with ESBLEC: Activation of apoptosis is linked to the marker proteins such as bax/bcl-2 and caspase-3, cleaved caspase-3 and β-Tubulin. All of these were detected by western blotting (Fig. 5A-E) and their expression was quantified by Image J software (Fig. 5F-G). The expression of bax/bcl-2 was upregulated with time and dose-dependent (Fig. 5F). Cleaved caspase-3 shows an increase tendency with the dosage increased; however, as the time going on, at the MOI=10 group, showed a decrease trend at 3 h and an increase tendency at 6 h and finally reached a high expression (Fig. 5G).

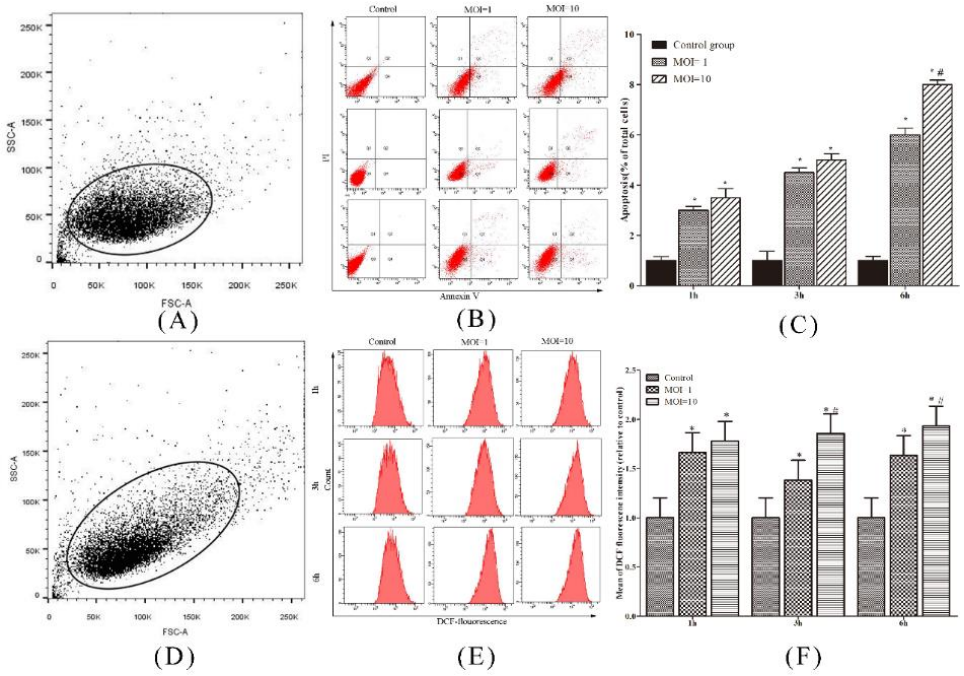

Fig. 3: Annexin V-PI and ROS production measured by flow cytometer as indicator of apoptosis in bMECs infected with 5 ESBLEC isolates. (A-B) Apoptosis ratio were upregulated with increasing MOI and time detected by flow cytometry. (C) The ratio has been quantified by a histogram. (D-E) Flow cytometer measures the content of intracellular ROS produced in bMECs infected with ESBLEC. (F) The quantification of intracellular ROS by a histogram. Bars with a * symbol represent significant differences with control group (P<0.05). Bars with a # symbol represent significant differences between MOI=1 and MOI=10 groups. Bars with a * symbol represent significant differences with control group (P<0.05). Bars with a # symbol represent significant differences between MOI=1 and MOI=10 groups.

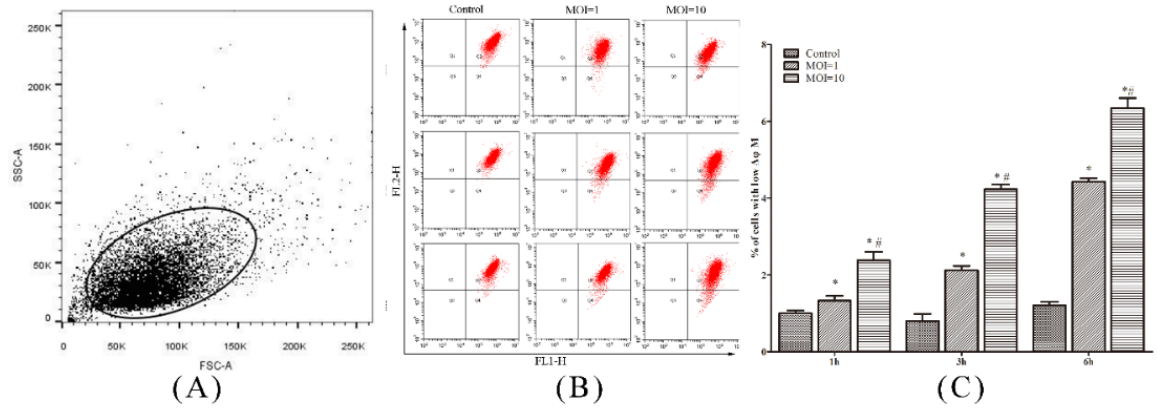

Fig. 4: The content of mitochondrial membrane potential (MMP) in bMECs infected with 5 ESBLEC isolates. After infected with ESBLEC, the MMP has decreased, which represent that the apoptosis occurred. With the MOI and time increased, the MMP decreased. (A-B) The flow cytometry image of MMP in bMECs infected with ESBLEC. (C) The ratio of low mitochondrial membrane potential has been quantized by a histogram. Bars with a * symbol represent significant differences with control group (P<0.05). Bars with a # symbol represent significant differences between MOI=1 and MOI=10 groups.

Morphological observation of apoptosis in bMECs infected ESBLEC: At MOI=10 the nucleus went through with nuclear membrane shrinkage, chromatin condensation and nuclear fragmentation (Fig. 6),morphological indications of apoptosis.

DISCUSSION

High prevalence of ESBLEC isolates was found in the present study, indicating the targeted research on this special group. Study on apoptosis mechanism in bMECs induced by ESBLEC has not been reported. Therefore, the current experiment was designed to determine the cytotoxic effect of LDH, the TUNEL staining, the ROS level, the ratio of Annexin V and MMP, the western blotting images of bcl-2, bax, caspase-3 and cleaved caspase-3, as well as to observe the morphological changes. Several (n=5) ESBLEC isolates were randomly selected to reduce the effects of experimental occasionality. On the whole, the study suggests that ESBLEC can induce apoptosis in bMECs through ROS/MMP/bax/bcl-2 axis.

The current study demonstrates that ESBLEC increase the amount of intercellular ROS in dose dependent manner. ESBLEC caused the loss of MMP in bMECs, possibly as a result of induced cellular injury. Intracellular ROS is in a dynamic equilibrium, with a lower concentration of ROS being essential for normal cellular signaling, and higher concentrations and long-time exposure of ROS resulting in damage to cellular macromolecules such as DNA, lipids and proteins, ultimately resulting in necrosis and apoptotic cell death (Singh *et al.*, 2019). This balance of ROS is negatively affected when the cell occurs *E. coli* to antimicrobial stress, infection, poisoning, and inflammation (Ung *et al.*, 2017; Hong *et al.*, 2019), with oxidative stress as a result. Mitochondrial dysfunction also can lead to ROS accumulation, which can be harmful for mitochondria (Jha *et al.*, 2017). MMP loss is an early initiation of apoptosis, and the main source of ROS is mitochondria (Zhang *et al.*, 2015). Therefore, the concentration of ROS reflects the level of damage to the mitochondria. In addition, the presented data indicates that apoptosis was increased by TUNEL stain in ESBLEC infected bMECs.

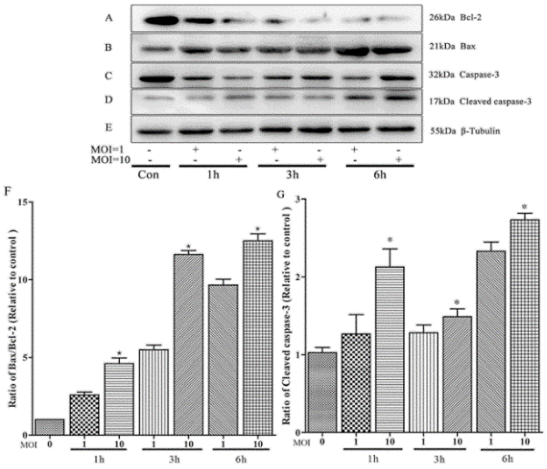

Fig. 5: Effects of ESBLEC on apoptosis protein marker levels of bMECs. (A-E) Western blotting image protein levels of bcl-2 (A), bax (B), caspase-3 (C), cleaved caspase-3 (D) and β-Tubulin (E) in bMECs treated with different supplements (+ represent supplying, - represents not supplying a certain amount of ESBLEC). (F) Quantitative analysis of bax/bcl-2 protein levels, and (G) Quantitative analysis of cleaved caspase-3 protein levels. Data represent means ± SD of three independent experiments. Bars with a * symbol represent significant differences between MOI=1 and MOI=10 groups (P<0.05).

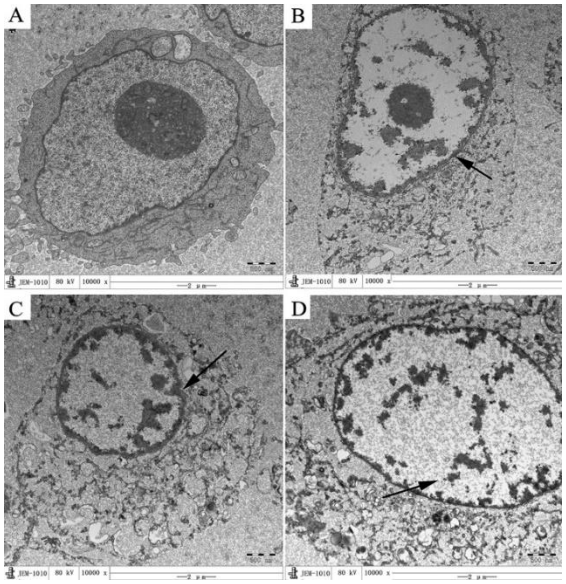

Fig. 6: Morphological features of apoptosis. Difference in bMECs morphology observed through transmission electron microscope between the control group (A) and the groups treated with ESBLEC (MOI=10) for 1h (B), 3h (C) and 6h (D). bMECs showed nuclear membrane shrinkage at 1h, depression at 3h, and complete degradation of contents at 6h.

In the present study, the bMECs morphology was observed by transmission electron microscope, shrinking nuclei characteristic of apoptosis were observed combined with the expression of cleaved caspase-3 and increased ratio of bax/bcl-2 after infected with ESBLEC, indicating induction of apoptosis, as previously reported (Del Puerto *et al.*, 2010; Peng *et al.*, 2016). Several studies indicated that bax, bcl-2 and caspase-3 are the key molecules involved in apoptosis in cancer cells (Del Puerto *et al.*, 2010; Peng *et al.*, 2016). Caspase-3 is a member of cysteine proteases family, which plays an essential role in the apoptotic process (Wall and McCormick, 2014). When cleaved caspase-3 actives, often accompanied by bax upregulating and bcl-2 downregulating (Thangarajan *et al.*, 2016).

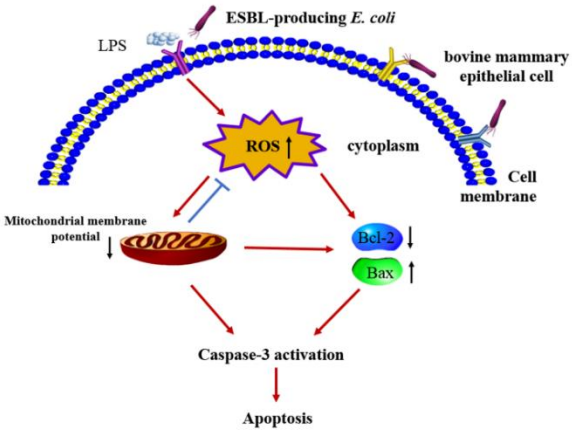

Fig. 7: Proposed signaling pathway of ESBLEC induced apoptosis in bMECs. ESBLEC increased bMECs intracellular ROS generation, mitochondrial superoxide overproduction and bax expression, decreased MMP, and activated caspase-3. The mediation of ESBLEC on the ROS/MMP/bax/bcl-2 pathway can be partially attributed to increased caspase-3 levels which promotes apoptosis.

The apoptosis process is associated with oxidative stress and MMP, with mitochondrial dysfunction showing up as an early sign of apoptosis (Bielaszewska *et al.*, 2013). MMP depolarization can lead to the changes in mitochondrial membrane permeability, eventually leading to the release of caspase-9 and ROS accumulation (Heaton *et al.*, 2013). Briefly, this approach can provide an evidence of apoptosis of ESBLEC in bMECs studies, offering the possibility to quickly obtain relevant information on the pathogenicity of isolates and on the progression of herd infections for a bovine mastitis of growing concern. ESBLEC increased bMECs intracellular ROS generation, mitochondrial superoxide overproduction and bax expression, decreased MMP, and activated caspase-3 and eventually promoted apoptosis (Fig. 7).

In the present study, we used 5 isolates to confirm ESBLEC can induce apoptosis in bMECs via ROS/MMP/ bax/bcl-2 pathway. However, apoptosis in bMECs induced by *E. coli* has various pathways to reveal the phenotype. Further study should be conducted to explore whether pathogenic effects and mechanisms of ESBLEC are the same *in vivo* or in other cell lines.

Conclusions: Twenty of 57 *E. coli* isolated from bovine clinical mastitis were defined as ESBL-producing isolates, indicating high prevalence of this multidrug-resistant phenotype. The cell infection model *in vitro* demonstrated that ESBLEC can induce apoptosis and related ultrastructural changes in bMECs. In addition, bMECs infected by ESBLEC had upregulated expression of bax/bcl-2 ratio and cleaved caspase-3. The findings of the current study suggest that ESBLEC has potential capability to damage the epithelial cells in mammary gland tissue when it causes bovine mastitis.

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Authors contribution: BH and JG conceived and designed the study. YS, PC, WH, XC and MS executed the experiment. YS, PC, WH, XC, MS, HB and JG analyzed the data. PC, BH, JG, JB and HB wrote, reviewed and edited the manuscript. All authors have approved the final version of the manuscript.

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