



Nocardia cyriacigeorgica from Bovine Mastitis Induced *In vitro* Apoptosis of Bovine Mammary Epithelial Cells via Activation of Mitochondrial-Caspase Pathway

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Nocardia is one of the causing agents of bovine mastitis and increasing prevalence of nocardial mastitis in shape of serious outbreaks has been reported from many countries. However, the mechanisms by which this pathogen damages the bovine mammary epithelial cells (bMECs) is not yet studied. Therefore, this study was designed with the aim to evaluate the apoptotic effects elicited by *Nocardia* and to investigate the pathway by which the *Nocardia* induce apoptosis in bMECs. Clinical *Nocardia cyriacigeorgica* strain from bovine mastitis was used to infect the bMECs for different time intervals, viz. 1, 3, 6, 12, and 18 h, and then the induced effects on bMECs were studied using adhesion and invasion assays, release of lactate dehydrogenase (LDH), apoptosis analysis by annexin V and propidium iodide (PI) double staining, morphological, and ultrastructural observations under scanning electron microscope (SEM) and transmission electron microscope (TEM), mitochondrial transmembrane potential ($\Delta\Psi_m$) assay using flow cytometry, and the protein quantification of mitochondrial cytochrome c and caspase-9 and caspase-3 by western blotting. The results of this study showed that *N. cyriacigeorgica* possessed the abilities of adhesion and invasion to bMECs. *N. cyriacigeorgica* was found to collapse mitochondrial transmembrane potential, significantly ($p < 0.05$) release mitochondrial cytochrome c and ultimately induce cell apoptosis. Additionally, it promoted caspase-9 ($p < 0.01$) and caspase-3 ($p < 0.05$) levels, significantly ($p < 0.01$) increased the release of LDH and promoted DNA fragmentation which further confirmed the apoptosis. Furthermore, *N. cyriacigeorgica* induced apoptosis/necrosis manifested specific ultrastructure features under TEM, such as swollen endoplasmic reticulum, cristae degeneration, and swelling of mitochondria, vesicle formation on the cell surface, rupturing of cell membrane and nuclear membrane, clumping, fragmentation, and margination of chromatin. The present study is the first comprehensive insight into patho-morphological ultrastructural features of apoptosis/necrosis induced by *N. cyriacigeorgica*, which concluded that the clinical *N. cyriacigeorgica* induced apoptotic changes in the bMECs through mitochondrial-caspase dependent apoptotic pathway.

Keywords: bovine mastitis, *Nocardia cyriacigeorgica*, bMECs, ultrastructural feature, mitochondria-dependent apoptotic pathway

INTRODUCTION

Nocardia species are gram-positive, aerobic, saprophytic, and widespread environmental actinomycetes, which have been reported as an opportunistic intracellular pathogen of human and animals (Sullivan and Chapman, 2010; Conville and Witebsky, 2011). *Nocardia* can cause localized or systemic nocardiosis with purulency or granulomas (Holland, 2010), which is probably transmitted by inhalation, ingestion or traumatic implantation, and can be disseminated through lymph and blood circulation (Ambrosioni et al., 2010). The most important species causing nocardiosis include *N. cyriacigeorgica*, *N. asteroides*, *N. brasiliensis*, *N. farcinica*, and *N. nova* (Ribeiro et al., 2008; Liu et al., 2011; Condas et al., 2013; Brown-Elliott et al., 2015; Hashemi-Shahraki et al., 2015). In human beings, the common manifestations of nocardiosis are pulmonary nocardiosis, central nervous system (CNS) nocardiosis, extrapulmonary nocardiosis, cutaneous, subcutaneous or lymphocutaneous nocardiosis, and nocardial bacteremia (Ambrosioni et al., 2010; Al Akhrass et al., 2011; Wilson, 2012). Whereas, in cattle, it is associated with farcy, abortion, pulmonary, and systemic nocardiosis (Beaman and Sugar, 1983; Bawa et al., 2010; Hamid, 2012). Nocardial bovine mastitis is the most important manifestation of nocardiosis and it has been reported from many countries (Dohoo, 1989; Hamid et al., 1998; Cook and Holliman, 2004; Brown et al., 2007; Pisoni et al., 2008; Ribeiro et al., 2008; Condas et al., 2013). Nocardial mastitis is characterized by the suppurative or granulomatous inflammation of the mammary gland followed an acute or chronic course (Böttig et al., 1989; Pisoni et al., 2008; Ribeiro et al., 2008). Moreover, its huge economic losses are mostly due to decrease milk production and culling of dairy cows (Cook and Holliman, 2004; Condas et al., 2013).

Bacterial adhesion and invasion are considered as important pathogenetic and virulence factors in the infection processes (Dego et al., 2002). Several *in vivo* and *in vitro* experiments demonstrated that *Nocardia* possessed the abilities to adhere to and invade into various types of cells, inducing cellular and tissue damages (Beaman and Beaman, 1998; Chapman et al., 2003; Beaman and Tam, 2008; Kohbata et al., 2009). When *Nocardia* attached to and rapidly penetrated through capillary endothelial cells (Beaman and Ogata, 1993), then entered the brain parenchyma, eliciting Lewy body inclusion in brain and Parkinson's symptoms in experimental animals (Chapman et al., 2003; Beaman and Tam, 2008). A previous study reported that *Nocardia* infection may induce macrophages and dendritic cells to differentiate into foamy cells (Meester et al., 2014). Furthermore, the invasion of *Nocardia* can even lead to the prevention of phagosome-lysosome fusion, inhibition of proteasome activity (Barry and Beaman, 2007), resistance to oxidative killing, blockage of phagosomal acidification, and alteration of lysosomal enzyme activity in macrophages (Beaman and Beaman, 1994). *Staphylococcus aureus* adhesion and invasion to bovine mammary epithelial cells (bMECs) has been proven to be the key events in the pathogenesis of bovine mastitis and the infected cells exhibited apoptotic morphology (Bayles et al., 1998; Dego et al., 2002); but for *Nocardia*, the adhesion and invasion ability to bMECs and the cell death effects are still unclear.

Cell death, the ultimate consequence of injury in host cells infection mainly includes apoptosis and necrosis. Necrosis is characterized by loss of cell membrane integrity, release of cellular contents and motivating inflammatory reaction; whereas, apoptosis is generally developed with cell membrane integrity, internucleosomal DNA fragmentation, formation of apoptotic bodies, no inflammatory reaction, and mediated through the intrinsic and extrinsic apoptosis pathway (Lamkanfi and Dixit, 2010). The extrinsic apoptosis pathways involve death receptors and caspase-8 signaling; whereas the intrinsic apoptotic pathway mainly targets the mitochondria (Lamkanfi and Dixit, 2010; Galluzzi et al., 2012).

In many bacterial pathogens, mitochondria-dependent apoptotic pathways have been well-recognized as a major pathogenic strategy (Ashida et al., 2011). During the process, apoptotic factors opens the mitochondrial permeability transition pore that ultimately result in loss of membrane potential and activation of cytochrome c (Yang et al., 2015; Xu et al., 2016). Then cytochrome c binds to apoptotic protease-activating factor I (Apaf-I) and tempts oligomerization. Consequently, apoptosome complex form by assembling of Apaf-1 oligomers with procaspase-9. Caspase-9 subsequently triggers apoptosis by caspase-3 activation (Elmore, 2007; Lamkanfi and Dixit, 2010). However, the specific mechanism through which capsase-9 activation triggers apoptosis in response to *N. cyriacigeorgica* in bMECs is unclear. *Nocardia* was shown to induce apoptotic death in dopaminergic cells, PC12 cells and HeLa cells; meanwhile, disruption of the mitochondrial membrane potential and caspase activation were involved in the apoptosis of HeLa cells (Barry and Beaman, 2007). However, the cell death effect of *Nocardia* on bMECs and the specific mechanisms involved in response to nocardial infection remain unknown.

Although, most of studies on *Nocardia* infections in various cells and laboratory animals were performed to demonstrate the pathogenicity and pathogenic mechanisms in central nervous system, respiratory system, and skin or cutaneous tissues (Barry and Beaman, 2007; Beaman and Tam, 2008; Meester et al., 2014; Lira et al., 2016). Nevertheless, there are rare studies focused on pathogenicity and mechanism underlying bovine mastitis caused by *N. cyriacigeorgica*. Therefore, the current study was designed with hypothesis that the *Nocardia* could adhere to and invade into bMECs, inducing apoptotic and necrotic cell death; in addition, *Nocardia* may regulate the cell apoptosis via mitochondrial-caspase pathway.

MATERIALS AND METHODS

Cell Culture

The bMECs line MAC-T was used in this study which was purchased from Shanghai Jingma Biological Technology Co., Ltd. China. Cells were cultured in DMEM/F-12 (HyClone, USA) supplemented with 10% heat-inactivated Gibco® Fetal Bovine Serum (FBS; HyClone, USA), 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C with 5% CO₂. Cells for adhesion and invasion assay were cultured in DMEM/F12 medium without antibiotics and while for other assays bMECs were cultured in DMEM/F12 medium with 4% FBS without antibiotics.

Bacterial Culture

N. cyriacigeorgica isolated previously from bovine mastitis was activated from frozen stocks by culture on tryptose soya agar (Difco™, Becton Dickison, Sparks, MD USA) supplemented with 5% defibrinated sheep blood and incubated at 37°C for 72 h, then sub-cultured in 7H9 broth to mid-log phase for the following experiments.

Adhesion Assay

Adhesion assay of clinical *N. cyriacigeorgica* to bMECs was performed according to previously described protocols (Pöhlmann-Dietze et al., 2000; Pereyra et al., 2016), with slight modifications as bMECs were infected with *N. cyriacigeorgica* at a multiplicity of infection (MOI, ratio of *N. cyriacigeorgica* to cells) of 50:1 for 10 min, 1, 2, and 3 h at 37°C with 5% CO₂. After incubation, the supernatants of infected cells were removed and cells were washed three times with phosphate buffer saline (PBS, pH 7.4) to remove non-adherent bacteria. Subsequently, cells were lysed by 1 mL PBS and 1 mL 1% Triton X-100 (0.5% v/v) to release adherent *Nocardia*. In control groups, both *Nocardia* suspensions (1 mL) and the cells were also treated with 1 mL Triton X-100. Finally, cell lysates and treated *Nocardia* supernatants were 10-folds serially diluted, plated onto sheep blood agar plates and incubated at 37°C for 48 h for enumeration of colony forming units (CFU). Adhesion rate of total *Nocardia* was expressed as:

$$\text{Adhesion rate of total } Nocardia = \frac{\text{Lysate of infected cells (CFU/mL)}}{\text{Lysate of } Nocardia \text{ supernatant and infected cells (CFU/mL)}} \times 100$$

The adhesion assay was repeated four times and each experiment was performed in quadruplicate.

Invasion Assay

In our previous study, we tested several dilutions of amikacin (25, 50, 75, and 100 µg/mL) with high concentration of *Nocardia* (1 × 10⁷ CFU/mL) and with different incubation time (1, 2, and 3 h). As a result, we found that 50 µg/mL of amikacin was enough to kill all *Nocardia* within 2 h; thus, this concentration of amikacin and incubation time was used for the following invasion assay. Invasion assay of *N. cyriacigeorgica* was performed according to the previous method with minor modifications (Beaman and Beaman, 1998; Pereyra et al., 2016). The bMECs were infected with *N. cyriacigeorgica* at a MOI of 50:1 for 10 min, 1, 2, and 3 h. Following incubation, cells were washed three times with PBS and treated with amikacin (50 µg/mL) for 2 h to kill extracellular *Nocardia*. The infected cells without amikacin treatment were used as control group. Then, cells were washed three times with PBS to remove non-adherent bacteria, further lysed with 0.5% Triton X-100 (v/v). Finally, cell lysates were 10-folds serially diluted for CFU determination. Invasion rate of adhered *Nocardia* was expressed as:

$$\text{Invasion rate of adhered } Nocardia = \frac{\text{Lysate of infected cells with amikacin treatment (CFU/mL)}}{\text{Lysate of infected cells without amikacin treatment (CFU/mL)}} \times 100$$

Invasion rate of total *Nocardia* was expressed as:

$$\begin{aligned} \text{Invasion rate of total } Nocardia &= \\ & \text{Adhesion rate of total } Nocardia \times \\ & \text{Invasion rate of adhered } Nocardia \end{aligned}$$

The invasion assay was repeated four times and each experiment was performed in quadruplicate.

Lactate Dehydrogenase (LDH) Release Assay

LDH, an enzyme, normally present in the cytoplasm and could release into the cell culture medium through damaged cell membrane after bacterial infection (Loeffler et al., 2004; Viguier et al., 2009). LDH release assay can be used to evaluate cytopathic effect of *N. cyriacigeorgica* on bMECs. Cells were infected with *N. cyriacigeorgica* at a MOI of 5:1 for 1, 3, 6, 12, and 18 h at 37°C with 5% CO₂. In addition, *Nocardia* suspensions in DMEM/F12 with 4% FBS and non-infected cells incubated for the same time were used as control. After incubation, the supernatants were collected and centrifuged at 18,000 g for 15 min. The supernatants were collected again for measurement of LDH release by cytotoxicity LDH Assay Kit-WST® (Dojindo Laboratories, Kumamoto, Japan). The absorbance at 490 nm was measured by a microplate reader.

DNA Ladder Analysis

As a characteristic feature for apoptotic cell death, DNA ladder was applied for the verification of cell apoptosis. The bMECs were infected with *N. cyriacigeorgica* in the same manner as described above. Moreover, cells without *Nocardia* treatment were used as control. Fragmented DNA was collected from cells according to the manufacturer's instructions of the DNA Ladder Extraction Kit (Beyotime, China). Moreover, this kit was also used to extract the DNA of *N. cyriacigeorgica* to determine the interference effect of *N. cyriacigeorgica* DNA. Finally, the DNA samples were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and photographed by gel documentation system (Alpha® Imager EC, SAN LEADRO, USA).

Apoptosis/Necrosis Analysis by Annexin V/Propidium Iodide (PI) Double Staining

Cell death was detected by the FITC Annexin V Apoptosis Detection Kit I (BD, USA) according to the manufacturer's instructions. *N. cyriacigeorgica* were incubated with bMECs at a MOI of 5:1 for 1, 3, 6, 12, and 18 h at 37°C with 5% CO₂. Cells without *Nocardia* treatment were used as the control groups.

After incubation, cells were harvested, washed twice with cold PBS and re-suspended in binding buffer at a concentration of 1×10^6 cells/mL, then 100 μ L of the cell suspension was stained by 5 μ L FITC Annexin V and 5 μ L PI. Following incubation for 15 min at room temperature, the samples were analyzed by the BD FACS Calibur flow cytometer (New Jersey, USA) within 1 h.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The bMECs adhered on coverslips were washed three times with cold PBS and fixed with 2.5% glutaraldehyde at 4°C for 1.5 h. After washing, cells were dehydrated through a graded series of ethanol (30, 50, 70, 80, 90, 100, and 100% ethanol) for 15 min in each at room temperature. Subsequently, cells were immersed into tert-butyl alcohol for 30 min. After lyophilization and gold coating, these cell samples were observed on a scanning electron microscope (Hitachi S-3000N, Japan).

For TEM, cells were harvested and the pretreatment of the cells before dehydration was similar to that for SEM. After dehydration by graded ethanol and acetone (three changes, for 10 min each), cells were sequentially embedded in epoxy resin-acetone mixtures (2:1) for 2 h and in pure resin overnight at 37°C. When the resin had polymerized, ultra-thin sections were cut by an ultramicrotome (Leica EM, Germany), stained with 1% uranyl acetate followed by lead citrate and viewed on a transmission electron microscope (Hitachi H-7650, Japan).

Mitochondrial Transmembrane Potential ($\Delta\Psi_m$) Assay

The bMECs were infected with *N. cyriacigeorgica* at a MOI of 5:1 for 1, 3, 6, 12, and 18 h at 37°C with 5% CO₂. Cells were collected for mitochondrial damage detection according to the changes of $\Delta\Psi_m$. The $\Delta\Psi_m$ was measured using a mitochondrial membrane potential assay kit with JC-1 (Beyotime, China) by flow cytometry. JC-1 was a dual-emission potential-sensitive probe and formed red-fluorescent aggregates in the mitochondria of the cells with higher potentials. But membrane potential collapse could result in the failure to red JC-1 in the mitochondria and the dye return to green-fluorescent monomer.

Western Blot Analysis

The bMECs were infected with *N. cyriacigeorgica* at a MOI of 5:1 for 1, 3, 6, 12, and 18 h at 37°C with 5% CO₂. Total proteins and cytoplasmic proteins without mitochondrion were respectively extracted from nocardial infected cells with RIPA lysis buffer (Beyotime, China) and Cell Mitochondria Isolation Kit (Beyotime, China). The protein concentrations were detected by the BCA method. Equivalent proteins from each sample were separated by SDS-PAGE gel, and then transferred onto PVDF membranes. Subsequently, the membranes were blocked in 5% BSA and incubated with the primary antibody for Cytochrome C (1:200, Santa, USA), caspase-9 (1:500, Santa, USA), caspase-3 (1:100, Santa, USA), and tubulin (1:1,000, Cell Signaling Technology, USA) overnight at 4°C followed by incubation with HRP-conjugated secondary antibody (1:5,000) for 1 h at room temperature. Finally, the bands were visualized using a BeyoECL Plus ECL Kit (Beyotime, China). Densitometric analysis of the

bands was quantified using Image J and these results were normalized using β -actin.

Apoptosis Analysis in Parallel with the Growth of Intracellular *N. cyriacigeorgica*

This experiment was carried out to evaluate the apoptosis and intracellular growth of *Nocardia* by killing the extracellular bacteria. The bMECs were infected with *N. cyriacigeorgica* at a MOI of 5:1 in DMEM/F12 for 2 h. Following co-cultures, cells were washed with PBS and treated with amikacin (50 μ g/mL in DMEM/F12) for 2 h to kill extracellular *Nocardia*. Then, cells were washed three times with PBS and cultured in DMEM/F12 medium with 4% FBS for 0 h (control group), 1, 3, 6, 12, and 18 h at 37°C with 5% CO₂ for the following steps: (i) for cell apoptotic analysis, cells were collected, after incubation, for apoptotic analysis by the FITC Annexin V Apoptosis Detection Kit I (BD, USA) as previously described; (ii) for total bacterial counting, the medium (1 mL) with infected bMECs was treated with 1 mL 1% Triton X-100 (v/v) for the CFU enumeration of total *Nocardia*. Simultaneously, the parallel infected bMECs were washed three times with PBS, further lysed with Triton X-100 and 10-folds serially diluted for CFU determination of the intracellular *Nocardia*. The number of *Nocardia* was expressed as Log₁₀ CFU/Well. The bacterial counting was repeated four times and each experiment was performed in quadruplicate; (iii) for observation of bacteria Gram staining was carried out. After washing with PBS, cells adhered on coverslips were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.2% Triton X-100 (v/v) for 10 min at room temperature. Finally, cells were washed, stained by Gram staining and observed under light microscope (Olympus, Japan).

Data Interpretation

Each experiment was repeated at least three times and the data were expressed as mean \pm standard deviation (SD). Statistical differences between groups were analyzed by One-way ANOVA followed by the Duncan and LSD multiple tests using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). *P*-value < 0.05 was regarded as statistically significant.

RESULTS

Adhesion and Invasion Assay

The preliminary quantitative study is shown in **Table 1**. The adhesion rate of total *N. cyriacigeorgica* increased from 4.5 to 42.8% within 3 h and this increase was time-dependent. Whereas, the invasion rate of total *Nocardia* also showed a time-dependent increase from 3.3 to 33.35% within 3 h. On the other hand, the invasion rate of adhered bacteria was persistent at a range of 64.4 to 77.9% within 3 h, indicating that more than 60% of the adhered *Nocardia* can invade into cells. Remarkably, these data showed the rapid adhesive and invasive capabilities of *N. cyriacigeorgica* with bMECs (**Table 1**).

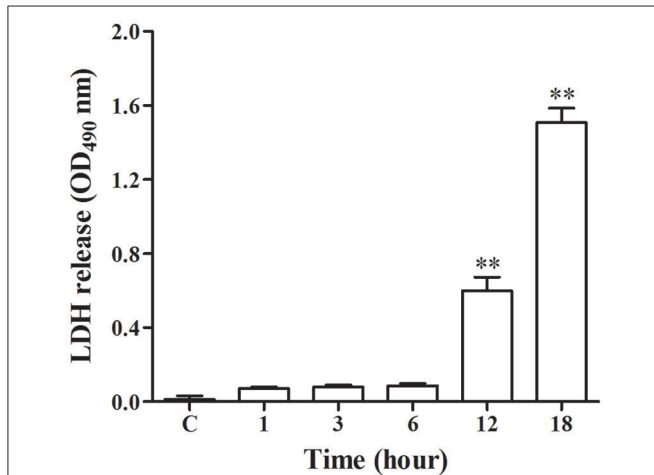
LDH Release Assay

As shown in **Figure 1**, there was release of LDH at 1, 3, and 6 h post-infection, however at 12 and 18 h post-infection there was a

TABLE 1 | Adhesion and invasion rate of *N. cyriacigeorgica* to bMECs.

Item	Rate (%)			
	10 min	1 h	2 h	3 h
Adhesion rate of total <i>Nocardia</i>	4.5 ± 0.7	22.0 ± 2.6	39.8 ± 2.4	42.8 ± 1.8
Invasion rate of adhered <i>Nocardia</i>	73.6 ± 8.0	64.4 ± 8.4	70.5 ± 8.5	77.9 ± 7.9
Invasion rate of total <i>Nocardia</i>	3.4 ± 0.8	14.3 ± 3.0	28.2 ± 4.9	33.4 ± 4.1

Invasion rate of total *Nocardia* = Adhesion rate of total *Nocardia* × Invasion rate of adhered *Nocardia*.

**FIGURE 1 | Lactate dehydrogenase (LDH) release assay in the medium.**

The LDH release assay was repeated three times and each experiment was performed in triplicate. Results were presented as Mean ± SD. ** $P < 0.01$ as compared to the control group.

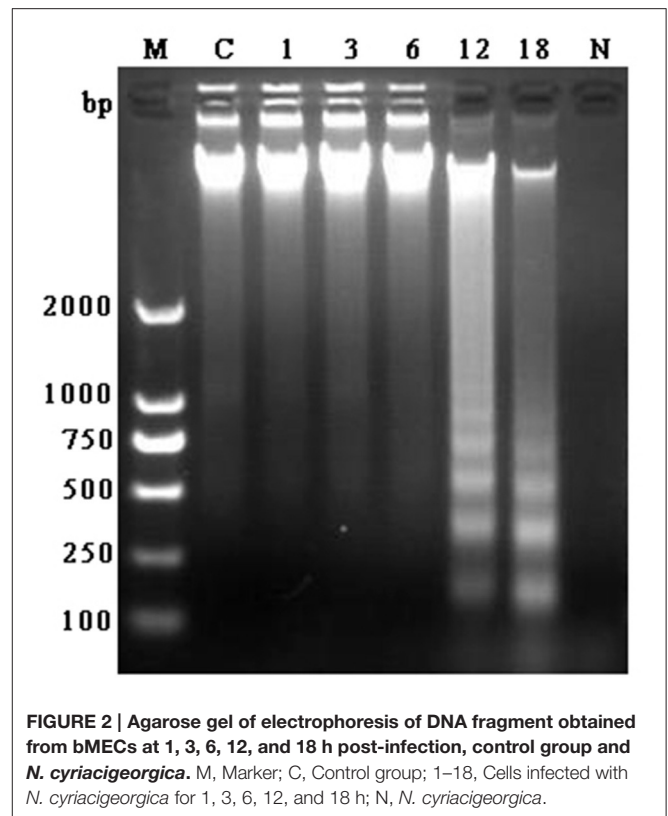
significant increase ($p < 0.01$) of LDH as compared with control group. This indicated that *N. cyriacigeorgica* seriously damage the cell membranes of bMECs with the passage of time.

DNA Ladder Assessment

DNA fragment is shown in **Figure 2**. There was no appearance of DNA ladder band in the bMECs of control group, 1, 3, and 6 h groups and also in *N. cyriacigeorgica* control group. But in the infected groups at 12 and 18 h, distinct typical DNA ladder bands were observed. These results indicated that *N. cyriacigeorgica* could induce apoptosis/necrosis in bMECs and the apoptotic DNA fragment was observed after 12 h post-infection.

Apoptosis/Necrosis Analysis

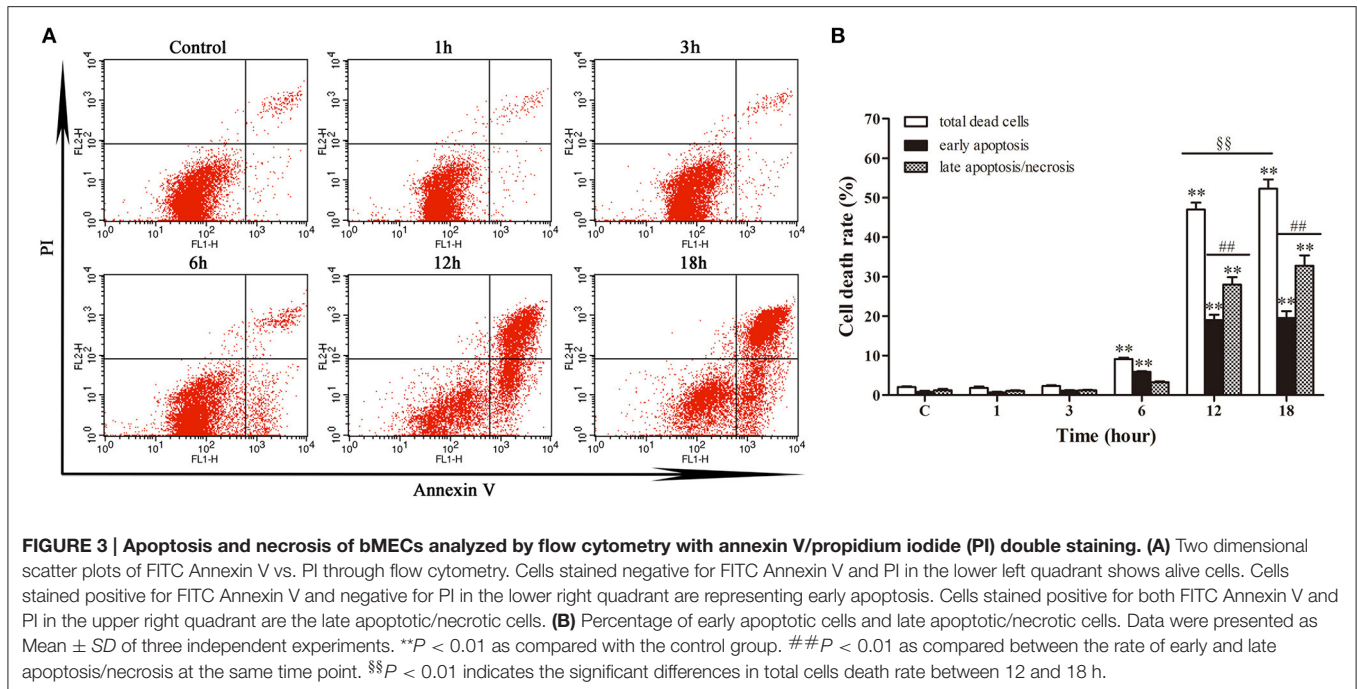
Annexin V/PI double staining was used to analyze the early apoptotic cells and late apoptotic/necrotic cells. **Figure 3** depicted limited apoptosis/necrosis at 1 and 3 h post-infection. The early apoptosis rate was increased significantly ($p < 0.01$) from 6 h post-infection compared with control group. There was a slight increase of apoptosis at 6 h post-infection; then the apoptotic/necrotic changes increased dramatically ($p < 0.01$) at 12 and 18 h post-infection in comparison with the

**FIGURE 2 | Agarose gel of electrophoresis of DNA fragment obtained from bMECs at 1, 3, 6, 12, and 18 h post-infection, control group and *N. cyriacigeorgica*. M, Marker; C, Control group; 1–18, Cells infected with *N. cyriacigeorgica* for 1, 3, 6, 12, and 18 h; N, *N. cyriacigeorgica*.**

control group. Furthermore, both at 12 h and 18 h, the late apoptosis/necrosis rate was much higher ($p < 0.01$) than early apoptosis rate. Additionally, the total death rate at 18 h (52.26%) was significantly higher ($p < 0.01$) than that at 12 h (46.99%). These data suggested that *N. cyriacigeorgica* could cause both apoptosis and necrosis, which was seriously intensified in time dependent manner.

Scanning Electron Microscopy (SEM)

To further confirm the adhesion, invasion, LDH release and cell death, SEM was used to test the interaction of bMECs infected with *N. cyriacigeorgica* and the cell damages. There were no morphological changes in control group (**Figure 4A**). At 6 h post-infection, morphological changes were observed as mild disruption of the some bMECs and short *Nocardia* filaments adhered on the surface of cells (**Figures 4B,C**). At 12 and 18 h post-infection, bMECs were obvious shrinkage, cytomorphosis, desquamation, and cell membrane breakage, with spherical protrusion appearance on some cell surface, abundant growing mycelium covering the cells and filaments penetrating into cell membrane both from the extracellular and intracellular (**Figures 4D–I**). Particularly, at 6, 12, or 18 h, SEM results demonstrated that microvilli on the cell surface were totally wrapped by *Nocardia* filaments (**Figures 4E,H,I**). Thus, these changes to the cellular morphology suggested that the penetration and viability of *N. cyriacigeorgica* may be responsible for aforesaid cell damages.



Transmission Electron Microscopy (TEM)

TEM was used to check the internalization of nocardial cells and ultrastructural changes in bMECs with *N. cyriacigeorgica* infection. The ultrastructure changes of infected cells are elaborated in **Figure 5**, which included swollen endoplasmic reticulum, cristae degeneration and swelling of mitochondria and also expansion of perinuclear space at 6 h post-infection (**Figures 5D–F**); while at 12 h of post infection, vesicle formation on the cell surface, rupturing of cell membrane, and nuclear membrane, chromatin clumping, fragmentation and margination of chromatin were noted (**Figures 5G–I**). Finally, serious disruption of cells, loss of organelles, and intramitochondrial dense granules accumulation were observed, at 18 h post-infection (**Figures 5J–L**). Furthermore, *Nocardial* cells were found both in cytoplasm and nucleus. These observations showed a process of progressive cell necrosis induced by clinical *N. cyriacigeorgica* infection.

Mitochondrial Transmembrane Potential ($\Delta\Psi_m$) Assay

Following a relatively stable state of $\Delta\Psi_m$ in the early stage (1–6 h) of nocardial infection, there was a remarkable decrease ($p < 0.01$) of $\Delta\Psi_m$ in the late stage of infection *viz.* 12–18 h in comparison with the control group (as shown in **Figure 6**).

Western Blot Analysis

The western blotting results of cytochrome c, caspase-9 and caspase-3 are depicted in **Figure 7**. A gradual increase in mitochondrial cytochrome c release was observed after 1 h post-infection ($p < 0.05$), with significantly higher caspase-9 activation ($p < 0.01$) from 1 h and markedly enhanced caspase-3 ($p < 0.05$) activation from 3 h of infection as compared to the control group.

Apoptosis Analysis in Parallel with the Growth of Intracellular *N. cyriacigeorgica*

The growth of intracellular *Nocardia* showed that the apoptosis rate was increased significantly ($p < 0.01$) from 12 h compared with control group (**Figures 8A,B**). At 12 and 18 h, most of the dead cells were at the early apoptotic stage ($p < 0.01$). In addition, the total death rate of bMECs at 18 h (19.70%) was significantly higher ($p < 0.01$) than at 12 h (15.80%). Strikingly, Gram staining showed that the *Nocardia* were short rod-shaped with no considerable growth during 1–6 h; nevertheless, they germinated into unique long mycelial form at 12 and 18 h, leading to pathomorphological changes of infected cells (**Figure 8C**). Bacterial counting presented an interesting result that there were no significant difference of the number of total *Nocardia* at different incubation time compared with control group. Similar results for the enumeration of intracellular *Nocardia* were also found within 12 h. This was in accordance with the apoptosis analysis, which presented that the apoptosis significantly increased at 12 and 18 h. The intracellular bacteria significantly decreased ($p < 0.01$) at 18 h compared with control group (**Figure 8D**). The unchanging number of *Nocardia* and the results of Gram staining revealed that both intracellular and extracellular *N. cyriacigeorgica* grew from small rods to mycelia rather than to reproduce during 18 h incubation (**Figures 8C,D**). During the robust growth phase of *Nocardia* at 12–18 h, cell apoptosis was aggravated remarkably (**Figures 8A–C**).

DISCUSSION

Bacterial adhesion and invasion played important roles in establishing infection. The quantitative analysis of adhesion and invasion of *N. cyriacigeorgica* and visualized results of

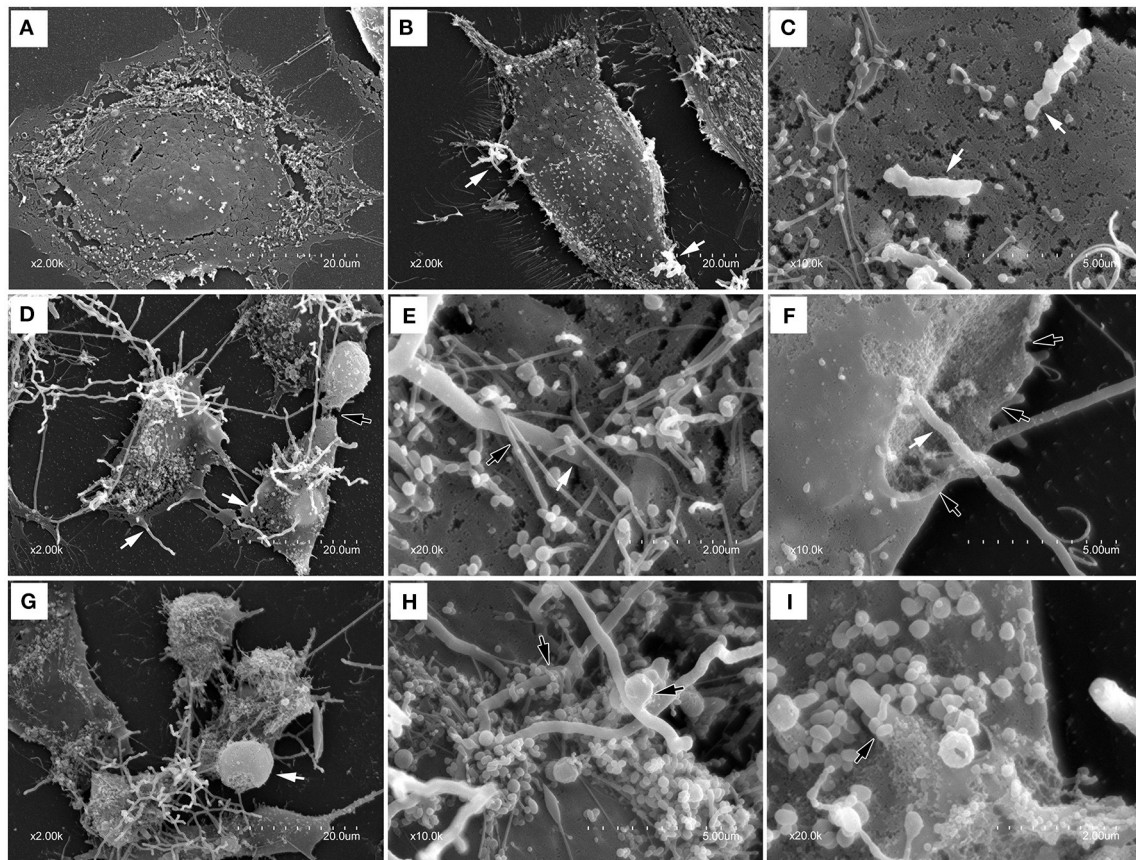


FIGURE 4 | Scanning electron photomicrographs showing interaction of bMECs infected with *N. cyriacigeorgica*. (A) demonstrates the normal cell without *N. cyriacigeorgica* infection that adherent to coverslip. (B) The cell with folding edge and several *N. cyriacigeorgica* filaments (white arrows) adhered on the cell surface at 6 h. (C) Apical attachment of *N. cyriacigeorgica* to cell surface at 6 h. (D) Atrophic and fragmented cells (black arrow) following long *N. cyriacigeorgica* filaments throughout cells (white arrows) at 12 h. (E) The white arrow showing *N. cyriacigeorgica* filament penetrating into the cell membrane and the black arrow presenting microvilli trapping *N. cyriacigeorgica* filament at 12 h. (F) A gap (black arrows) on the cell membrane with *N. cyriacigeorgica* filaments inside it (white arrows) at 12 h. (G) Shrinking cells coated with numerous *N. cyriacigeorgica* filaments. A nucleus-like spherical protrusion observed on the cell surface (white arrow) at 18 h. (H) Microvilli wrapped *N. cyriacigeorgica* filaments at 18 h. (I) A tip of intracellular *N. cyriacigeorgica* filament penetrated through the cell membrane at 18 h.

SEM and TEM showed that *N. cyriacigeorgica* possess the adhesion and penetration abilities. Significantly, we observed that *N. cyriacigeorgica* could promptly adhere to and penetrated into the bMECs within 10 min. These results were in accordance with a previous study which reported 68% adherence rate and 70.8% penetration rate of *N. asteroides* GUH-2 (*N. cyriacigeorgica* GUH-2) to endothelial cells in brain within 15–25 min exposure period (Beaman and Ogata, 1993). The findings of this study and some previous studies suggested that *Nocardia* has the capability of adhesion, invasion and viability within various host cells both *in vitro* and *in vivo* (Beaman and Ogata, 1993; Beaman and Beaman, 1994, 1998).

In the log-phase *N. cyriacigeorgica* depicted strong penetrative and adhesion ability than the stationary-phase and only live log-phase organisms can penetrate into cells (Beaman and Beaman, 1998). *Nocardia* can adhere to cells through surface adhesion molecules, live, and heat-killed *Nocardia* both can bind to the cell surface. Most of studies demonstrated that a filamentous tip associated 43-kDa protein of *N. asteroides*

GUH-2 played a dominant role in attachment and invasion to pulmonary epithelial cells and HeLa cells (Beaman and Beaman, 1998). The antiserum against the 43-kDa antigen inhibited apical adhesion and penetration to pulmonary epithelial cells, and prevented spread to the brain. Through gene sequence analysis of *N. cyriacigeorgica*, a series of putative mammalian cell entry proteins (mce) of *N. cyriacigeorgica* (Vera-Cabrera et al., 2013; Zoropogui et al., 2013) were found and these proteins were essential for pathogens to attach, enter and survive in the host cells (Zhang and Xie, 2011). Additionally, adhesion of *Nocardia* was associated with the cell surface structures, such as microvilli. Cell surface provides a direct connection with the bacteria. As evident from the SEM results, this presented that the microvilli of bMECs can capture *Nocardia*. This phenomenon was also observed previously on other cells (Beaman and Beaman, 1994, 1998).

After exposure to bMECs, *N. cyriacigeorgica* infection led to perforation in cell membranes, release of LDH, fragmentation of

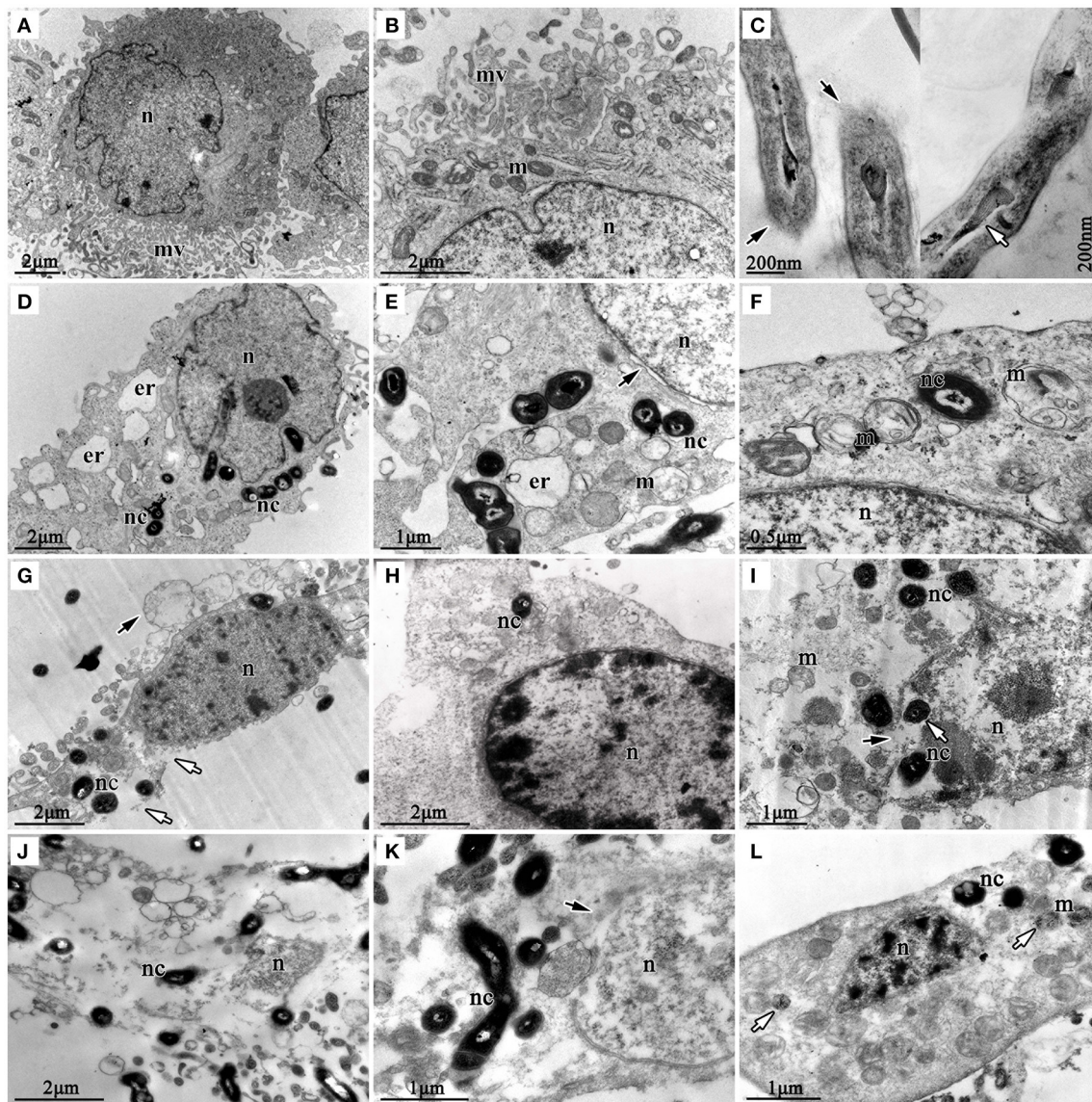
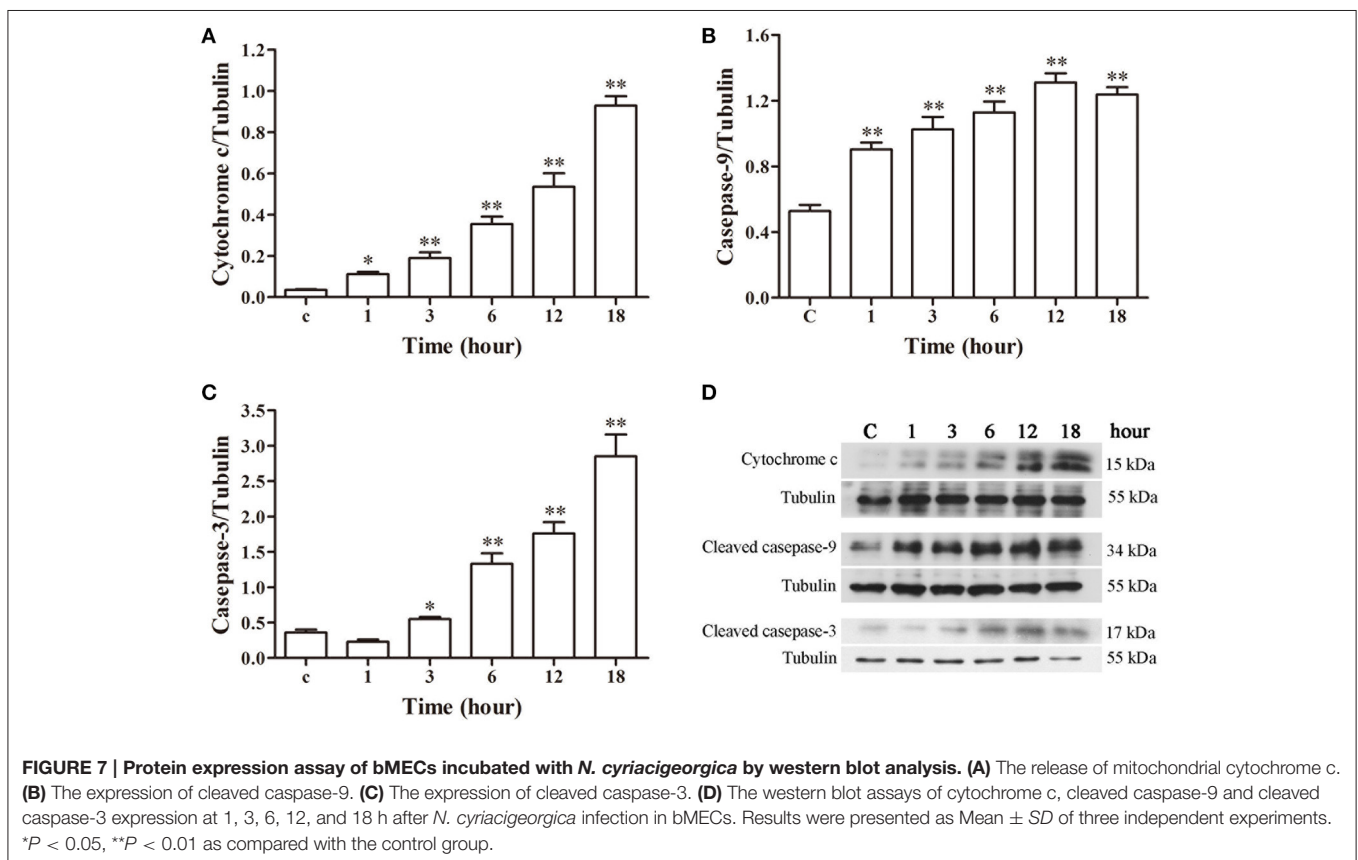
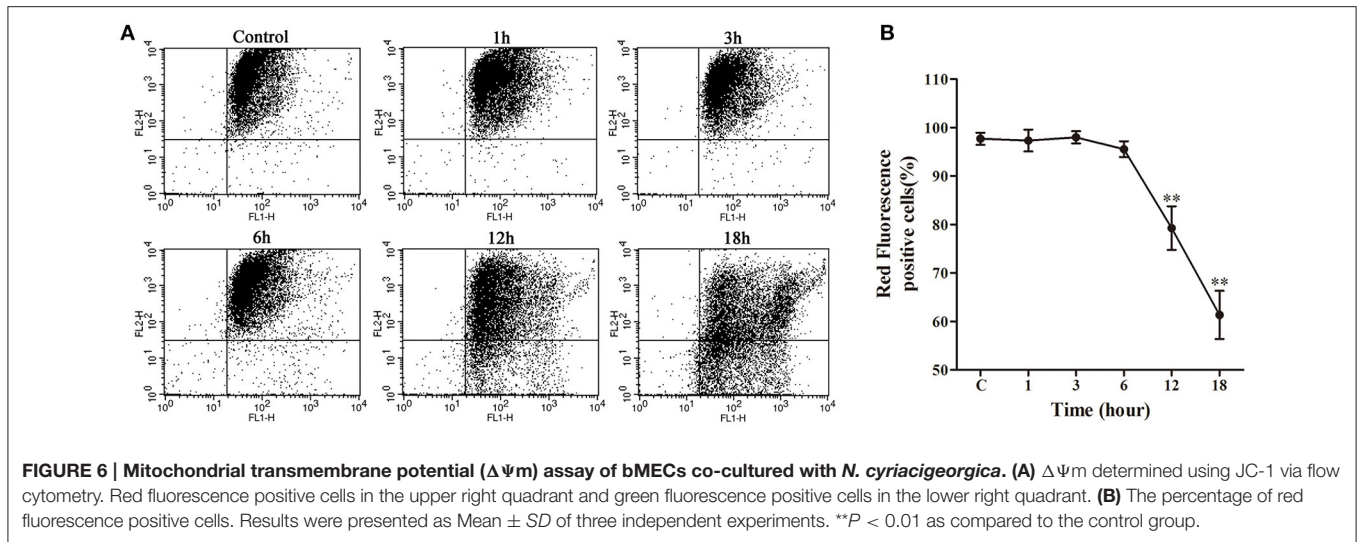


FIGURE 5 | Transmission electron photomicrographs showing ultrastructural pathological changes in bMECs co-cultured with *N. cyriacigeorgica*. (A,B) are the non-infected cells that show rich microvilli on cell surface and abundant mitochondria in cytoplasm, non-infected cells. (C) is the longitudinal sections of *N. cyriacigeorgica* incubated in DMEM/F12 with 4% FBS which depict the tips of log-phase *N. cyriacigeorgica* presenting hazy cell wall (black arrows) compared with that in the trunk. The white arrow is showing tubular-like nucleus of *N. cyriacigeorgica*. (D) Nocardial cells in cytoplasm and swollen endoplasmic reticulum at 6 h. (E) Flocculent densities in swollen and round mitochondria, expansion of perinuclear space (black arrow) at 6 h. (F) Mitochondria with electron density decreasing and crista degeneration at 6 h. (G) Vesicles on the cell surface (black arrow), chromatin condensation and fragmentation, cell membrane breakage (white arrows) at 12 h. (H) Chromatin condensation and accumulation at 12 h. (I) Mitochondria disruption, nuclear membrane rupture (black arrow); nocardial cells in cell nucleus (white arrow) at 12 h. (J) Cell disruption with loss of organelles at 18 h. (K) The nuclear membrane ruptures and disruption (black arrow) at 18 h. (L) Mitochondrial pyknosis and intramitochondrial dense granules accumulation, chromatin condensation and fragmentation, loss of microvilli (white arrow) at 18 h. nc-*N. cyriacigeorgica*, n-nucleus, m-mitochondrion, mv-microvillus, er-endoplasmic reticulum.

nuclear membrane, chromatin condensation and mitochondrial degeneration, which can caused untimely collapse of the entire cell. Previous studies suggested that the pathogenicity of *Nocardia* is dependent on the virulence factors and toxins. The putative virulence factors of *N. cyriacigeorgica* included catalase, superoxide dismutase, hemolysin, invasion, protease, mammalian cell proteins (mec), mycolic acids, nitrate reductase,

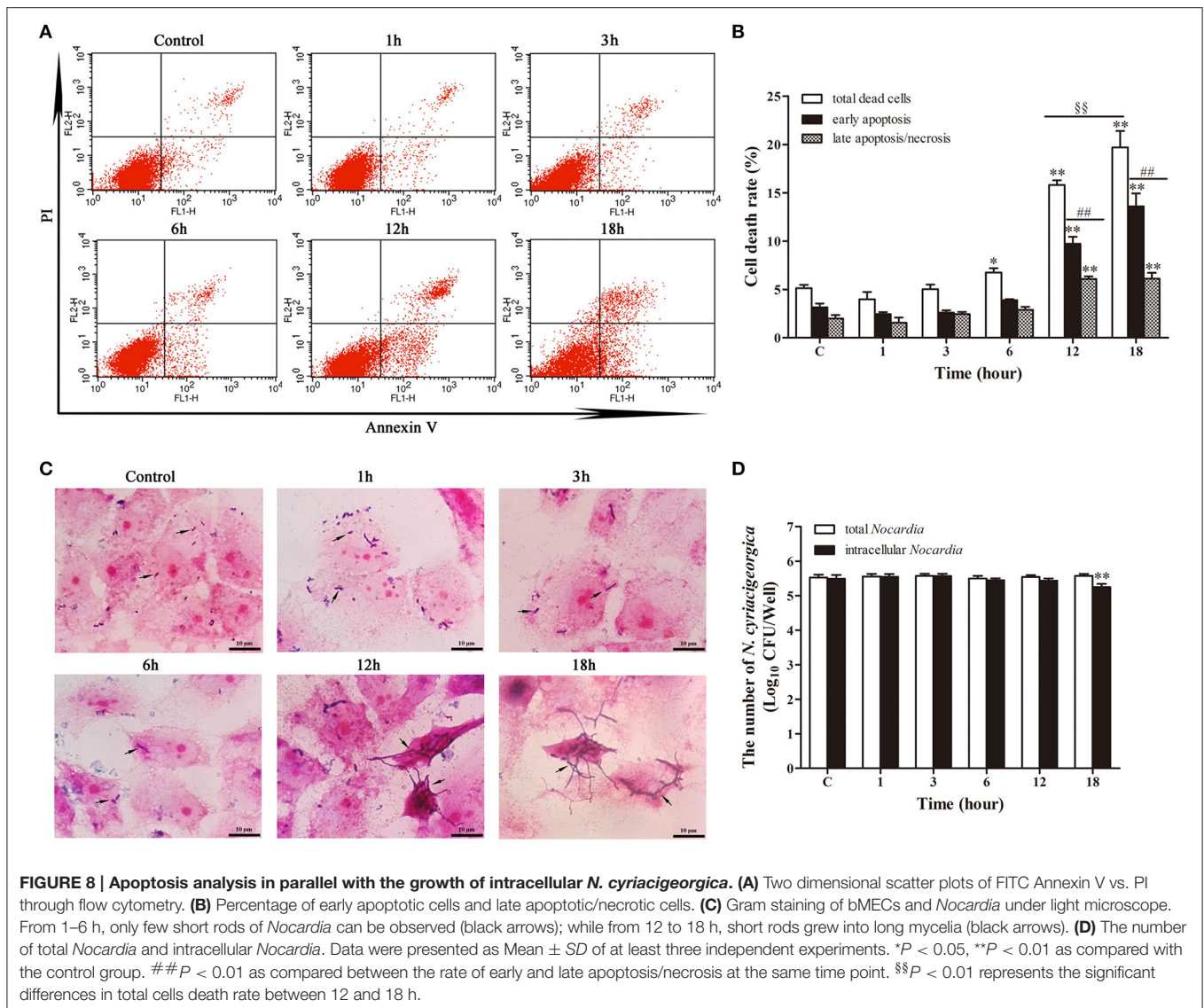
and PE/PPE/PGRS family proteins through gene analysis (Vera-Cabrera et al., 2013; Zoropogui et al., 2013), among most of them had been proved. Catalase and superoxide dismutase of *N. cyriacigeorgica* probably acted important roles in defense against deleterious superoxide and reactive oxygen species (ROS) during intracellular killing by phagocytes (Wu et al., 2006). The presence of nitrate reductase for



N. cyriacigeorgica suggested an ability to grow under low-oxygen conditions in stimulated macrophages (Zoropogui et al., 2013). Significantly, mycolic acids, the major and specific lipid components of cell envelope, have important implications in the pathogenesis of *Mycobacterium*, *Nocardia* and *Rhodococcus*, and *Corynebacterium* (Elamin et al., 2012; Verschoor et al., 2012). The cell wall-associated lipids of *Nocardia* induced the

production of the proinflammatory cytokines and inhibited important macrophage microbicidal effects (Trevino-Villarreal et al., 2012).

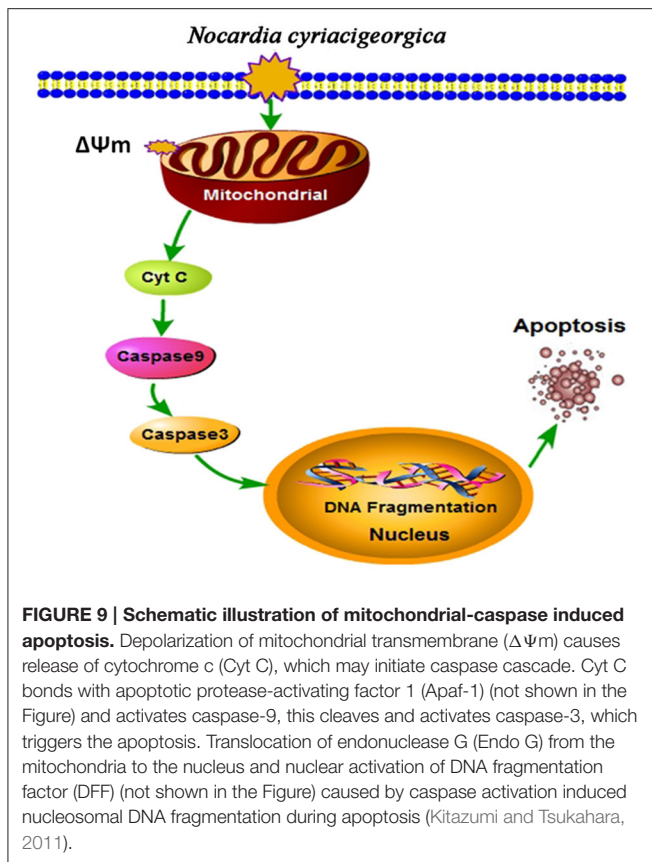
Bacteria often elicited cell apoptosis as a survival strategy. Pathogens have evolved a series of toxins and virulence factors to modulate host cell death (Lamkanfi and Dixit, 2010). During the first 6 h of *Nocardia* infection, there were only several



short nocardial filaments on the cell surface imparting mild morphological changes in bMECs, and there were also no obvious cytotoxic effects and $\Delta\Psi_m$, following no or low number of dead cells. Nevertheless, during the late stage of infection, short nocardial mycelia grew into long and strong mycelia, which can invade and penetrate into the bMECs, and even grew within cells; simultaneously, *N. cyriacigeorgica* was found to significantly elicit cell apoptosis, and exacerbated changes in cell morphology and ultrastructure, with a typical fragmentation of DNA and a collapse of $\Delta\Psi_m$ from 12 h. Besides, there was robust release of LDH from 12 h, which was associated with the serious cell membrane breakage and the large number of late apoptotic/necrotic cell in this period. A previous study demonstrated that live *Nocardia* can invade into cells and significantly increase in apoptosis rate in infected cells compared to heat-killed *Nocardia* (Barry and Beaman, 2007). Considering these above results, we can assume that the penetration and

aggressive growth of *N. cyriacigeorgica* mycelia might be one of the factors to induce death of bMECs. Additionally, on PC12 cells, *Nocardia* culture filtrate was found to induce apoptotic morphology after 24 h-treatment and increased release of LDH after 48 h (Loeffler et al., 2004), indicated that the metabolites of *Nocardia* are toxic to cells.

An extra experiment was performed to know the cytopathic and apoptotic effects of only intracellular *Nocardia*, which was coupled with study regarding the growth of intracellular bacteria. For this purpose the extracellular bacteria were killed with amikacin and apoptosis and growth of the intracellular bacteria was evaluated. Interesting, the results showed that apoptosis and bacterial growth have a parallel relationship. These effects were significantly exhibited during 12 and 18 h. Importantly, there was no significant changes in the total number of *Nocardia* but the bacteria grew into mycelial form, imparting cell damage and apoptosis. At 12 and 18 h of infection, a rapid



growth of *Nocardia* was noted without reproduction, which indicated that the aggressive growth of intracellular *Nocardia* may act as an important role in the cell injury leading to cell death.

These data suggested that *N. cyriacigeorgica* infection and invasion can induce both apoptotic and necrotic changes in bMECs after 6 h, corroborating with the previous findings in which *Nocardia* induced apoptosis in PC12 cells and HeLa cells (Tam et al., 2002; Loeffler et al., 2004; Barry and Beaman, 2007). In HeLa and PC12 cells, live *Nocardia* promoted a significant DNA fragmentation during 6 h-exposure period (Tam et al., 2002; Barry and Beaman, 2007). Previous studies also showed that *Nocardia* localized in substantia nigra and then underwent a rapid growth, inducing apoptosis of dopaminergic cells and failure of inflammatory response in 24 h (Kohbata and Beaman, 1991; Tam et al., 2002). Cell apoptosis is a double-edged sword both for host and bacteria during infection. For the host, cell apoptosis is a defense mechanism against pathogenic bacteria to prevent the release of intracellular bacteria and the spread of bacteria. Whereas, for bacteria, they induce cell apoptosis to block the spilling of cellular contents, to suppress the motivation of inflammatory reaction and then to evade host defenses. Several bacterial pathogens can cause cell apoptosis; whereas, many intracellular pathogens, such as *Mycobacterium tuberculosis*, can protect infected cells from apoptosis for its survival (Faherty and Maurelli, 2008; Butler et al., 2012). However, in the process

of infection, *N. cyriacigeorgica*, being an intracellular pathogen, caused apoptosis and necrosis in bMECs.

The ultrastructural pathology of TEM showed the mitochondrial damage. To further validate our results, the collapse of $\Delta\Psi_m$ and release of mitochondrial cytochrome c were determined, presenting a decrease of $\Delta\Psi_m$ and an increase of cytochrome c release. On the other hand, western blot analysis suggested the promotion of caspase-9 and caspase-3 activation. Likewise, similar effects on mitochondria and caspase-3 activity have been proved in the *Nocardia*-induced HeLa apoptosis at a MOI of 5:1 co-cultured for 5 h (Barry and Beaman, 2007). Altogether, from these findings we can draw the conclusion that *N. cyriacigeorgica* can induce apoptosis of bMECs mediated by a mitochondria-caspase dependent pathway. Mitochondrial dysfunction to release proteins from the intermembrane space into the cytosol is the pivotal focus in the process of apoptosis (Wang and Youle, 2009). As for apoptotic signaling, cytochrome c was a critical apoptogenic factor and was capable of initiating the caspase cascade (Kagan et al., 2009). Normally, cytochrome c is localized in the mitochondrial intermembrane space. During the infection of *N. cyriacigeorgica*, the opening of the mitochondrial permeability transition pore induced depolarization of mitochondrial transmembrane $\Delta\Psi_m$, release of apoptogenic factors and loss of oxidative phosphorylation (Tait and Green, 2010). Cytochrome c transposed from the mitochondrial membrane space to the cytosol; then bond to apoptotic protease-activating factor 1 (Apaf-1), triggered the formation of the apoptosome, inducing recruitment and activation of caspase-9. Caspase-9 cleaves and activates executioner caspase-3, which triggers the apoptosis (Ow et al., 2008; Tait and Green, 2010). Simultaneously, translocation of endonuclease G (Endo G) from the mitochondria to the nucleus and nuclear activation of DNA fragmentation factor (DFF) caused by caspase activation induced nucleosomal DNA fragmentation during apoptosis (Kitazumi and Tsukahara, 2011). DNA fragmentation is a hallmark of apoptosis and is one of the last consequences of apoptosis (Kitazumi and Tsukahara, 2011). The major schematic representation of mitochondrial-caspase induced apoptotic pathway studied in this work is elucidated in **Figure 9**.

In summary, these results of the present study demonstrated that clinical *N. cyriacigeorgica* was able to adhere and invade the bMECs, causing disruption of cell membrane and mitochondrial degeneration. Our data supports the hypothesis that *N. cyriacigeorgica* induced host cell death via an apoptotic mechanism and the later stage of *N. cyriacigeorgica* infection followed mitochondrial-dependent apoptotic pathway in bMECs. These findings provide that the apoptosis of bMECs was induced via triggering the caspase cascade and might present important insights into the mechanisms of *N. cyriacigeorgica* infections in bovine mammary tissues.

ETHICS STATEMENT

The present study was conducted in accordance with the ethical guide lines of China Agricultural University (CAU), Beijing.

Furthermore, prior to the initiation of this work, proper approval was granted by the departmental committee of College of Veterinary Medicine, CAU.

AUTHOR CONTRIBUTIONS

WC planned, performed the experiments, and then wrote manuscript. XG and YL performed the experiments, LZ and TA helped in analysis of flow cytometry experiments, GL collaborated in writing manuscript,

MS and TA revised and corrected the manuscript, JG analyzed the data and BH designed and evaluated the research. All authors have read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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