N-Acetylcysteine can Suppress Mitochondria-Dependent Apoptosis of Macrophages Caused by *Burkholderia pseudomallei* Infection

**Abstract**

Melioidosis, a zoonotic infection primarily caused by *Burkholderia pseudomallei* infection, suppresses the host immune response and induces apoptosis. Excessive Reactive Oxygen Species (ROS) can trigger mitochondrial-dependent apoptosis and oxidative stress may be an important mechanism in *B. pseudomallei*-mediated cytotoxicity. In this study, the changes of ROS in macrophage apoptosis induced by *B. pseudomallei* were investigated. Our results showed that *B. pseudomallei* infection (MOI = 0, 1, 5, 10) for 24 h induces ROS overproduction, regulating protein expression involved in oxidative stress and leading to mitochondrial apoptosis. Furthermore, pretreatment with antioxidant N-Acetylcysteine (NAC) for 2 h significantly reduced oxidative damage and mitochondrial damage overproduction. In summary, our results emphasize the effects of ROS in *B. pseudomallei* pathogenesis, and NAC inhibits mitochondria-dependent apoptosis of macrophages induced by *B. pseudomallei* infection by reducing ROS production.

**Keywords:** Burkholderia pseudomallei; Apoptosis; Mitochondrial damage; Oxidative stress; Infection

**Introduction**

Melioidosis is a systemic infectious disease and is mainly prevalent in tropical regions such as Southeast Asia, infected by *Burkholderia pseudomallei* [1-4]. *B. pseudomallei* has virulence factors that promote its survival inside host cells, resulting in high mortality rates associated with the disease [5-7]. Modeling estimates melioidosis worldwide at about 165,000 cases and 89,000 deaths annually [6,8]. Humans and animals can acquire *B. pseudomallei* infections naturally through broken skin, inhalation, or ingestion [9,10]. Bacteria can evade host innate immune defenses using their virulence factors and replicate within cells [11].

In studies using a mouse macrophage model, *B. pseudomallei* has been shown to be able to escape the phagosome using the Type III Secretion System (TTSS), replicate within the cytoplasm, spread between cells to infect neighboring cells, and ultimately lyse the host cell [6]. When *B. pseudomallei* invades phagocytic cells like neutrophils and macrophages, these cells activate defense mechanisms such as apoptosis to eliminate the bacteria [12]. ROS are closely involved in apoptosis [13]. During bacterial infection, neutrophils and macrophages recognize invading bacteria and rapidly produce ROS and Reactive Nitrogen Species (RNS) that render the engulfed bacteria inactive [1,14]. Mouse model studies of melioidosis have suggested that ROS plays a more significant role than RNS in controlling bacterial infection in phagocytic cells [15]. Excessive ROS accumulation can cause oxidative stress, resulting in damage to cellular organelles and triggering the apoptosis pathway [16,17].

ROS is a byproduct of oxidative phosphorylation that mainly occurs in mitochondria [18]. The balance of ROS metabolism is crucial for normal cellular function [19]. Under normal physiological conditions, the regulation of ROS levels relies on the antioxidant enzyme system, with SOD being the primary intracellular antioxidant enzyme [20]. Exogenous antioxidants like vitamin C and
glutathione can also be utilized to inhibit excess ROS production [21]. N-Acetylcysteine (NAC), as a precursor of reduced glutathione, produces an indirect antioxidant effect that reduces ROS levels in the body [22-25].

This study intends to construct a mouse macrophage (RAW264.7 cell) model of *B. pseudomallei* infection and investigate the host cell apoptosis mechanism using this model with the aid of the antioxidant NAC. Specifically, we focused on the mechanism of ROS in the apoptosis of RAW264.7 cells infected by *B. pseudomallei* and whether NAC can regulate the mitochondria-dependent apoptosis of macrophages induced by *B. pseudomallei* infection. Exploring the role of ROS in *B. pseudomallei*-induced cell apoptosis provides theoretical support for inhibiting *B. pseudomallei*-induced cell apoptosis using NAC, and presents novel therapeutic strategies for addressing *B. pseudomallei*-induced cell toxicity.

Materials and Methods

**Bacterial strains and Cell lines**

*B. pseudomallei* (HN Burkholderia pseudomallei 001) was isolated from a patient with melioidosis in Hainan (GenBank accession numbers CP038805) [26]. Bacterial culture was performed using Luria-Bertani (LB) medium. The bacterial growth curve and viable numbers were obtained at 37°C and 220 rpm for 16 h to 18 h, until the OD=0.88 [27]. RAW264.7 cell grown at 37°C, 5% CO₂ with 10% FBS in liquid LB medium at 37°C and 220 rpm for 16 h to 18 h, until the OD=0.88 [27].

**Cell viability and cell proliferation analysis**

Cell viability and cell proliferation analysis

Cell Counting Kit 8 (C0037, Beyotime, China) and BeyoClickTM Cell Counting Kit 8 (C0037, Beyotime, China) was used to detect cell viability and cell proliferation of RAW264.7 cells. The infected RAW264.7 cells were grown in a 10 cm dish (7 × 10⁶) for 24 h and then treated based on experimental needs after reaching 80% confluence. The pancreas digests and centrifuges the cells. Reagents were added as directed and incubated at Room Temperature (RT) for 15 min in darkness. We analyzed the cells using BD Accuri™ C6 Plus Flow Cytometry within 1 h and determined the apoptosis rate using FlowJo™ v10 software.

**ROS Measurement**

We measured ROS levels using the Reactive Oxygen Species Assay Kit (S0035S, Beyotime, China). RAW264.7 cells were grown in 24-well plates (2 × 10⁴). When the respective reagents were added as per instructions, they were observed under a fluorescence microscope or analyzed by BD Accuri™ C6 Plus flow cytometry.

**ATP and SOD measurement**

Based on previous studies, ATP levels were measured using an ATP Assay Kit (S0026, Beyotime, Nantong, China) [27]. Cell lysis with ATP lysis was first followed by ATP detection reagent and detected by Infinite 200 PRO Multimode Microplate Reader (TECAN, Switzerland).

The total SOD inhibition rate was determined by Total Superoxide Dismutase Assay Kit with NBT (S0109, Beyotime, China). RAW264.7 cells were grown in a 10 cm dish (7 × 10⁴) and treated as per experimental requirements when they reached 80% confluence. Cells were lysed with SOD lysis, 13,000 g, 4°C centrifuged for 5 min to collect the supernatant. 20 μL of supernatant was mixed with SOD working solution, incubated at 37°C for 30 min, and detected by Multimode Microplate Reader.

**Real-Time quantitative PCR of mtDNA**

RNA was extracted from the cells using TRIZOL Reagent (15596026CN, Thermo Fisher, USA), and cDNA was prepared according to the relevant protocols using HiScript III All in One RT Super Mix Perfect for qPCR (R333-01, Vazym E, China). Real-time quantitative PCR was performed using the ChamQ Universal SYBR qPCR Master Mix (Q711-02, VazymE, China). The RT-qPCR was performed using mtDNA primers as previously reported [29]. F: TGCGACATCATATTGGGAGGAGG, R: GTAGCGTCGTTGGTACTCCTGA, and GAPDH internal reference primer (F: GAGGGGCCCATCACAGCTTTC; R: CATCACATCTCCAGAGAGC).

**Indirect Immunofluorescence Assay (IFA)**

The infected RAW264.7 cells were washed with pre-cooled PBS, fixed in 4% paraformaldehyde for 15 min; permeabilized with 0.1%
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Triton X-100 for 10 min, and blocked with 1% BSA for 1 h [30]. Add COX IV Rabbit mAb (1:250 dilution) and Cyt C Antibody (1:20 dilution) and place at 4°C to incubate for 8 h to 12 h. After washing with PBST, Alexa Fluor 488-Labeled Goat Anti-Rabbit IgG (P0176, Beyotime, China) was mixed and incubated in darkness for 1 h. Stained samples were viewed and photographed on a fluorescence microscope (Nikon). The fluorescence intensity of COX IV and Cyt C was analyzed by Image J according to the obtained photos. The average fluorescence intensity was analyzed by GraphPad Prism v8.02.

Western blot analysis

RIPA lysate was used to lyse cells on ice, and the supernatant was centrifuged at 12,000 g for 30 min. Protein concentration was measured using a BCA protein quantitation kit (P0012S, Biyuntian, China). The protein samples were blended with 1X Loading Buffer (P0015, Biyuntian, China) and denatured at 95°C for 10 min in a metal bathtub. The protein samples were electrophoresed in 10% to 15% SDS-PAGE, then transferred to a polyvinylidene fluoride membrane. It was incubated with the indicated antibody at 4°C overnight. TBST was washed and then incubated with a secondary antibody for 1 h. Finally, the membrane was exposed to BeyoECL Plus (P0018S, Biyuntian, China) and imaged using the ChemiScope 6100 imaging system.

Transmission electron microscopy (TEM)

The infected RAW264.7 cells were washed with pre-cooled PBS. Followed by fixation with 2.5% glutaraldehyde overnight at 4°C and then fixation with 2% osmium tetroxide at RT for 1.5 h. It was embedded in uranium acetate/lead citrate slices, stained, and photographed using transmission electron microscopy.

Statistical analyses

All data are expressed as mean ± Standard Deviation (SD) of at least 3 independent experiments. Differences between treatment and control groups were analyzed using one-way ANOVA. P<0.05 was considered statistically significant unless otherwise stated.

Results

B. pseudomallei induced apoptosis in RAW264.7 cells, diminished by NAC

RAW264.7 cells were infected with B. pseudomallei at MOIs of 0, 1, 5, 10, 50, 200, and 1000, and found that the cell viability was significantly reduced (Figures 1A-1C). Further, apoptosis was detected. As a result, the infected cells showed a higher apoptosis rate (Figure 1D). These results indicated that B. pseudomallei infection caused apoptosis and increased in a dose-dependent manner.

Interestingly, when RAW264.7 cells were pretreated with NAC significantly enhanced the cell viability and EdU positive rate and reduced the apoptosis rate induced by B. pseudomallei (Figures 1E-1H). The results demonstrated that NAC could effectively reduce B. pseudomallei-induced apoptosis in RAW264.7 cells.

NAC inhibits oxidative stress induced by B. pseudomallei infection

As shown in Figure 2A-2C, we found that the ROS and the inhibition rate of SOD of B. pseudomallei-infected RAW264.7 cells was significantly increased in a dose-dependent manner, suggesting that B. pseudomallei infection induces oxidative stress in RAW 264.7 cells. It is worth noting that NAC pretreatment for 2 h significantly

Figure 1: NAC diminished the apoptosis caused by B. pseudomallei. (A) EdU detects cell proliferation. Proliferating cells were stained with Azide 555 dye (red) and the nucleus with DAPI dye (blue). (B, F) Statistical analysis of the EdU positive rate was performed. C. Cell viability of B. pseudomallei was detected using CCK8. (D, G) Analysis of apoptosis was performed using BD flow cytometry. E. Co-treatment of different NAC and B. pseudomallei concentrations was detected using CCK8.

*P<0.05, **P<0.01, ***P<0.001
reduced the ROS level and SOD inhibition rate induced by *B. pseudomallei*.

To address the role of NAC in oxidative stress induced by *B. pseudomallei*, the characterization of Bax, Bcl2, Caspase-3, Caspase-9, and p53 expression was further detected. As a result, the expression of p53, Caspase-9, and the activation of Caspase-3 showed a dose-dependent and significant increase in the *B. pseudomallei*-infected cells. Besides, the expression ratio of Bcl-2/Bax was found to decrease (Figure 3A, 3B). However, NAC pretreatment also significantly reduced the damage of apoptosis proteins induced by *B. pseudomallei* (Figure 3C, 3D). All these results implied that NAC protected against *B. pseudomallei*-induced oxidative stress.

**NAC inhibits mitochondrial apoptosis induced by *B. pseudomallei***

To determine if *B. pseudomallei* infection induced mitochondrial damage in RAW264.7 cells, we further investigated the structure of mitochondria in infected cells using TEM. *B. pseudomallei*-infected RAW264.7 cells showed mitochondrial swelling and disruption of the cristae (Figure 4A).

Furthermore, we found a significant decrease in mtDNA copy number levels and ATP levels (Figure 4B, 4C). It confirmed that *B. pseudomallei* infection induces mitochondrial damage. Whereas, pretreatment with NAC attenuated the mitochondrial damage caused by infection (Figures 4D-4F).

In addition, we found a significant increase in Cyt C expression and decrease in COX IV expression (Figure 5A, 5B). It confirmed that *B. pseudomallei* infection induces mitochondrial apoptosis. However, pretreatment with NAC can decrease Cyt C expression while increasing COX IV expression (Figure 5C, 5D). Therefore, these results indicated that NAC could inhibit mitochondrial apoptosis induced by *B. pseudomallei*.

**Discussion**

Due to the neglect of melioidosis, its clinical diagnosis becomes difficult, thus delaying the treatment. Without an effective vaccine, there is a need for alternative drugs for treating melioidosis [31]. In this study, our results demonstrate that *B. pseudomallei* infection induces mitochondrial-dependent apoptosis through oxidative stress, which can be alleviated by reducing intracellular ROS levels with NAC. These findings provide valuable insights into the development of a potential cure strategy for *B. pseudomallei* infection.

Research has demonstrated that apoptotic cell death is one of the host cells’ responses to *B. pseudomallei* infection [32,33] consistent with the previous studies. Some research has shown that bacterial infection can induce oxidative stress [34,35]. Our results proved that...
B. pseudomallei induced RAW264.7 cells apoptosis via oxidative stress.

To understand how B. pseudomallei is related to oxidative stress, we observed that B. pseudomallei-infected RAW264.7 macrophages can activate oxidative stress by upregulating ROS levels. Considering that oxidative stress is known to play a significant role in other infectious diseases, NAC is an antioxidant that is clinically used to treat acetaminophen poisoning by eliminating ROS [22] and is also of great significance in tuberculosis and chronic bronchitis [36]. This indicates that NAC plays a significant role in clinical treatment and may have potential significance in the treatment of melioidosis. Recently, NAC has been reported to inhibit apoptosis by inhibiting oxidative stress, and our study is in agreement with previous studies [37]. We also observed that NAC inhibits oxidative stress, thereby reducing apoptosis of the mitochondrial pathway.

Previously, only one study showed that NAC increases intracellular GSH in diabetic patients with melioidosis, but B. pseudomallei infection is not ameliorated [38]. Therefore, the effectiveness of NAC as a treatment for melioidosis requires further investigation. Although the mechanisms underlying the reciprocal relationship between bacterial infection and oxidative stress are complex and require further investigation in the future.

These results emphasize that B. pseudomallei infection induces macrophage apoptosis and oxidative stress, and NAC can relieve mitochondrial pathway apoptosis induced by B. pseudomallei by inhibiting oxidative stress.

**Conclusion**

In conclusion, our study shows that NAC can inhibit mitochondria-dependent apoptosis in B. pseudomallei-infected macrophages by reducing ROS levels. A concise model illustrating the role of mitochondrial apoptosis in macrophages induced by B.
Figure 4: NAC inhibits mitochondrial damage induced by *B. pseudomallei*.
(A, D) Mitochondrial ultrastructure was observed by TEM. The red arrows indicate the mitochondria in RAW264.7 cells. An enlarged view is shown in the red box. (B, E) ATP levels were detected in different populations. (C, F) Real-time fluorescence quantitative PCR was used to detect mtDNA copy number levels.

*P<0.05, **P<0.01, ***P<0.001

Figure 5: NAC inhibits *B. pseudomallei*-induced mitochondrial apoptosis.
(A, C) COX IV was detected using fluorescence microscopy and Western blot. (B, D) Fluorescence microscopy and Western blot detection Cyt C. *P<0.05, **P<0.01, ***P<0.001
pseudomallei has been presented and is shown in Figure 6.

High levels of ROS can activate p53, which is involved in mitochondrial apoptosis. P53 positively regulates Bax, negatively regulates Bcl2, promotes cytochrome C release, causes an apoptotic cascade (Caspase9/3), and leads to apoptosis. On the other hand, NAC can significantly reduce B. pseudomallei-induced apoptosis by inhibiting intracellular ROS production.

**Funding**

This study was supported by the Major Science and Technology Program of Hainan Province (ZDK[202003, ZDK]2021036), the National Natural Science Foundation of China (81960002, 82272431), the State Key Research Development Program of China (2022YFC2305000, 2022YFC2305003), the Graduate Innovative Research of Hainan Medical University (HYYS2020-17), the Innovation and Entrepreneurship Training Program for college students of Hainan Medical University (X20201180080), and the Hainan Province Clinical Medical Center, Science, and Technology Plan of Hainan Province (Clinical Research Center).

**References**


