N-acetylcysteine (NAC) Attenuating Apoptosis and Autophagy in RAW264.7 Cells in Response to Incubation with Mycolic Acid from Bovine *Mycobacterium tuberculosis* Complex

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Abstract

Bovine tuberculosis is an airborne infectious disease caused by organisms of the *Mycobacterium tuberculosis* (MTB) complex. Mycolic acid (MA) is the main lipid component of the cell membrane of MTB. It is non-enzymatically reduced by NAD(P)H and further produces reactive oxygen species (ROS), which can cause oxidative stress in human cells. N-acetylcysteine (NAC) is a synthetic precursor of glutathione (GSH) and exhibits anti-ROS activity. However, the underlying mechanisms of its protective properties remain uncertain. Herein, after pre-incubation of RAW264.7 cells with NAC, the factors associated with apoptosis and autophagy were measured. Mechanistically, NAC could reduce MA-induced expression of pro-apoptotic and pro-autophagy proteins. At the mRNA level, NAC can inhibit AMPK and activate mTOR expression. The results indicate that NAC might regulate autophagy in RAW264.7 cells through the AMPK/mTOR pathway. To further prove the effect of NAC on MA, ICR mice were used to evaluate the lung injury. Hematoxylin-eosin (HE) staining was performed on the lung. The results show that NAC could reduce cell injury induced by MA. In conclusion, our research showed that NAC attenuates apoptosis and autophagy in response to incubation with mycolic acid.

K e y   w o r d s: N-acetylcysteine, mycolic acid, apoptosis, autophagy

Introduction

Bovine tuberculosis is an airborne infectious disease caused by organisms of the MTB complex (Baker et al. 2019). MTB complex often colonizes immunocompromised hosts, then induces inflammation, and disrupts the host immune response. MTB can produce many substances to exert its virulence to mammalian cells. One of them is mycolic acid (MA). MA is a unique lipid component of the MTB cell wall and plays an essential role in the process of binding MTB to macrophages (Lehmann et al. 2018). Previous studies have shown that MTB lacking MA is less pathogenic *in vivo*, suggesting that MA plays an important role in MTB's infection (Slama et al. 2016). Furthermore, MTB promotes bacterial survival by inducing oxidative stress in macrophages (Mohanty et al. 2016). Therefore, the

strategy to alleviate the virulence of MTB is to inhibit the biosynthesis of MA, neutralize the oxidative stress produced by MA, and reduce the damage of ROS to intracellular substances.

N-acetylcysteine (NAC) is a synthetic precursor of intracellular cysteine and glutathione (GSH) (Aldini et al. 2018), and its anti-ROS activity is attributed to the ability to react with oxygen ions. Some researchers believe that inhaled GSH or NAC can increase the level of GSH and reduce the oxidative damage that MTB causes to the host (Amaral et al. 2016). Several reports show that NAC protects against oxidative stress-induced cell death (Wang et al. 2014). The self-protective mechanism of intracellular MTB produces free radicals, which increases the toxicity to cells, so whether antioxidants are beneficial for MTB infection is still under investigation. As a marker of MTB cell

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envelope (Lederer et al. 1975), does MA affect cells? Although a large body of literature has elucidated the structural mechanism of MA (Marrakchi et al. 2014), it is unclear whether NAC affects autophagy and apoptosis of MA-incubated RAW cells.

In this study, RAW264.7 macrophages were used to explore further the immunological response and cell damage of host cells after incubation with MA. Animal experiments were performed to investigate the role of NAC in antagonizing the effects of MA in the induction of apoptosis and autophagy.

Experimental

Materials and Methods

Animals. Male ICR (Institute of Cancer Research) mice aged six weeks were purchased from the Chinese Academy of Sciences (Shanghai, China). The Ethics Committee approved all experimental procedures for Laboratory Animals at the Ningxia University. Mice were kept under conditions of a pathogen-free facility and given free access to food and tap water. After the mice were acclimated for one week, the mice were divided into the following four treatment groups: 1) the control group (administered 50 µl, 0.9% saline by intranasal route for 7 days); 2) the MA group (received 50 µl MA [50 µg/ml] for 7 days); 3) the MA + NAC group (received 50 µl MA [50 µg/ml] by intranasal route 3 hours post NAC protection for 7 days); and 4) the NAC group (received intranasal NAC for 7 days).

Histological evaluation. Mice were sacrificed by cervical dislocation at indicated time points. Lungs were extracted from mice, washed once with PBS, fixed in 4% paraformaldehyde for 24 hours, and stained with hematoxylin and eosin. The fixed tissue was embedded in paraffin and cut into 4-µm-thick sections using a microtome.

Cell culture. The RAW264.7 cell line was cultured in DMEM medium supplemented with 10% FBS and incubated at 37°C and 5% CO_2. Tissue-culture plasticware was acquired from Corning (Tewksbury, MA, USA). Before incubation, the cell line was seeded at a density of 1×10^6 cells per well in 6-well plates. The total cell number was determined with an automated vision-based cell counter.

Assessment of cell viability. To assess the viability rate of cells, we used the MTT assay (Sigma-Aldrich, Saint Louis, MO, USA). The RAW264.7 cells were seeded at a density of 5×10^3 cells per well in 96-well plates. They were subsequently treated with MA (50 µg/ml) for 0, 6, 12, and 24 hours. Another cells were pretreated with NAC at a concentration of 600 mg/ml for 2 hours before treatment with MA. After 2 hours, the culture supernatant was replaced with fresh DMEM. MA (50 µg/ml) was added to a culture medium. After 6, 12, and 24 hours of incubation, the MTT solution (50 µl) was added to each well. The plates were transferred to the incubator at 37°C and 5% CO_2 for 4 hours. After the incubation, the DMSO solution (200 µl) was added to each well. The absorbance was measured at 560 nm with a microplate reader (Bio-Rad Laboratories Inc, Hercules, CA, USA). The reading was converted to the percentage of the controls.

Quantification of IL-6 levels by ELISA. The RAW264.7 cell culture supernatant in each group was collected for the measurement of pro-inflammatory cytokine IL-6 levels. According to the manufacturer's instructions, the IL-6 levels were assayed by the cytokine ELISA (Wuhan Boster Biological, Wuhan, China).

Real-time quantitative PCR analysis. The total RNA was extracted using the MiniBEST Universal RNA Extraction Kit (Takara Biotechnology, Dalian, China). RNA was reverse transcribed into cDNA according to the experimental procedure of the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The reverse transcription products were used as templates and subjected to quantitative PCR (Q-PCR). The obtained cDNA was subjected to PCR amplification in an ABI 7500 Fast thermocycler (Applied Biosystems, USA) using a SYBR-Green PCR Kit (Takara Biotechnology, Dalian, China). A two-step PCR cycle procedure was used: 95°C for 30 seconds, then 40 cycles for 5 seconds at 95°C, followed by an annealing and extension step at 60°C for 15 seconds. After completion of PCR amplification, DNA melting curve analysis was performed. The primers were synthesized by Shanghai Sango Company (Shanghai, China). Specific primer sequences for each gene are shown in Table I.

Table I
Primer sequences.

Gene name	Forward primer	Reverse primer
TNF-α	CGCTGAGGTCAATCTGC	GGCTGGGTAGAGAATGGA
Caspase-9	AGCGATTCTGCCTTTCAC	TGGAGATTTTGTGGTCAGC
mTOR	CTGGGGCTGCTTTCTGT	ACGGTTTTCTGCCTCTTGT
AMPK	CATCCCCAAACCTGTCC	ACAAGCCCCGAACAAAA

Western blot analysis. Total protein was extracted from the different treatment groups using the M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). The protein sample (20 μg) was mixed with 6* loading buffer and baked for 5 minutes. The proteins from each treatment group at the equal concentrations were separated by electrophoresis on 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Mini-PROTEAN Tetra Cell, Bio Rad, Hercules, CA, USA). Membranes were blocked in 5% milk, immersed in the primary antibody solution, and incubated overnight at 4°C. It was thoroughly washed six times with 0.5% TBS-Tween, for 5 minutes each time. Then, the membrane was incubated with the secondary antibody for 1 hour at room temperature. Protein bands were displayed using a chemiluminescence (ECL) kit (UVP, Upland, CA, USA). β-actin was used as an internal control. The band intensity of the protein was quantified by using the ImageJ program. Antibodies used for blotting were BAX, LC3, and beclin-1 (at the dilution of 1:2000).

Statistics. Results are expressed as mean ± standard deviation. One-way ANOVA using the Student's t-test was used for multiple comparisons in SPSS 16.0 software. $P < 0.05$ was considered to represent a statistically significant difference.

Results

NAC decreases RAW264.7 cell viability during incubation with MA. The viability of RAW264.7 macrophages incubated with MA was determined with the MTT assay. The cells were treated with MA (50 μg/ml) for 0, 6, 12, and 24 hours. Other groups of cells were pretreated with NAC at a concentration of 600 mg/ml. The results showed a significant reduction of the RAW264.7 viability after treatment with MA when compared to the control after 6 hours of incubation, and the survival rate of the cells incubated with MA continued to decline within 36 hours (Fig. 1A). Pretreatment with NAC provided a significant increase in viability when compared to the MA-treated cells.

The IL-6 level in the supernatant was measured with an ELISA to elucidate further the cytokine level in the MA-induced cell damage and the effect of NAC on RAW264.7 macrophages. The results confirmed that MA could reduce the secretion of IL-6 compared with the control group (Fig. 1B). In contrast to the MA-treated cells, the treatment with NAC reduced the secretion of IL-6 significantly.

NAC reduces MA-induced apoptosis in RAW264.7 macrophages *in vivo*. To determine whether NAC regulates the apoptosis of RAW264.7 cells exposed to MA, the Western blot was used to analyze the relative expression of the BAX gene, which encodes an important protein for the regulation of apoptosis. As shown in Fig. 2B, MA induced a decrease in the BAX level compared with the control. Combined treatment of NAC further exaggerated this effect, indicating that NAC reduced apoptosis in RAW264.7 cells treated with MA.

Then, the caspase-9 mRNA level was pronouncedly decreased when compared to MA in the MA + NAC group (Fig. 2D). The level of TNF-α mRNA was found to decrease when compared to MA (Fig. 2C). This observation further proved the protective effect of NAC on RAW364.7 cells.

NAC reduces MA-induced autophagy in RAW264.7 cells via the AMPK/mTOR signaling pathway. To further explore the upstream events that
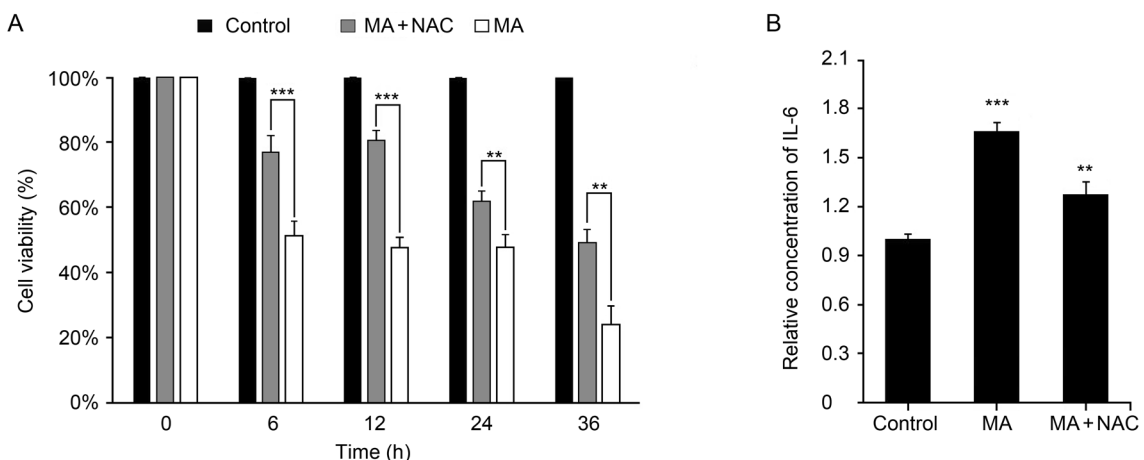

Fig. 1. Effect of NAC on the viability and cytokine levels of RAW264.7 cells during incubation with MA.
(A) Different groups of RAW264.7 cells were treated with MA (50 μg/ml) for 6, 12, 24, and 36 h and then analyzed for viability with MTT. Changes in the optical density (OD) values at 560 nm were recorded with a microplate reader. (B) The cell culture supernatants were collected after 24 h in the MA group and NAC + MA group; the levels of secreted cytokines in the samples were quantified. The graphical representation shows the relative concentration of the secreted IL-6. (Mean ± standard deviation, n = 3 experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student's t-test).
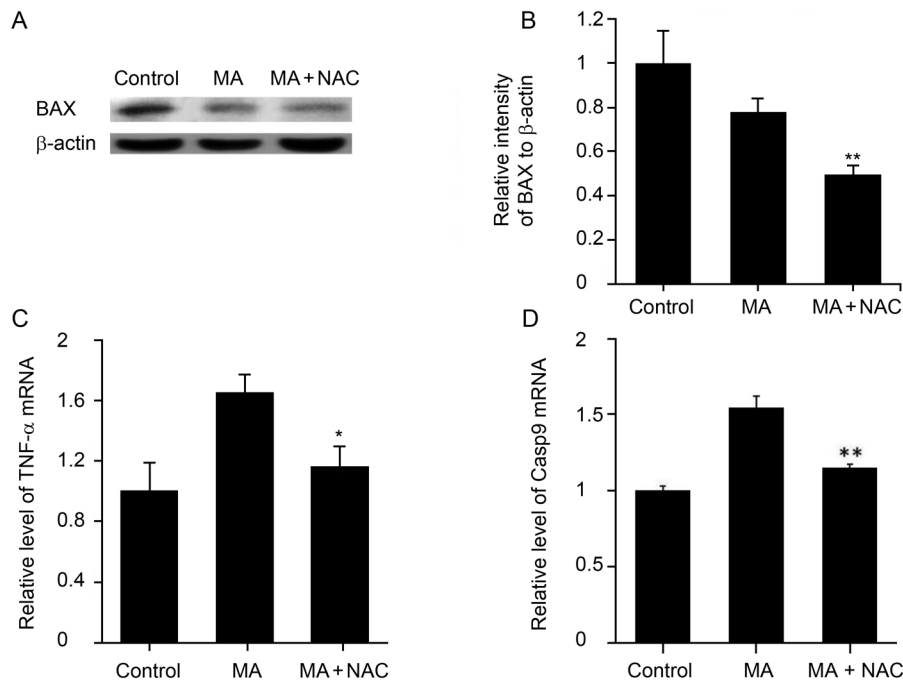
Fig. 2. Effects of NAC on apoptosis-related factors in RAW264.7 cells during incubation with MA.

The MA group was treated with MA (50 μg/ml) for 24 h. MA + NAC group was pre-treated with NAC at a concentration of 600 mg/ml for 2 h before treatment with MA. The results are representative of three separate experiments. (A) Representative Western blot of BAX in RAW264.7 treated with MA or MA + NAC (n = 3 experiments, *$p < 0.05$, **$p < 0.01$ by image J). (B) Quantification of BAX levels, as represented in A it was quantified by densitometry and standardized to the β-actin level. (C, D) The levels of TNF-α and Casp9 mRNA was standardized by the double delta CT method. (Mean ± standard deviation, n = 3 experiments, *$p < 0.05$, **$p < 0.01$ by Student's t-test).

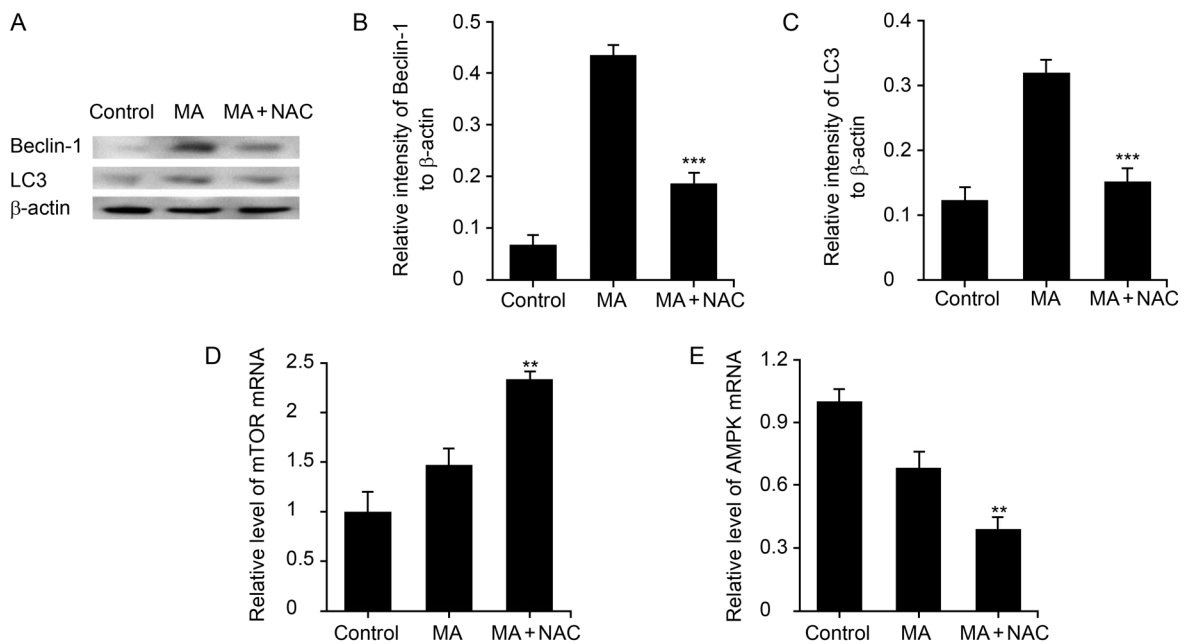

Fig. 3. Effects of NAC on autophagy-related factors in RAW264.7 cells during incubation with MA.

The MA group was treated with MA (50 μg/ml) for 24 h. MA + NAC group was pre-treated with NAC at a concentration of 600 mg/ml for 2 h before treatment with MA. (A) Representative Western blot of beclin-1 and LC3 in RAW264.7 macrophages treated with MA or MA + NAC (n = 3 experiments). (B, C) Quantification of beclin-1(b) and LC3(c) levels, as represented in A. (D, E) The levels of mTOR and AMPK mRNA was quantified by densitometry, and standardized to the β-actin level. (Mean ± standard deviation, n = 3 experiments, *$p < 0.05$, **$p < 0.01$ by Student's t-test).

were induced by the combination of MA and NAC, we measured the expression of autophagy-related factors. LC3 and beclin-1 are essential cytokines involved in the regulation of autophagy. As shown in Fig. 3A and B, pretreatment with NAC significantly suppressed the expression of LC3 and beclin-1 under MA treatment. This result indicates that NAC decreased autophagy in MA-treated RAW264.7 cells (Fig. 3A-C).
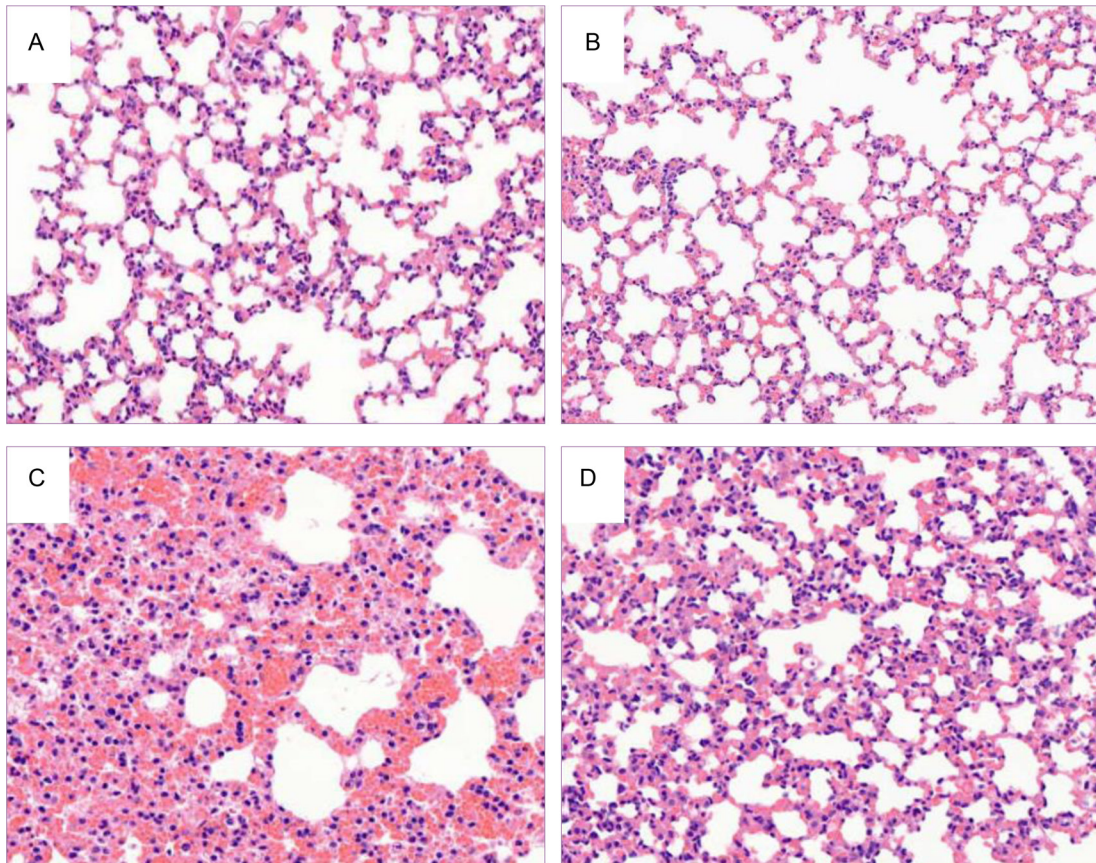
Fig. 4. Morphological changes in the lung tissue (H&E staining) in ICR mice treated with MA and induced by NAC. Representative photomicrographs illustrated characteristic lesions in 8 week-old C57BL/6 mice. The lung tissues, which had not been lavaged, were fixed with 4% paraformaldehyde solution and routinely processed for pathological slices. The morphological change of the lung tissue was observed after H&E staining. (A) The control group. (B) The NAC group. (C) The MA group. (D) The MA + NAC group. (Scale bar 50 μm).

Next we sought to examine AMPK and mTOR, important components in autophagy. The results indicated that NAC induced an increase in the level of mTOR mRNA when compared with MA only (Fig. 3D). As shown in Fig. 3E, NAC induced a decrease in the level of AMPK mRNA when compared to the MA group.

NAC reduces lung damage caused by MA. Finally, to confirm the immune suppression function of MA and NAC's protection, we examined the histological changes in the lungs of mice after their treatment by the intranasal route with NAC and MA. Lung tissues of mice were analyzed by hematoxylin-eosin (HE) staining. Lung tissue sections from the control group and NAC group displayed normal structure and no pathologic changes under the light microscope; no inflammatory cell infiltration was observed (Fig. 4A, B). In the MA group, the lung tissue of the mice was obviously damaged, the interstitial lung was congested, and apparent inflammatory cell infiltration was observed (Fig. 4C). In the MA + NAC group, the degree of alveolar wall damage was reduced compared with the MA group, and the infiltrating inflammatory cells were also significantly reduced (Fig. 4D).

Discussion

Bovine MTB complex is the primary causative agent of bronchiectasis and chronic and recurrent lung infections in patients. MA is a key compound of MTB infection and a significant contributor to lung injury in chronic MTB infection (Liu and Nikaido 1999). Early studies have shown that MA damage is primarily mediated by the generation of ROS and the destruction of host antioxidant defense mechanisms (Li et al. 2015). Consistent with previously published studies, we show here that RAW264.7 cells in response to incubation with MA reduce the relative expression of protein engaged in apoptosis and autophagy.

In the current study, RAW264.7 cells were first treated with NAC for 2 hours and then treated with MA for 24 hours. Here, we firstly showed that pretreatment with NAC significantly attenuated the changes in the level of the cell apoptosis protein BAX that were induced by the MA treatment. Compared with the MA group, the expression of the BAX gene was obviously reduced in the NAC + MA group. In addition, the mRNA levels of TNF-α and caspase-9 were also decreased.

BAX protein is known to be pro-apoptotic by regulating the permeability of the mitochondrial outer membrane (Lin et al. 2019). Caspase-9 cleaves the inactive pro-forms of effector caspases in apoptosis (Liu et al. 2014). When the mitochondrial membrane permeability changes, it promotes caspase-9 activation to induce apoptosis through TNF-α. The data obtained in the present study showed that NAC resulted in the simultaneous downregulation of caspase-9 and TNF-a, and the subsequent translocation of BAX to mitochondria. These results supported the hypothesis that NAC reduced MA-induced cell apoptosis of RAW264.7 cells, and it was mediated by the intrinsic mitochondrial pathway.

Under certain stress conditions (such as oxidative damage), autophagy plays a vital role in cell survival by scavenging proteins and damaged organelles to maintain cell homeostasis and integrity (Gutierrez et al. 2004). We examined the gene and protein expression of autophagy through quantitative real-time PCR and Western blot analysis. Besides, IL-6 concentration was evaluated with ELISA. From a mechanistic standpoint, LC3 can specifically accumulate in neonatal autophagosomes, so it is the most extensive and useful specific marker of autophagy. In contrast to the marker LC3, which forms the final autophagosome, beclin-1 is involved in the early stages of autophagy, releasing phosphorylated beclin-1, synthesizing autophagic vesicles, and recruiting proteins (Menon and Dhamija 2018). They are widely monitored as autophagy-related proteins. Our results show that incubation with MA increased the expression of autophagy-related protein. Remarkably, NAC inhibited the expression of the LC3 and beclin-1-encoding genes.

Furthermore, there are many signaling cascades involved in autophagy regulation in response to different stimuli. AMPK is the primary sensor of energy stress and is normally activated in response to various intracellular and extracellular stresses (Hardie 2014). It can also be activated by intracellular oxidative stress. The AMPK/mTOR signaling pathway is a classical upstream pathway regulating autophagy. Indeed, the reduction in ROS inhibits AMPK, thereby activating mTOR, the negative regulator of autophagy, and subsequently inhibiting autophagy (Cerni et al. 2019). Our results demonstrate that NAC might inhibit the expression of the autophagy-related LC3 and beclin-encoding genes via the reduction of intracellular ROS production.

In conclusion, our research showed that NAC attenuated the expression of the genes encoding for proteins of apoptosis and autophagy in RAW264.7 cells in response to incubation with MA. Mechanistically, an infection of MTB induced intracellular ROS production and subsequently promoted cell apoptosis. NAC attenuated MA-induced suppression of the activation of BAX and the production of IL-6. At the genetic

level, NAC inhibits the expression of the TNF-α and caspase-9 genes and reduces the translation of apoptotic proteins. On the other hand, to investigate its possible autophagy mechanism, some autophagy-related proteins were measured. Our current study demonstrated that MA-induced the reduction of the cell autophagy-relative protein LC3, beclin-1, and the cell apoptosis-relative protein BAX. However, NAC could efficiently inhibit this reduction.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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