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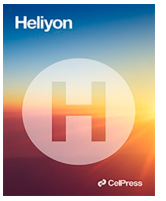

Research article

Acacetin protects against acute lung injury by upregulating SIRT1/ NF-κB pathway

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ABSTRACT

Acacetin is one of the natural flavone components found in many plants and possesses diverse pharmacological activities. The anti-inflammatory properties and definite mechanism of acacetin remains incompletely illuminated. Here, we evaluated the efficacy of acacetin on lipopolysaccharide (LPS)-induced acute lung injury *in vivo* and TNF-α-stimulated cellular injury *in vitro*. As indicated by survival experiments, acacetin reduced mortality and improved survival time of LPS-induced acute lung injury in mice. 50 mg/kg of acacetin obtained higher survival (about 60 %), and 20 mg/kg of acacetin was about 46.7 %. In addition, 20 mg/kg of acacetin rescued lung histopathologic damage in LPS treated mice, lowered lung-to-body weight and lung wet-to-dry ratios, suppressed myeloperoxidase activity in lung tissue, the contents of protein, the numbers of total cells and neutrophils in bronchoalveolar lavage fluid (BALF), and the contents of inflammatory cytokines such as TNF-α, IL-6, IL-17 and IL-1β in BALF. Acacetin also increased the activity and expression of SIRT1, thereby suppressing acetylation-dependent activation NF-κB. Similarly, *in vitro*, acacetin increased cell viability, reduced levels of TNF-α, IL-6, IL-17, and IL-1β, increased NAD^+ levels as well as NAD/NADH ratio, and then up-regulated the activity and expression of SIRT1, and restrained acetylation-dependent activation NF-κB in TNF-α-stimulated A549 cells, which could be abolished by SIRT1 siRNA. Collectively, the current study showed that acacetin exerts a protective effiect on acute lung injury by improving the activity and expression SIRT1, thereby suppressing the acetylation-dependent activation of NF-κB-p65 and the release of downstream inflammatory cytokines.

1. Introduction

Acute lung injury (ALI) is a critical condition characterized by rapid onset of inflammation and damage to the lung tissue, leading to impaired gas exchange and respiratory failure. It is common and devastating for critically ill ICU patients to suffer from acute lung injury and acute respiratory distress syndrome [1,2]. Severe hypoxia, extensive accumulation of inflammatory cells in the lung, and the release of cytokines and activation of the inflammatory process are characteristically hallmarks of acute lung injury and acute respiratory distress syndrome [1]. Current treatments for ALI primarily focus on supportive care, including mechanical ventilation, fluid

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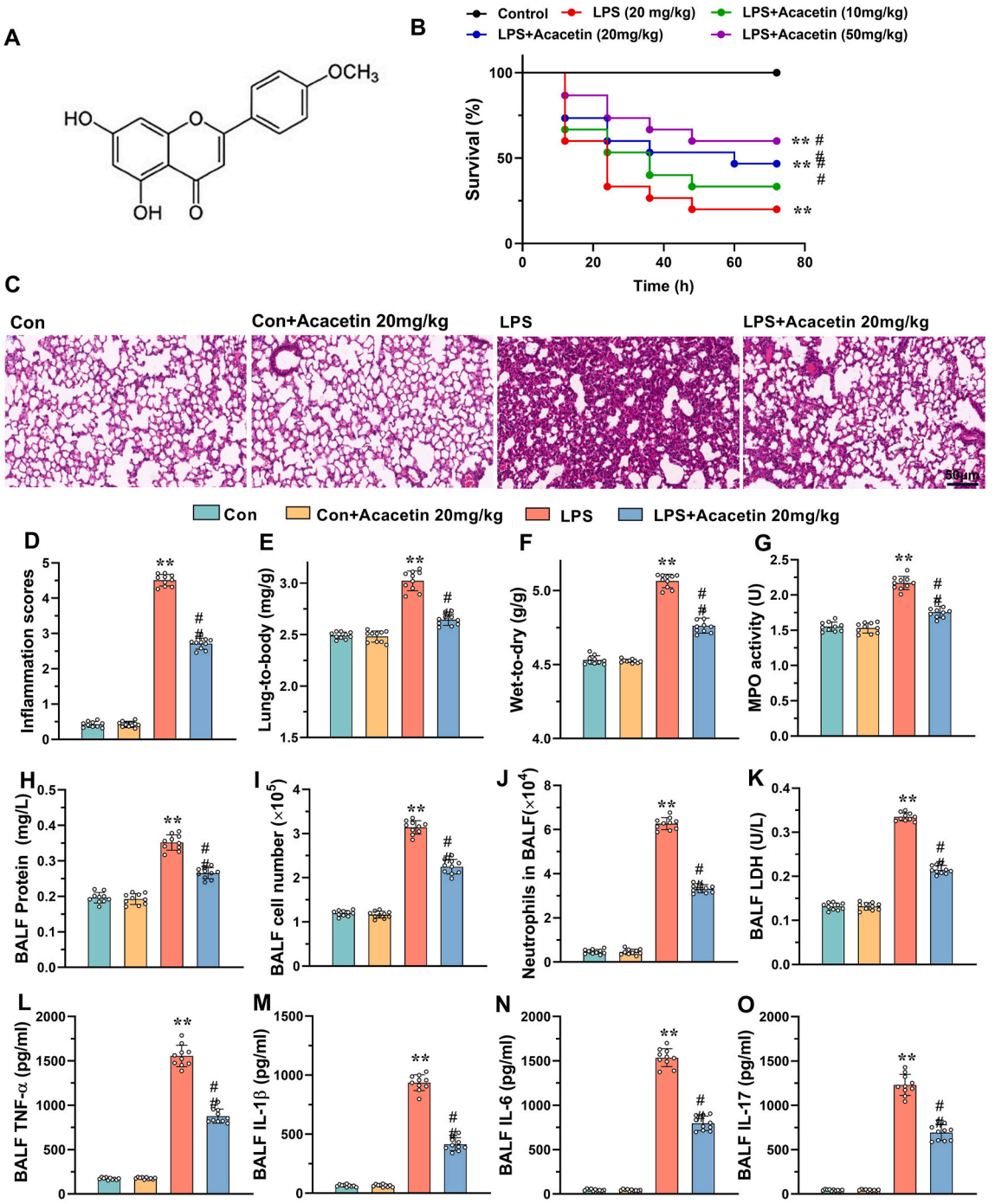

Fig. 1. Acacetin improved the accumulative mortality and attenuated acute lung injury induced by LPS. (A) the molecular structure of acacetin. (B) The effect of acacetin on LPS-induced accumulative mortalities was checked. Mice were intraperitoneally treated with or without different doses of acacetin treatment (10, 20, or 50 mg/kg) 1 h after being challenged intraperitoneally with 20 mg/kg of LPS. The living condition of mice were closely observed, and the mortality of mice was recorded every 12 h for 3 days after the LPS injection. Littermate mice were used in the survival study and each group contained 15 animals. Survival data was evaluated by the Kaplan Meier method followed by the log-rank test for multiple comparisons. $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. LPS group. (C–D) The examination of lung histopathologic changes was performed to assess inflammation scores. The bar represents 50 μm. Effects of acacetin on lung-to-body weight ratios (E), lung wet-to-dry weight ratios (F), MPO activity (G) in lung tissues were evaluated respectively. (H–O) Protein amount, total cell, neutrophil counts, and the content of LDH, TNF-α, IL-1β, IL-6 and IL-17 in bronchoalveolar lavage fluid (BALF) were investigated. Data were means ± S.D., $n = 10$ mice/group. $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. LPS group.

management, and the administration of vasopressors to stabilize hemodynamics. However, these approaches address the symptoms rather than the underlying pathophysiology. The common effective treatments such as anti-inflammatory medication application, and mechanical ventilation and oxygen inhalation are used widely in the patients with acute lung injury and acute respiratory distress syndrome. Stem cells and molecular targeted therapies also have achieved certain therapeutic efficacy and are expected to become potential treatment methods [1]. But even though, the morbidity and mortality rate of acute lung injury and acute respiratory distress syndrome remains especially high [3]. It still needs enormously to explored the underlying pathophysiological mechanisms of acute lung injury and acute respiratory distress syndrome for prevention and therapeutic strategies.

SIRT1, a NAD + -dependent deacetylase, has been implicated in the regulation of inflammation and cell death [4]. SIRT1 produce a marked anti-inflammatory effect through its deacetylation of a variety of factors participating in inflammation pathways such as NF-κB, P38 and MAPK, among others [5,6]. Specially, the activation of SIRT1/NF-κB signaling pathway are vital processes in the pathogenesis of acute lung injury and acute respiratory distress syndrome, presents a promising therapeutic target [7,8]. However, research on the SIRT1/NF-κB axis in the context of ALI is still in its infancy. Further exploration is needed to understand the mechanisms and effects by which SIRT1 modulates NF-κB activity and to develop novel therapeutic strategies that can harness this interaction for the treatment of ALI. Furthermore, in accordance with this property of SIRT1, some drugs, exhibit great effect on upregulating SIRT1 function, are drawing enough attention for their clinical practice in the prevention and cure of inflammatory diseases and acute lung injury and acute respiratory distress syndrome [9–12].

Acacetin is a natural flavone existed in many plants, such as *Carthamus tinctorius* L., *Robinia pseudo acacia*, *Calea urticifolia,* and *Bitola pendula*[13]. It exerts multiple biological activities, such as anti-inflammatory, anti-oxidation, anti-aging, anti-viral, anti-cancer, anti-microbial, and anti-obesity, and has been frequently used in traditional Chinese medicine for myocardial ischemia/reperfusion injury, diabetes, neuroinflammation, arthritis, obesity, viral-mediated infections as well as hepatic protection [13,14]. Furtherly, acacetin is considered as a novel SIRT1 activator because of its ability of enhancing SIRT1 function then regulating its multiple downstream pathways [15]. However, the efficacy and potential mechanism of acacetin on acute lung injury remains not fully investigated. Herein, we investigated the efficacy and the potential mechanism of acacetin on lipopolysaccharide (LPS)-induced acute lung injury *in vivo* and TNF-α-stimulated cellular injury *in vitro* in this study. Results showed that acacetin cut down the mortality and improved the survival time of LPS-induced mice, ameliorated acute lung injury by upregulating the activity and nuclear expression SIRT1, and thereby suppressing the acetylated activation of NF-κB-p65 and the release of downstream inflammatory cytokines.

2. Materials and methods

2.1. Animals and reagents

Adult male C57BL/6J mice (weighed 18–22 g), acquired from the animal center of Fourth Military Medical University, were maintained in a conventional facility with 12 h dark-light cycles. All animal experiments were approved by Animal Ethical Experimentation Committee of the Fourth Military Medical University in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (No. IACUC-20200901).

Acacetin (the molecular structure shown in Fig. 1A, #480-44-4), CelLytic™ NuCLEAR™ extraction kit (#NXTRACT), NAD^+/NADH Assay Kit (#MAK460), SIRT1 activity assay kit (#CS1040-1 KT) and LPS (*Escherichia coli* lipopolysaccharide, 055: B5, #L2880) were acquired from Sigma-Aldrich Inc. (St. Louis, MO, USA). The myeloperoxidase (MPO) activity assay kit (#ab105136) was purchased from Abcam (Cambridge, UK). Anti-acetyl-NF-κB-p65 (Lys310, #AF1017), anti-NF-κB-p65 (#BF8005), anti-SIRT1 (#DF6033) and anti-histone H3 (#BF9211) antibodies were obtained from Affinity Biosciences Inc. (Jiangsu, China). NF-κB-p65 Transcription Factor Assay Kit (#10007889) was purchased from Cayman Chemical (Michigan, USA). The enzyme-linked immunosorbent assay (ELISA) kits for TNF-α (#DY410-05), IL-1β (#SMLB00C), IL-6 (#SM6000B), and IL-17 (#SM1700) were purchased from R&D Systems Inc. (Minneapolis, MN, USA). LDH activity test kit (#MAK066), BCA Protein Assay Kit (#23227), DMEM medium (#21068028), fetal bovine serum (#12484028), TRIzol reagent (#10296028), and cDNA Reverse Transcriptor Kit (#4368813) were purchased from ThermoFisher Scientific (USA). One Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (#RR064A) were purchased from TaKaRa Biotechnology (Dalian, China). A549 cells, were acquired from American Type Culture Collection (#CCL-185, Rockville, MD, USA). Control siRNA (#sc-37007), SIRT1 specific siRNA (#sc-40986), siRNA Transfection Reagent (#sc-29528) were purchased from Santa Cruz Biotechnology (USA).

2.2. Survival study

Mice were intraperitoneally treated with or without various dosages of acacetin (10, 20, or 50 mg/kg) 1 h after being challenged intraperitoneally with 20 mg/kg of LPS. The living condition of mice were closely observed, and the mortality of mice was documented twice a day and continued for 3 days after the LPS injection. Littermate mice were used in the survival study and each group contained 15 animals. Acacetin was soluble in 5 % DMSO at a dose of 100 mg/ml before use. Equivalent saline or DMSO were used as control.

2.3. Model and grouping

Mice were assigned randomly into 4 groups ($n = 10$): Control group and Acacetin group: mice were intraperitoneally injected equivalent saline or 20 mg/kg of acacetin. LPS group: mice were intraperitoneally injected 10 mg/kg of LPS. LPS + Acacetin group: mice were intraperitoneally administered 20 mg/kg of acacetin 1 h after LPS injection. The whole mice were sacrificed by cervical

dislocation at 6 h after LPS or saline administration to take lung specimens for subsequent experiments or to make tracheal intubation for bronchoalveolar lavage fluid (BALF) collection.

2.4. Histological study

The upper lobe of the right lung was chosen for routine formalin fixation and paraffin embedment, which then were made into paraffin sections to stain with hematoxylin-eosin. Microscopic evaluation was performed in a double-blind method to evaluate the severity of acute lung injury. And inflammation scores were calculated according to the varying severe degrees of hemorrhage, edema, neutrophil infiltration and hyaline membrane formation [16].

2.5. Myeloperoxidase (MPO) activity assay

The lower lobe of the right lung was obtained to measure MPO activity. In a nutshell, the weighed lung tissue samples were homogenized in PBS, and then performed subsequent experimental operations according to the manufacturer's instructions. The amount of MPO activity was quantified the optical density at the wavelength 412 nm by using of a microplate reader.

2.6. The ratios of lung-to-body weight and lung wet-to-dry weight assessment

The body weights of all mice were weighed. At the end of the experiment, the whole lung tissues were weighed to calculated the ratios of lung-to-body weight. And then the left lung tissues were isolated and weighed again (wet weight), which were parched up at 50 °C for 72 h and weighed one more time (dry weight). The ratios of lung wet-to-dry weight were worked out to quantify the severity of pulmonary edema.

2.7. Bronchoalveolar lavage fluid (BALF) preparation and measurements

Make tracheal intubation to collect BALF (1 ml ice-cold PBS three times, 3 ml in total per mouse) for all group. For each mouse, the recycling ratio of the total injected volume was about 75 % (2.25 ml). The gathered BALF was centrifuged at 1000 g for 20 min at 4 °C to obtain the cell pellets, which were resuspended again in PBS and taken for cell counting using a hemocytometer to estimate the total cell number in BALF [16]. Next, the cell pellets were resuspended one more time in 1 ml of red blood cell-lysis buffer to eliminate red cells. The remaining part was white cells, which were re-pelleted by centrifugation at 520*g* for 20 min at 4 °C, and then made cytospin specimen to stain with Wright-Giemsa. These cells were counted and classified under a microscope to estimate the fraction of neutrophils [17]. Additionally, the content of protein in BALF was measured by BCA method with bovine serum albumin as a standard.

2.8. Cell culture and treatment

A549 (RRID: CVCL_0023) cell lines were purchased from the American Type Culture Collection (ATCC). A549 cells were cultured in DMEM medium replenished with 10 % fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5 % CO_2 and 95 % air. Exponentially growing A549 cells (about 80 % confluence) after being deprived of FBS for 12 h, were challenged with 10 ng/ml of TNF-α in the presence or absence of acacetin (0, 0.3, 1, 3 μM) for 24 h. At the end of experiment, the supernatant of the culture medium was collected to detect the levels of inflammatory cytokines by ELISA. The cells were collected to carry out MTT assay, SIRT1 activity or Western blot analysis, respectively. Acacetin was soluble in 5 % DMSO at a concentration of 3 μM before use. Equivalent DMEM medium or DMSO were used as control.

2.9. RNA interference of SIRT1

A549 cells (80 % confluence) were transfected with control siRNA or SIRT1 siRNA for 48 h by using of siRNA Transfection Reagent, siRNA Transfection Medium and siRNA Dilution Buffer according to the manufacturer's instructions [18]. Then the cells were challenged with 10 ng/ml of TNF-α in the presence or absence of acacetin (3 μM) for another 24 h. At the end of experiment, the supernatant and the cells were collected for further experiments.

2.10. Methyl thiazolyl tetrazolium (MTT) assay

A549 cells were seeded into 96-well plates at 1×10^5 cells/ml. After being deprived of FBS for 12 h, the cells were activated 10 ng/ml of TNF-α in the presence or absence of acacetin for another 24 h. Then 5 μl of MTT solution in PBS (5 mg/ml) was added to each well and hatched for 4 h. Discarded carefully the MTT solution in each well, and added 100 μl of pure dimethyl sulfoxide into each well to make the formazan crystals soluble. The amount of MTT formazan was measured by spectrophotometrical methods at the absorbance of 550 nm.

2.11. Lactate dehydrogenase (LDH) assay

According to the manufacturer's instructions, the contents of LDH in BALF and A549 cells supernatants were determined with a

corresponding detection kit and represented spectrophotometrically at the absorbance of 460 nm.

2.12. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TNF-α, IL-6, IL-17 and IL-1β in both BALF samples and A549 cells supernatants were determined by using commercially available ELISA kits according to the manufacturer's instructions, respectively.

2.13. NAD^+/NADH assessment

A commercialized NAD^+/NADH Assay Kit were used to measure the levels of NAD^+ and NADH both in lung tissue and A549 cells. Total NAD^+ and NADH were quantified according to the manufacturer's instructions [18].

2.14. SIRT1 activity assessment

SIRT1 activity both in lung tissue lysate and A549 cells lysate were detected by a commercialized fluorometric assay kit following the manufacturer's instruction [19]. In this kit, fluorophore and quencher are coupled to amino terminal and carboxyl terminal of substrate peptide, respectively, and before reaction of deacetylase, the fluorescence cannot be emitted. However, if SIRT1 performs deacetylation, substrate peptide will become cut by the action of protease added simultaneously, quencher will separate from fluorophore, and fluorescence will be emitted. Deacetylase enzyme activity is measured by measuring this fluorescence intensity. The fluorescence intensity was subsequently tested in microplate reader at Ex 350 nm/Em 460 nm. Enzyme activity was presented as relative fluorescence intensity and standardized to the control lung tissue or A549 cells.

2.15. Quantitative real-time polymerase chain reaction (qRT-PCR)

The mRNA expression of SIRT1 both in lung tissues and A549 cells were detected by meanings of qRT-PCR. TRIzol reagent was used to pick up total RNA from tissues or cells. Reverse transcription was performed to obtain the complementary Deoxyribose Nucleic Acid (cDNA). Afterwards, 1 μL of cDNA and SYBR Green was used for PCR measurement. The primer sequences were: GAPDH (Mouse, F: 5′-AACTTTGGCATTGTGGAAGG-3′, R: 5′-ACACATTGGGGGTAGGAACA-3′). GAPDH (Human, F: 5′-TGAAGGTCGGAGTCAACGGATTTG-3′, R: 5′-ATGTGGGCCATGAGGTCCACCAC-3′). SIRT1 (Mouse, F: 5′-CACATGCCAGAGTCCAAGTT-3′, R: 5′-AAATCCA-GATCCTCCAGCAC-3′). SIRT1 (Human, F: 5′-GCCTCATCTGCATTTTGATG'-3, R: 5′-TCTGGCATGTCCCACTATCA'-3). The relative expression levels of SIRT1 mRNA were analyzed via the $2^{-\Delta\Delta CT}$ method.

2.16. Nuclear extract preparation and western blot analysis

The nuclear protein fractions of lung tissue and A549 cells were extracted in accordance with the protocol of CelLytic™ NuCLEAR™ extraction kit as previously described [16]. The content of protein was measured by using BCA method. SDS-PAGE was used to separate an equal amount of protein, which then were transferred to the PVDF membrane. Next, the PVDF membrane was hatched with antibodies, HRP-conjugated IgG, and enhanced chemiluminescence solution in sequence. A gel imaging system (Bio-Rad, CA, USA) was used to analyze the immunoblots. The expression levels of SIRT1, acetyl-NF-κB-p65 and NF-κB-p65 in the nucleus were detected. H3 was used as a loading control for nuclear extract.

2.17. NF-κB-DNA binding activity assessment

NF-κB p65-DNA binding activity in lung tissue or A549 cell lysate were assessed following the manufacturer's instruction. The nuclear extract from lung tissue or A549 cell lysate were added into a microporous plate precoated with specific double-stranded DNA (dsDNA) sequence containing the NF-κB response element. This step ensured that the nuclear extract could combine with NF-κB response element and easily be detected by NF-κB specific antibody and HRP labeled secondary antibody in subsequent. After adding enzyme substrate, the absorbance at 450 nm was measured by spectrophotometrical methods.

2.18. Statistical analysis

Data were presented as means ± S.D., and the statistical differences were performed with analysis of variance (one-way ANOVA) followed by Dunnett's test for multiple comparisons. Survival data was evaluated by the Kaplan Meier method followed by the log-rank test for multiple comparisons. A statistical difference was accepted as significant if $P < 0.05$.

3. Results

3.1. Acacetin improved the accumulative mortality of mice induced by LPS

As shown in Fig. 1B, acacetin significantly lowered the accumulative mortality of mice induced by LPS. The accumulative mortalities during 3 days in LPS group were about 80 %, which were observably higher than that in 50 mg/kg of acacetin (about 60 %), and

in 20 mg/kg of acacetin (about 46.7 %). Additionally, 10 mg/kg of acacetin failed to protect against death. Thus, 20 mg/kg of acacetin was used for the subsequent experiments.

3.2. Acacetin attenuated LPS-induced acute lung injury in mice

Firstly, the pulmonary histological changes 6 h after LPS stimulation were detected. The normal pulmonary and alveoli histological structure were observed in the control group and acacetin group. LPS instillation resulted in caused alveolar damage, pulmonary edema, and infiltration of inflammatory cells, which in turn increased the inflammation score of lung tissue. However, these pulmonary histological alterations were less outstanding compared with those in LPS group after acacetin treatment (Fig. 1C–D).

Secondly, as the indexes of lung edema, the ratios of lung-to-body weight and lung wet-to-dry weight were checked (Fig. 1E–F). The ratios of lung-to-body weight and lung wet-to-dry weight in LPS group were dramatically increased compared with those in the control group, which were markedly reduced after acacetin administration.

Next, MPO activity in the lung tissues were measured to assess the neutrophil accumulation. As Fig. 1G shown, LPS brought out a significant increase in MPO activity compared with that in the control group. Acacetin treatment indeed inhibited MPO activity induced by LPS.

Finally, we investigated the protein amount, total cell, neutrophil counts, LDH content and inflammatory cytokines content (TNF-α, IL-6, IL-17 and IL-1β) in bronchoalveolar lavage fluid (BALF) to assess the severity of lung injury. Results indicated that LPS administration increased protein amount, elicited a significant recruitment of total cells and neutrophils, and increased the content of LDH, TNF-α, IL-6, IL-17 and IL-1β in BALF. However, acacetin significantly reversed these effects of LPS administration, suppressed the protein leakage, and the accumulation of leukocytes in the alveolar spaces (Fig. 1H–O). These results suggest that LPS-induced acute lung injury correlates with inflammatory cell infiltration, edema, exudation, and dysfunction is significantly improved in mice treated

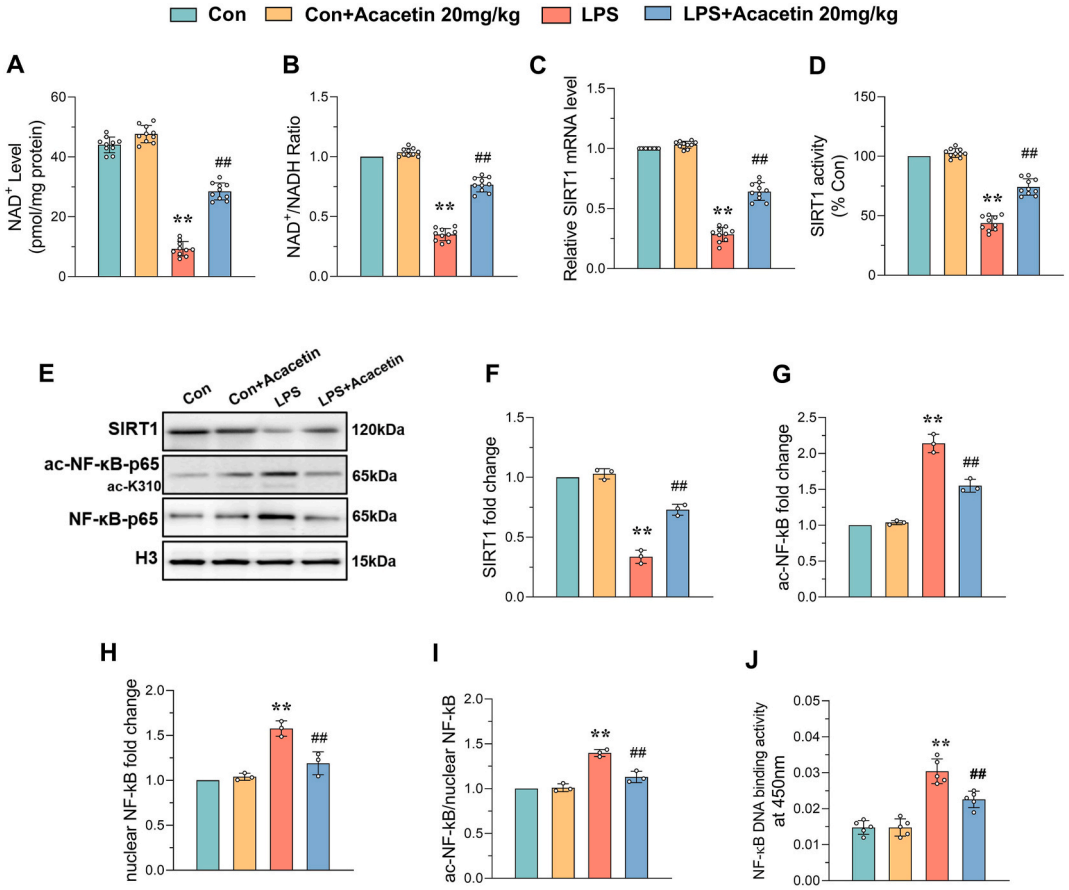

Fig. 2. Acacetin improved the function of SIRT1 and inhibited the acetylated activation of NF-κB-p65. The levels of NAD^+ (A), the ratio of NAD^+/NADH (B), the mRNA expression of SIRT1 (C), and the activity of SIRT1 (D) were detected in the lung tissue from LPS-treated mice with or without acacetin administration. The representative immunoblots of SIRT1, acetylated NF-κB-p65, and NF-κB-p65 in the nuclear protein, H3 was used as a loading control (E). The levels of SIRT1 (F), acetylated NF-κB-p65 (G), nuclear NF-κB-p65 (H), the ratio of acetylated NF-κB-p65/nuclear NF-κB-p65 (I) were analyzed ($n = 3$ independent experiments). The DNA binding activity of NF-κB (J) were assessed ($n = 5$ independent experiments). Data were means ± S. D., $n = 10$ mice/group. $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. LPS group.

with acacetin.

3.3. Acacetin improved the function of SIRT1 and inhibited the acetylated activation of NF-κB-p65

We further examined the activity and expression of SIRT1, and the acetylation of NF-κB-p65 in lung tissue to clarify the potential mechanism that acacetin possessed protective effects against LPS-induced ALI. Since SIRT1 is a NAD^+-dependent protein deacetylase, its activity is heavily influenced by the NAD+/NADH ratio. A higher ratio of NAD^+/NADH results in a higher SIRT1 activity [4,20]. Besides, introducing NAD^+/NADH exogenously promotes SIRT1 activity [21]. So we examined NAD^+ levels and NAD^+/NADH ratio in the lung tissue. Results showed that LPS administration significantly decreased NAD^+ levels and NAD^+/NADH ratio (Fig. 2A–B). Accordingly, the mRNA and nuclear protein expression of SIRT1, as well as SIRT1 activity were markedly declined after LPS administration compared with that in the control group (Fig. 2C–F). Not only that, the nuclear protein expression of NF-κB-p65, the acetylated form of NF-κB-p65 (K310), the ratio of ac-NF-κB-p65/nuclear NF-κB-p65 and the DNA binding activity of NF-κB were significantly elevated in LPS treated mice (Fig. 2E–G-J). However, acacetin treatment efficiently improved NAD^+ levels and the ratio of NAD^+/NADH (Fig. 2A–B), reversed the mRNA and nuclear protein expression of SIRT1 and the activity of SIRT1 (Fig. 2C–F), and reduced the nuclear protein expression of NF-κB-p65, the acetylated form of NF-κB-p65 (K310), the ratio of ac-NF-κB-p65/nuclear NF-κB-p65 and the DNA binding activity of NF-κB (Fig. 2E–G-J). Moreover, to evaluate the binding affinity of acetyl acetin for SIRT1, molecular docking assays were conducted. The results showed that acacetin and SIRT1 the best binding energy of −8.927 kcal/mol, indicating highly stable binding (Supplementary Fig. 1). These results indicate that the therapeutic effect of acacetin in LPS-induced acute lung injury is involved in upregulating SIRT1 function and thereby suppressing the acetylated activation NF-κB.

3.4. Acacetin reversed TNF-α-induced reduction of A549 cell viability and reduced TNF-α-induced inflammatory cytokines release

Based on research reports from other scholars and our preliminary experiments, 10 ng/ml of TNF-α, the optimal concentration to induce the smallest cell injury, were used to establish A549 inflammatory injury model [22–26]. MTT assay was performed to investigate the protective effects of acacetin on the viability of A549 cells. As shown in Fig. 3A, TNF-α prominently reduced the viability of A549 cells. But acacetin turned over the reduction of A549 cells viability in a concentration-dependent manner. LDH content is considered to be an indicator of cell injury. So we assessed the efficacy of acacetin on the content of LDH in A549 cells'

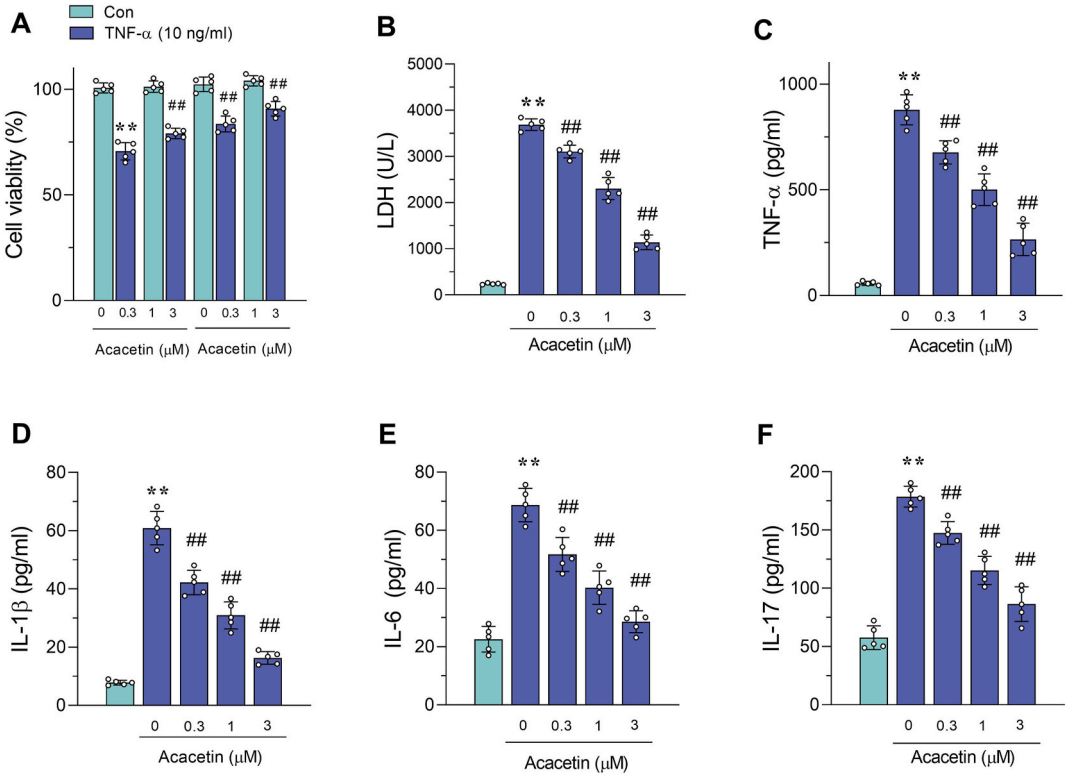

Fig. 3. Acacetin reversed TNF-α-induced reduction of A549 cell viability and reduced TNF-α-induced inflammatory cytokines in A549 cells' supernatant. (A) A549 cells were treated with different doses of acacetin (0, 0.3, 1, 3 μM) after administration with or without TNF-α (10 ng/ml). The cell viability was measured by MTT assay. The contents of LDH (B), TNF-α (C), IL-1β (D), IL-6 (E), and IL-17 (F) in the supernatant were tested. Data were means ± S. D., $n = 5$ independent experiments. $^{**}P < 0.05$ vs. control group, $^{\#\#}P < 0.05$ vs. TNF-α treated group.

supernatant. We found that TNF-α increased LDH content in the supernatant, which was reduced concentration-dependently by acacetin treatment (Fig. 3B). Meanwhile, TNF-α strongly increased the contents of TNF-α, IL-6, IL-17 and IL-1β in the supernatant. Compared with the TNF-α treated group, acacetin concentration-dependently lessened the contents of TNF-α, IL-6, IL-17 and IL-1β (Fig. 3C–F).

3.5. *Acacetin up-regulated SIRT1 function and restrained NF-κB-p65 acetylated activation in A549 cells*

As shown in Fig. 4A and B, acacetin administration significantly increased NAD^+ levels as well as NAD/NADH ratio in A549 cells treated with TNF-α. Similarly, acacetin exert powerful effects on reversing the mRNA and nuclear protein expression of SIRT1 and the activity of SIRT1 (Fig. 4C–F), and reducing the nuclear protein expression of NF-κB-p65 and acetyl-NF-κB-p65, the ratio of ac-NF-κB-p65/nuclear NF-κB-p65 and the DNA binding activity of NF-κB (Fig. 4E–G-J).

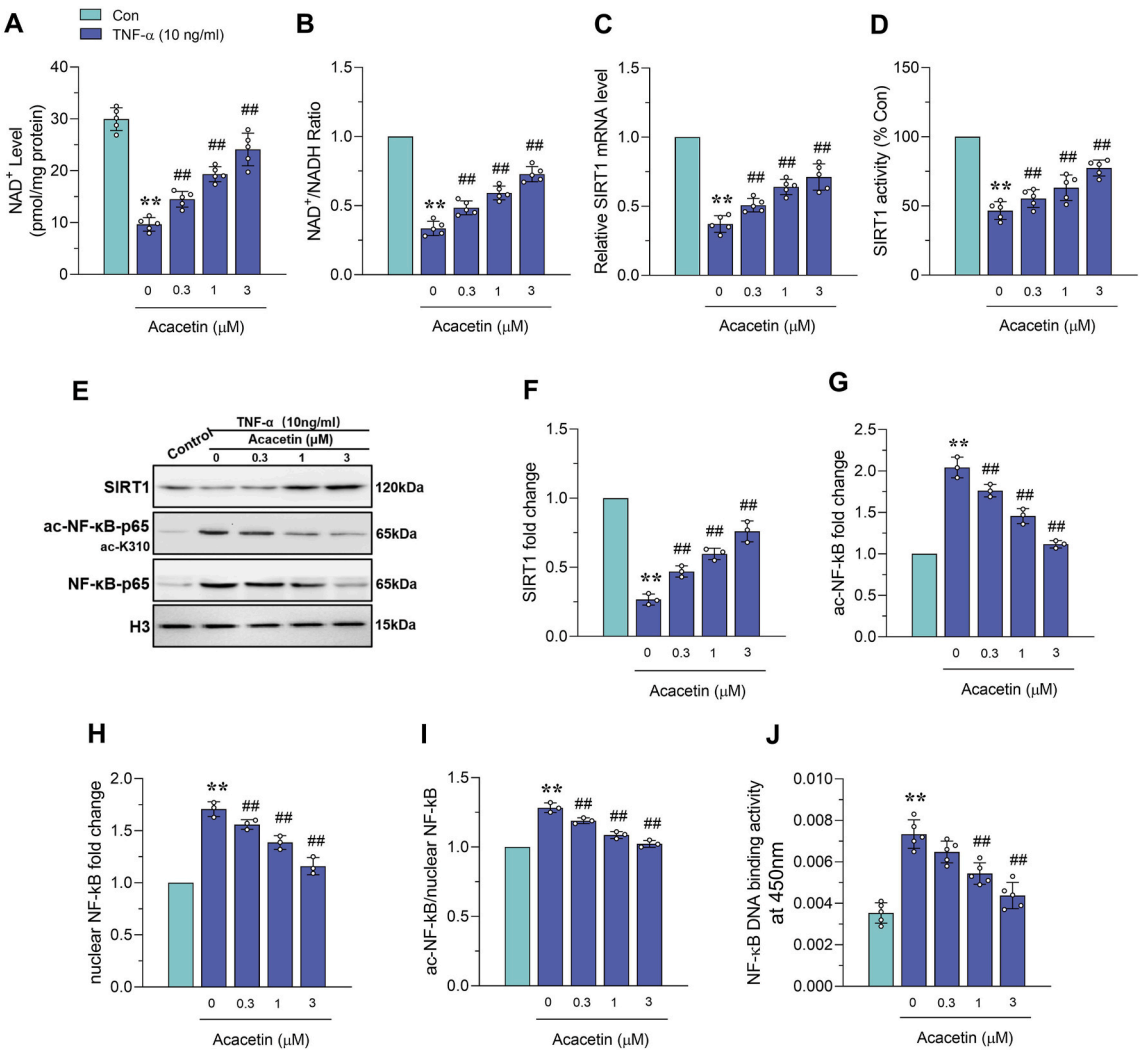

Fig. 4. Acacetin up-regulated SIRT1 function and restrained NF-κB-p65 acetylated activation in A549 cells. A549 cells were treated with TNF-α (10 ng/ml) in the presence or absence of acacetin (0, 0.3, 1, 3 μM) for 24 h. The levels of NAD^+ (A), the ratio of NAD^+/NADH (B), the mRNA expression of SIRT1 (C), and the activity of SIRT1 (D) were detected. The representative immunoblots of SIRT1, acetylated NF-κB-p65, and NF-κB-p65 in the nuclear protein, H3 was used as a loading control (E). The levels of SIRT1 (F), acetylated NF-κB-p65 (G), nuclear NF-κB-p65 (H), the ratio of acetylated NF-κB-p65/nuclear NF-κB-p65 (I) were analyzed ($n = 3$ independent experiments). The DNA binding activity of NF-κB (J) were assessed. Data were means ± S. D., $n = 5$ independent experiments. $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. TNF-α treated group.

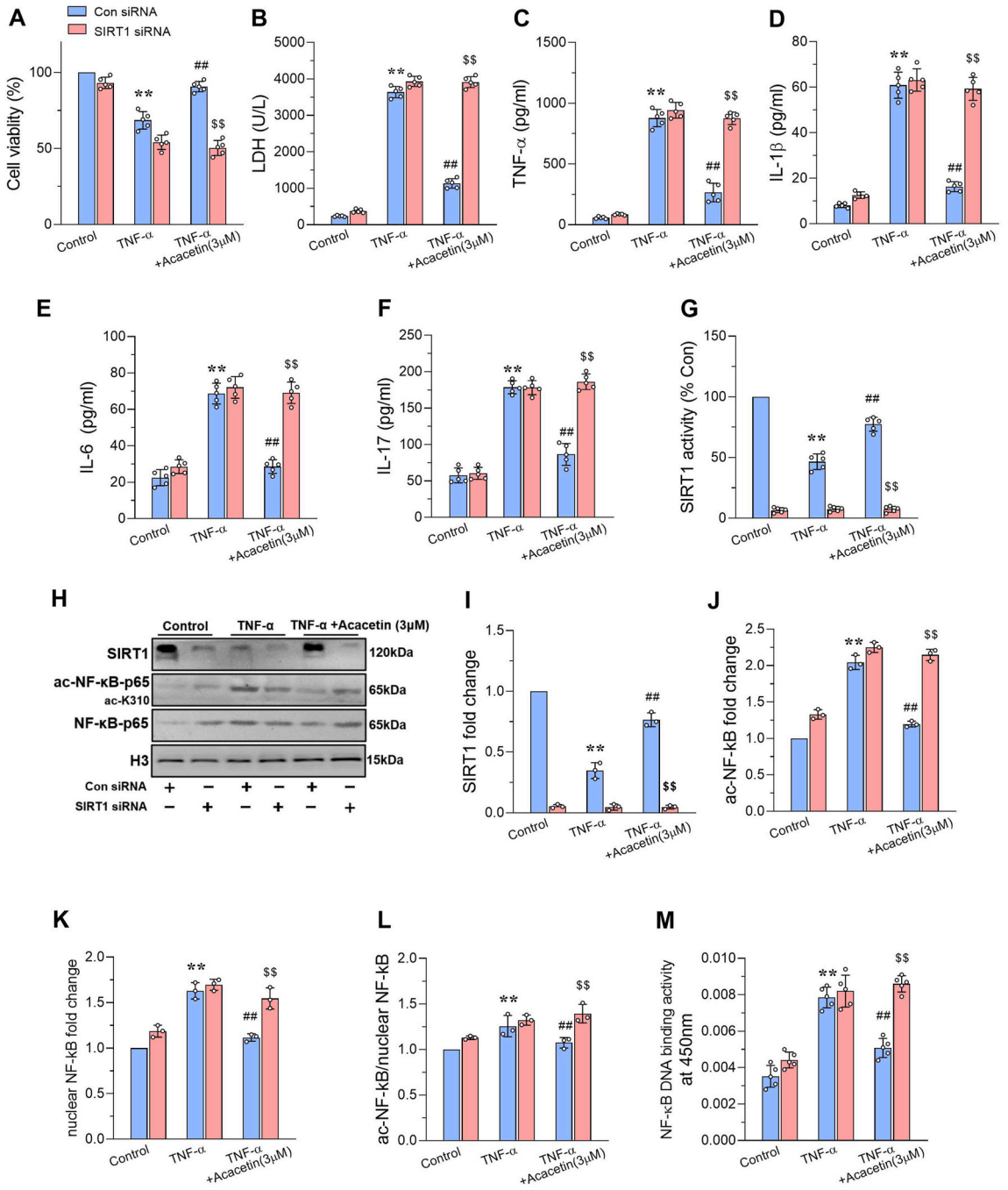

Fig. 5. Silencing SIRT1 abolished the efficacy of acacetin for improving SIRT1 function and restraining NF-κB-p65 acetylated activation induced by TNF-α. A549 cells were transfected with Control siRNA or SIRT1 siRNA to further investigate the relationship of acacetin and SIRT1. (A) A549 cells viabilities were measured by MTT assay. (B–E) The contents of LDH, TNF-α, IL-1β, IL-6, and IL-17 in the supernatant of A549 cells were evaluated. (F) The activity of SIRT1 were detected. (G) The representative immunoblots of SIRT1, acetylated NF-κB-p65, and NF-κB-p65 in the nuclear protein, H3 was used as a loading control. The levels of SIRT1 (H), acetylated NF-κB-p65 (I), nuclear NF-κB-p65 (J), and the ratio of acetylated NF-κB-p65/nuclear NF-κB-p65 (K) were analyzed ($n = 3$ independent experiments). (L) The DNA binding activity of NF-κB were assessed. Data were means ± S. D., $n = 5$ independent experiments. $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. TNF-α treated group, $^{\$\$}P < 0.01$ vs. TNF-α+Acacetin group.

3.6. Silencing SIRT1 abolished the effects of acacetin on improving SIRT1 function and restraining NF-κB-p65 acetylated activation induced by TNF-α

To further verify whether SIRT1 plays a key role in acacetin inhibition on the inflammatory injury of A549 cells induced by TNF-α, siRNA molecule targeting SIRT1 were utilized. As shown in Fig. 5A, the viability of A549 cells transfected with SIRT1 siRNA were reduced lightly. While TNF-α instillation further lowered the viability of A549 cells transfected with SIRT1 siRNA, which were not altered by acacetin application. Moreover, transfection with SIRT1 siRNA abolished the efficacy of acacetin on improving the content of LDH, TNF-α, IL-6, IL-17 and IL-1β in the supernatant of A549 cells (Fig. 5B–E). Also of note is that SIRT1 activity and the nuclear protein expression of SIRT1 were remarkably reduced in cells transfected with SIRT1 siRNA but not control siRNA. TNF-α instillation or acacetin application cannot alter SIRT1 activity levels and the nuclear protein expression of SIRT1 in the cells transfected with SIRT1 siRNA (Fig. 5F–H). SIRT1 activity and the nuclear protein expression of SIRT1 observed in the cells transfected with control siRNA was decreased with TNF-α instillation, which was reversed by acacetin (Fig. 5F–H). Meanwhile, the nuclear protein expression of NF-κB-p65 and acetyl-NF-κB-p65, the ratio of ac-NF-κB-p65/nuclear NF-κB-p65 and the DNA binding activity of NF-κB in the cells transfected with control siRNA were significantly increased after TNF-α instillation, which were markedly improved with acacetin application (Fig. 5I–L). But these parameters mentioned above in the cells transfected with SIRT1 siRNA were ulteriorly increased after TNF-α instillation, and were not reversed by acacetin at all (Fig. 5I–L). These results confirm that acacetin inhibits TNF-α-induced inflammatory injury of A549 cells is mediated by SIRT1/NF-κB pathway.

4. Discussion

In the present study, we showed that acacetin possessed the protective efficacy on acute lung injury. Results showed that acacetin lowered the mortality of mice, attenuate acute lung injury by improving the activity and expression SIRT1, thereby suppressing the acetylated activation of NF-κB-p65 and the release of downstream inflammatory cytokines, which indicated that acacetin, as a new agonist for SIRT1, exerts an important therapeutic potential for the treatment of acute lung injury.

Previous studies reported that acacetin has satisfactory effects on prevention and treatment of various cardiovascular diseases [13,14]. Acacetin exhibits selective anti-atrial fibrillation properties by preferentially blocking atrial potassium currents (I_{Kur}, I_{KACh}, I_{sKCa} and I_{to}) [27]. Additionally, acacetin has protective efficacy against myocardial ischemia/reperfusion injury by suppressing inflammation, oxidation, and apoptosis via upregulating AMPK/Nrf2 signaling pathway [28,29]. Particularly, the antioxidant activities and anti-inflammatory effects of acacetin were assessed on LPS-induced acute lung injury in mice and LPS-stimulated RAW 264.7 macrophage [30,31]. But the potential mechanism of acacetin on acute lung injury remains not fully investigated.

In this study, we found that 20 mg/kg of acacetin significantly cut down the mortality of mice, and prolonged the survival time of mice, relieved the severity of acute lung injury, and attenuated inflammatory exudation and pulmonary edema. Besides, there were great improvements in the lung histopathologic changes after acacetin treatment. The increased of lung-to body weight ratio, wet-to-dry ratio, MPO activity and the release of inflammatory cytokines were also markedly decreased after acacetin treatment. Meanwhile, acacetin concentration-dependently protected A549 cells against TNF-α-induced injury as revealed by MTT assay. And the inflammatory cytokines, such as LDH, TNF-α, IL-6, IL-17 and IL-1β in the supernatant, were all concentration-dependently lessened by acacetin treatment. The above results indicated the key role of acacetin in the protection of acute lung injury.

It is well known that SIRT1/NF-κB pathway is one of the important mediators contributing to acute lung injury [5]. SIRT1 directly inhibits the activity of NF-κB and restrain NF-κB-mediated downstream signal pathway via blocking the acetylated activation of NF-κB-p65 at lysine310 [32]. Additionally, SIRT1 suppresses the nuclear aggregation of NF-κB-p65, subsequently downgrades the DNA binding activity of NF-κB [33]. Thus, SIRT1 has strong anti-inflammatory effects. However, there is mounting evidence both *in vivo* and *in vitro* showing that the hindered activity and downregulated expression of SIRT1 are involved in the pathogenesis of acute inflammation related diseases [34], which are also an important reason for the progression of acute lung injury. Accordingly, some drugs, such as resveratrol, targeted SIRT1 and increased its expression and activity, exhibit great anti-inflammatory activity for the treatment of acute inflammation related diseases [35,36]. As a novel agonist for SIRT1, acacetin has been reported that it can increase SIRT1 activity and upregulate SIRT1 expression [15,28,37–39]. In this study, we found that LPS administration *in vivo* or TNF-α irritation *in vitro* reduced NAD $^{+}$ levels and NAD^{+}/NADH ratio**,** thereby influenced the activity of SIRT1, as well as the mRNA and nuclear protein expression of SIRT1. Not only that, the nuclear protein expression of NF-κB-p65, the acetylated form of NF-κB-p65 (K310), the ratio of ac-NF-κB-p65/nuclear NF-κB-p65 and the DNA binding activity of NF-κB were prominently elevated in LPS treated mice or TNF-α stimulated A549 cells. Acacetin treatment efficiently improved NAD^{+} levels and the ratio of NAD^{+}/NADH, reversed the mRNA and nuclear protein expression of SIRT1 and the activity of SIRT1, and reduced the nuclear protein expression of NF-κB-p65, the acetylated form of NF-κB-p65 (K310), the ratio of ac-NF-κB-p65/nuclear NF-κB-p65 and the DNA binding activity of NF-κB both *in vivo* and *in vitro*. However, SIRT1 siRNA significantly abolished the protective effect of acacetin on A549 cells stimulated by TNF-α. These results fully explain that the therapeutic effect of acacetin in acute lung injury is involved in upregulating SIRT1 function and thereby suppressing the acetylated activation NF-κB.

There were still some limitations of this study. First, SIRT1-mediated deacetylation profoundly influences multiple biological processes during inflammation. We only covered the SIRT1/NF-κB pathway involvement in the therapeutic effects of acacetin on acute lung injury, and the other potential molecules or signaling pathways couldn't be excluded. Second, in the complex landscape of ALI, a multitude of cell types, including alveolar type I and type II epithelial cells, alveolar macrophages, and pulmonary microvascular endothelial cells, contribute to the intricate regulatory network. For instance, NF-κB requires acetylation mediated by p300 to produce inflammatory mediators such as TNF-α, IL-6, and IL-17. SIRT1 activation inhibits this step and also modulates glycolysis in

macrophages. Our study primarily elucidated the protective mechanism of acacetin in A549 cells against ALI. To comprehensively delineate the cellular mechanisms underlying acacetin's ameliorative effects in ALI, future research should extend to cell-specific *in vitro* studies, encompassing alveolar macrophages and pulmonary microvascular endothelial cells, to enrich our understanding of its therapeutic potential.

5. Conclusion

Collectively, our results indicated that acacetin treatment prominently reduced the rate of lethality in LPS-treated mice, attenuating the severity of LPS-induced acute lung injury *in vivo* and TNF-α-stimulated cellular injury *in vitro*, which have a bearing on upregulating SIRT1 function thereby suppressing the acetylated activation NF-κB and the release of downstream inflammatory cytokines. Hence, as a new agonist for SIRT1, acacetin exerts an important therapeutic effect for the treatment of acute lung injury.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Compliance with ethical standards

All animal experiments were approved by Animal Ethical Experimentation Committee of the Fourth Military Medical University in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (No. IACUC-20200901).

CRediT authorship contribution statement

Lanxin Gu: Investigation, Formal analysis, Data curation. **Yue Yin:** Methodology, Investigation, Funding acquisition, Formal analysis. **Manling Liu:** Writing – review & editing, Writing – original draft, Funding acquisition. **Lu Yu:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition.

Declaration of competing interest

No potential conflict of interest was reported by the author(s).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37083.

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