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Oleuropein alleviates sepsis-induced acute lung injury *via* the AMPK/Nrf-2/HO-1 signalingShan-Hu Wang, Yang-Yang Wu, Xiao-Jiao Xia 

Department of Pediatrics, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325027, China

ABSTRACT

Objective: To explore the effect of oleuropein on sepsis-induced acute lung injury (ALI) *in vitro* and *in vivo* and investigate the underlying mechanism.

Methods: In an lipopolysaccharide (LPS)-mediated cell model of sepsis-induced ALI and a cecal ligation and puncture-induced mouse model of septic ALI, CCK-8 assay and flow cytometry analysis were used to detect cell activity and apoptosis. ELISA and relevant assay kits were used to measure the levels of inflammatory cytokines and oxidative stress, respectively. Western blot was applied to determine the expression of apoptosis- and AMP-activated protein kinase (AMPK)/nuclear factor erythroid 2-related factor-2 (Nrf-2)/heme oxygenase-1 (HO-1) signaling-associated proteins. JC-1 staining, adenosine triphosphate (ATP) assay kit, and MitoSOX Red assays were performed to detect mitochondrial membrane potential, ATP content, and mitochondrial ROS formation, respectively. Moreover, lung injury was evaluated by measuring lung morphological alternations, lung wet-to-dry ratio, myeloperoxidase content, and total protein concentration.

Results: Oleuropein reduced inflammatory reaction, oxidative damage, and apoptosis, and ameliorated mitochondrial dysfunction in LPS-exposed BEAS-2B cells and mice with septic ALI. Besides, oleuropein activated the AMPK/Nrf-2/HO-1 signaling pathway. However, these effects of oleuropein were abrogated by an AMPK inhibitor compound C.

Conclusions: Oleuropein can protect against sepsis-induced ALI *in vitro* and *in vivo* by activating the AMPK/Nrf-2/HO-1 signaling, which might be a potential therapeutic agent for the treatment of sepsis-induced ALI.

KEYWORDS: AMPK/Nrf-2/HO-1 signaling; Inflammatory response; Lung damage; Mitochondrial dysfunction; Oleuropein; Oxidative stress; Sepsis

1. Introduction

Sepsis is caused by dysregulation of immune reaction to infection[1]. As a severe complication of sepsis, acute lung injury (ALI) comprising varied acute respiratory failure disorders may develop into acute respiratory distress syndrome[2], which is also increasingly regarded as a potential contributor to multiple organ injury and even death[3]. It is estimated that 25%-50% of sepsis patients may develop sepsis-induced ALI[4]. Given that the etiology of septic ALI is complex and the patient's prognosis is dismal, the mechanisms underlying sepsis-induced ALI need to be investigated to develop effective therapeutic strategies.

Oleuropein, a potent antioxidant prevalent in the Oleaceae family, possesses a spectrum of pharmacological properties including anti-inflammatory, anti-tumor, antiviral, and hypoglycemic effects, along

Significance

Previous studies have disclosed the protective activity of oleuropein against lung damage, but its role in sepsis-induced acute lung injury remains unclear. In the present study, oleuropein relieved inflammation, oxidative damage, apoptosis, as well as mitochondrial dysfunction *in vitro* and *in vivo* *via* the activation of AMPK/Nrf-2/HO-1 signaling. However, its efficacy needs further investigations.

 To whom correspondence may be addressed. E-mail: Xiaxiajiao@163.com

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with cardioprotective effects[5]. Existing research has underlined the efficacy of this compound in treating diverse human disorders, such as diabetes complications, cardiovascular complications, neurodegenerative diseases, cancers, *etc*[6,7]. Moreover, emerging studies suggested that oleuropein may exert protective effects on lung damage[8–10]. Nonetheless, there are few studies regarding the specific role and mechanism of this compound in septic ALI.

Nuclear factor erythroid 2-related factor-2 (Nrf-2)/heme oxygenase-1 (HO-1) signaling is a classical antioxidant pathway extensively involved in human diseases[11,12]. AMP-activated protein kinase (AMPK) is a kinase that functions in the upstream of Nrf-2[13]. Emerging evidence has established that the AMPK/Nrf-2/HO-1 signaling is closely related to the process of sepsis-mediated ALI[14–16]. Additionally, oleuropein has been proposed to play a protective role in human diseases by motivating the AMPK signaling and the Nrf-2/HO-1 signaling[17–19]. However, whether oleuropein functions in sepsis-related ALI through modulating the AMPK/Nrf-2/HO-1 signaling remains unclear. Therefore, this research was conducted to explore the effect of oleuropein on septic ALI and its underlying mechanism.

2. Materials and methods

2.1. Cell culture and treatment

Human lung epithelial cells BEAS-2B obtained from Cell Bank of Typical Culture Preservation Committee of Chinese Academy of Science (Shanghai, China) were incubated in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 100 U/mL penicillin/streptomycin (Hyclone, USA) in a humidified incubator with 5% CO₂ at 37 °C. After the establishment of a cellular model induced by lipopolysaccharide (LPS) at a concentration of 10 µg/mL[20], BEAS-2B cells were treated with oleuropein at varying concentrations (5, 10 and 20 µM; MedChemExpress, USA)[10]. In addition, cells were exposed to an AMPK inhibitor compound C (10 µM, MedChemExpress, USA)[21].

2.2. Cell Counting Kit-8 (CCK-8)

Following treatment, BEAS-2B cells were inoculated into 96-well culture plates (5×10^3 cells/well). The cells were further incubated with 10 µL of CCK-8 solution (Selleck Chemicals, USA) for 2 h. Cell activity was determined by measuring the OD value at 450 nm under a microplate reader (BioTek, Winooski, VT).

2.3. ELISA

Tumor necrosis factor-alpha (TNF-α, human cat. no. RJ14857;

mouse cat. no. RJ17929), interleukin-6 (IL-6, human cat. no. RJ11850; mouse cat. no. RJ16958), interleukin-8 (IL-8, human cat. no. RJ11853; mouse cat. no. RJ23791), and interleukin-1 beta (IL-1β, human cat. no. RJ11824; mouse cat. no. RJ16944) concentrations in cell supernatants or mouse lung tissues were calculated by relevant ELISA kits (RENJIEBIO, Shanghai, China) according to the manufacturer's instructions. Under a microplate reader, the OD values were recorded at 450 nm.

2.4. Estimation of oxidative stress markers

Malondialdehyde (MDA; cat. no. A003-1-2), superoxide dismutase (SOD; cat. no. A001-3-2), as well as glutathione (GSH; cat. no. A006-2-1) contents in the cell supernatants or mouse lung tissues were respectively quantified by corresponding assay kits from Nanjing Jiancheng Inc. (Nanjing, China). Under a microplate reader, the OD values were recorded at 532 nm, 450 nm, and 420 nm, respectively.

2.5. Flow cytometry

For the measurement of cell apoptosis, Annexin V-FITC Apoptosis Detection Kit (Bestbio, China) was used. After PBS washing, the harvested BEAS-2B cells were then gently resuspended in a binding buffer. Subsequently, cells were labeled by Annexin V-FITC for 10 min in the dark on ice and then stained with propidium iodide (PI) for 15 min. The apoptotic rate was assessed by using a flow cytometer (Beckman Coulter, Inc.) according to the manufacturer's instructions, and quantified by Expo32 v1.2 software (Beckman Coulter, Inc.).

2.6. 5, 5', 6, 6' - Tetrachloro - 1, 1', 3, 3' - tetraethylbenzimidazolocarbo-cyanine iodide (JC-1) staining

BEAS-2B cells were inoculated in 6-well plates (2×10^5 cells/well) before designated treatment. Subsequently, BEAS-2B cells were stained by JC-1 dye working solution (2.5 µg/mL; Molecular Probes, Life Technologies) in pre-warmed serum-deprived medium and then incubated for 30 min at 37 °C in the dark. Under a fluorescence microscope (Olympus, Japan), mitochondrial membrane potential (MMP) was examined.

2.7. Determination of adenosine triphosphate (ATP) generation

ATP content was detected using an ATP assay kit (cat.no. ab113849; Abcam). After lysis, BEAS-2B cells (1×10^4 cells per well) were exposed to ATP substrate solution and the luminescence was examined by a microplate reader.

2.8. MitoSOX Red assay

BEAS-2B cells were spread onto a 96-well black opaque plate (1×10^5 cells/mL) before designated treatment. Afterward, the cells were processed with 10 μ M MitoSOX red working solution (Thermo Fisher Scientific) at 37 °C for 20 min. After PBS washing and trypsinization, the fluorescent intensity (Ex/Em=510/580 nm) was estimated with the application of a fluorescence spectrophotometer (Perkin-Elmer, USA).

2.9. Establishment of a mouse model of septic ALI with cecal ligation and puncture (CLP)

A total of 18 C57BL/6 male mice (8-12 weeks) were procured from Shanghai Sipple-Bikai Laboratory Animal Co., Ltd (Shanghai, China). The experimental animals were fostered individually in a humidity-controlled (60%-65%) and temperature-controlled (22-25 °C) environment under a 12-h light/dark cycle with unlimited access to water and food. The experimental animals were randomly divided into 3 groups: the sham group, the CLP group, and the CLP + oleuropein 50 mg/kg group ($n = 6$). After narcotization of mice with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), the cecum of mice in the CLP group was exposed and the abdominal wall was incised 2 cm. Next, the cecum was penetrated at the distal end of the ligation using a 21-gauge needle. After the extrusion of a small amount of feces, the cecum was repositioned and the abdominal cavity was sutured. Mice in the sham group underwent the same surgical procedures without puncture or ligation. Mice in the CLP + oleuropein 50 mg/kg group were intraperitoneally injected with oleuropein (50 mg/kg) following CLP surgery[8]. After the sacrifice of mice using pentobarbital sodium (150 mg/kg), mouse lung tissues were harvested.

2.10. Hematoxylin-eosin (H&E) staining

After being immobilized by formalin and embedded with paraffin, mouse lung tissues were sliced into 4 μ m-thick sections. After dewaxing, the slices were processed with hematoxylin (Solarbio, Beijing, China) for 5 min and eosin (Solarbio, Beijing, China) for 2 min. Following the block with a neutral resin, the prepared sections were observed using a light microscope (Nikon, Tokyo, Japan). The lung injury score was also calculated.

2.11. Lung tissue wet/dry weight ratio

To determine the wet/dry weight ratio, the mice's lungs were weighed immediately and dried in an oven at 60 °C for 48 h.

2.12. Myeloperoxidase (MPO) activity assay

After the homogenization of mouse lung tissues, MPO activity was examined with an MPO assay kit (Nanjing Jiancheng Co., Ltd., China). Under a microplate reader, the OD value was read at 460 nm.

2.13. Evaluation of total protein concentration in bronchoalveolar lavage fluid (BALF)

Following the gentle lavage of lung tissues three times, BALF was prepared and centrifuged at 250 $\times g$ at 4 °C for 10 min. The protein concentration was calculated using the BCA method (Pierce, Rockford, IL, USA).

2.14. Western blot

Following the homogenization of both cells and tissues in RIPA buffer (Hangzhou Fude Biotechnology Co., Ltd., China), protein concentrations were quantified utilizing the BCA method. Subsequently, these proteins that were subjected to electrophoresis were transferred onto a PVDF membrane. The membranes were immersed in 5% BSA for blocking and immunoblotted with primary antibodies (Bcl-2, 1:1 000; #AF6139; Bax, 1:1 000; #AF0120; cleaved caspase 3, 1:1 000; #AF7022; caspase 3, 1:1 000; #AF6311; Nrf2, 1:1 000; #AF0639; histone H3, 1:1 000; #AF0863; HO-1, 1:1 000; #AF5393; AMPK, 1:1 000; #AF6423; p-AMPK, 1:1 000; #AF3423; GAPDH, 1:3 000; #AF7021), which were all obtained from Affinity Biosciences. Subsequently, the membranes were probed with secondary antibodies linked with HRP (ab6721; 1:2000; Abcam). The bands were visualized using ECL reagent (Pierce, Rockford, IL, USA) and then analyzed with Quantity One 1-D Analysis Software (Bio-Rad, USA).

2.15. Statistical analysis

The data were presented as mean $\pm$ standard deviation (SD) and analyzed by one-way ANOVA, followed by Tukey's *post-hoc* test. $P < 0.05$ indicated statistical significance. GraphPad Prism 9.5 software (La Jolla, USA) was employed for statistical analysis.

2.16. Ethical statement

All experiments involving animals complied with the international guidelines for care and use of laboratory animals (National Institutes of Health Publication No. 85-23, revised 1996) and were ethically approved by the Animal Ethics Committee of Wenzhou Medical University (Approval No. 14301).

3. Results

3.1. Oleuropein mitigates inflammatory reaction and oxidative damage in LPS-induced BEAS-2B cells

CCK8 assay showed that different concentrations of oleuropein (5, 10, and 20 μM) produced no effect on BEAS-2B cell viability (Figure 1A). Subsequently, a cell model of sepsis-induced ALI was induced by LPS, and the viability of BEAS-2B cells was diminished following the treatment of LPS. An increase in cell viability was observed after treatment with oleuropein at all concentrations (Figure 1B). In ELISA assay, LPS significantly increased TNF- α , IL-6, IL-1 β , and IL-8 levels, which were all concentration-dependently declined by oleuropein (Figure 1C). Additionally, MDA activity was

raised while SOD and GSH concentrations were depleted in LPS-challenged BEAS-2B cells. However, administration with oleuropein concentration-dependently reduced MDA activity and increased SOD and GSH activities in LPS-stimulated BEAS-2B cells (Figure 1D). Overall, oleuropein possesses anti-inflammatory and anti-oxidative stress properties in LPS-induced BEAS-2B cells.

3.2. Oleuropein suppresses LPS-induced apoptosis in BEAS-2B cells

To unveil the effects of oleuropein on the apoptosis of LPS-exposed BEAS-2B cells, flow cytometry analysis was conducted. As portrayed in Figure 2A, the LPS-induced apoptosis level in BEAS-2B cells was alleviated by oleuropein in a concentration-dependent manner. Further, Western blot analysis showed that in BEAS-2B

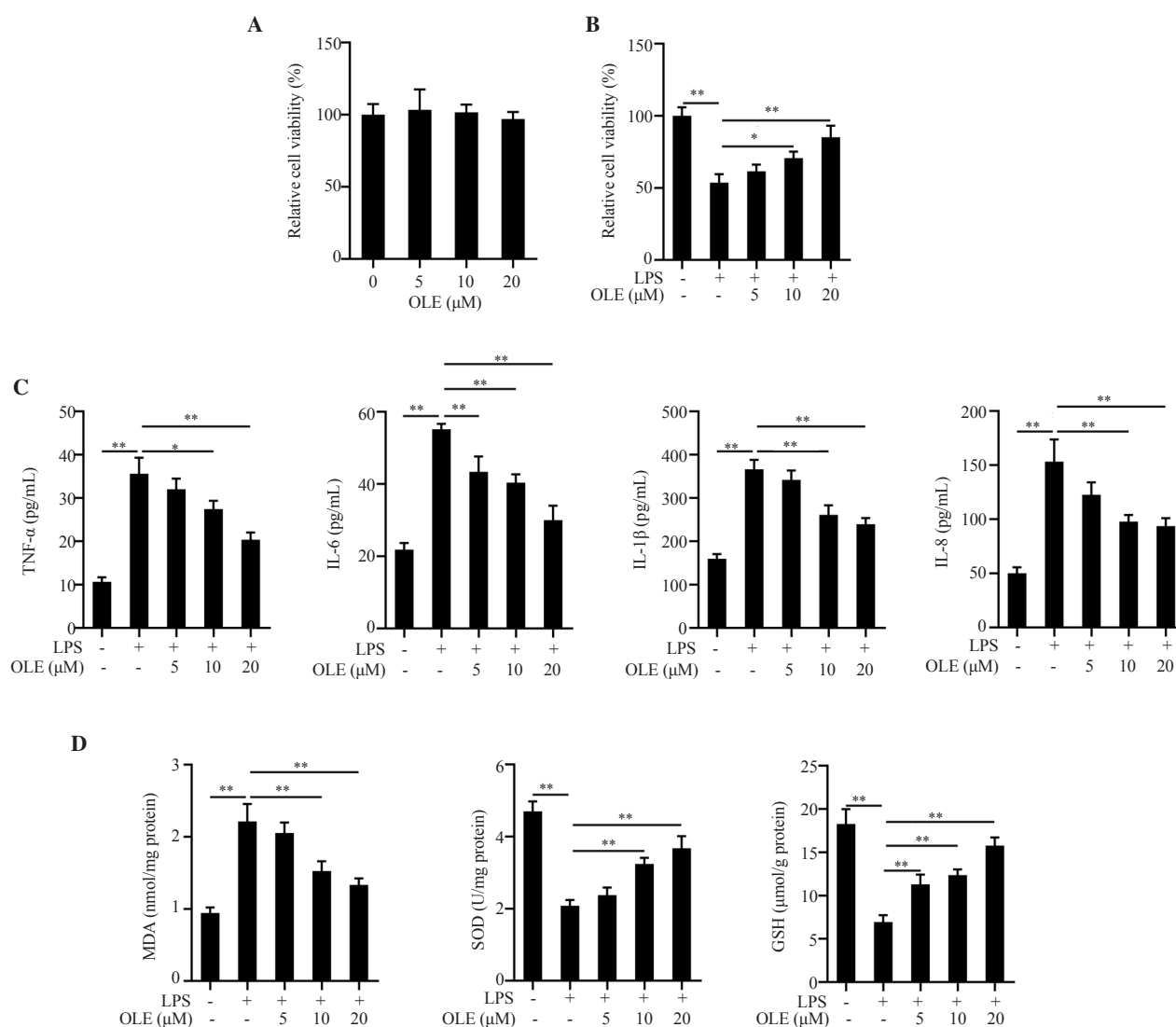


Figure 1. Oleuropein mitigates inflammation and oxidative stress in LPS-induced BEAS-2B cells. BEAS-2B cells were exposed to 10 $\mu\text{g/mL}$ LPS and then treated with oleuropein at 5, 10 and 20 μM . (A-B) CCK-8 assay was used to detect the viability of BEAS-2B cells exposed to LPS and varying concentrations of oleuropein. (C) The inflammatory levels were determined by ELISA assay. (D) The activities of oxidative stress markers were detected using corresponding assay kits. The data are presented as mean $\pm$ standard deviation (SD) of triplicate experiments and analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. * $P < 0.05$ and ** $P < 0.01$. OLE: oleuropein; LPS: lipopolysaccharide; MDA: malondialdehyde; SOD: superoxide dismutase; GSH: glutathione.

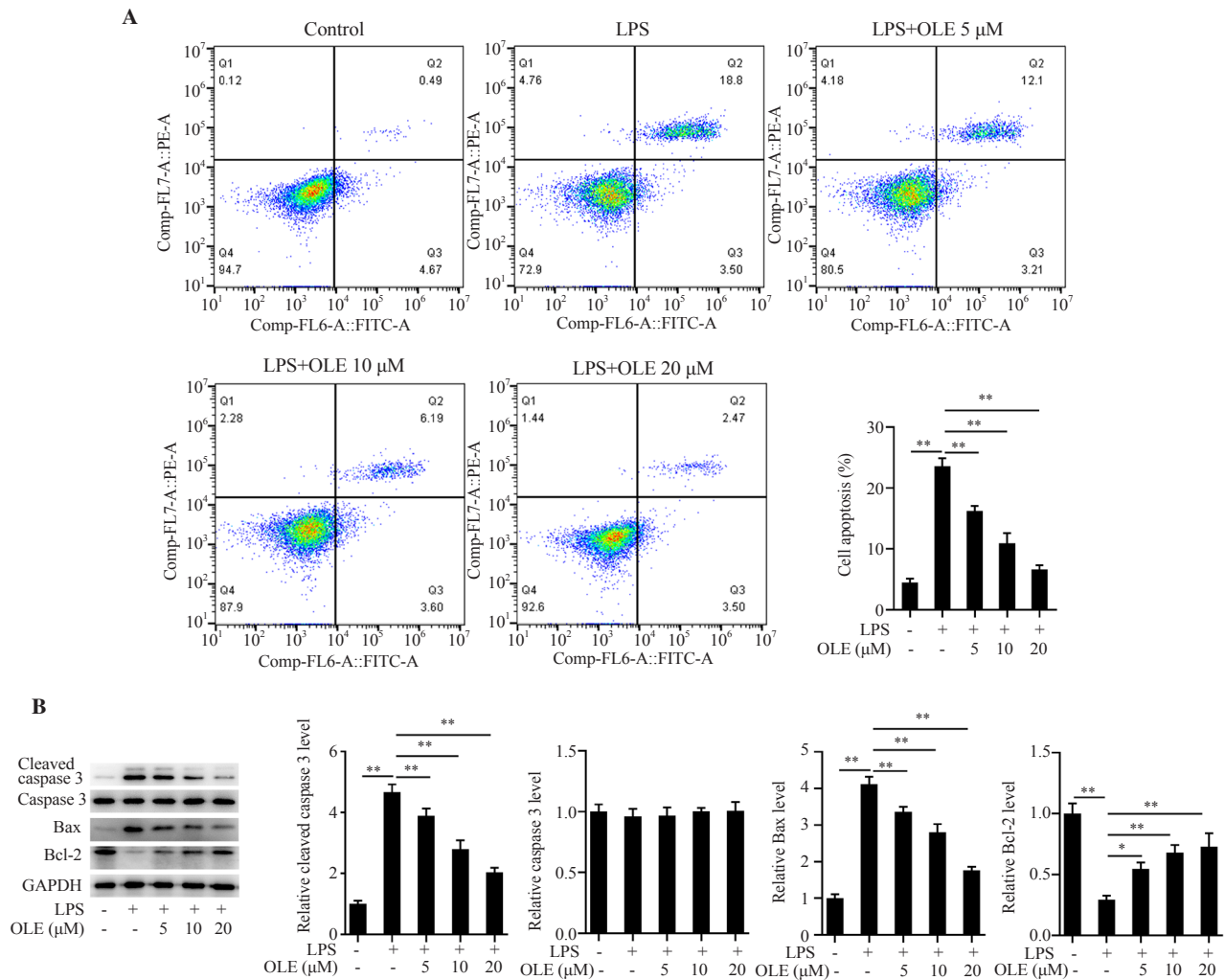


Figure 2. Oleuropein suppresses LPS-induced apoptosis in BEAS-2B cells. (A) Flow cytometry analysis was used to detect cell apoptosis. (B) Western blot was used to detect the expression of proteins associated with apoptosis. The data are presented as mean $\pm$ SD of triplicate experiments and analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. * $P < 0.05$ and ** $P < 0.01$.

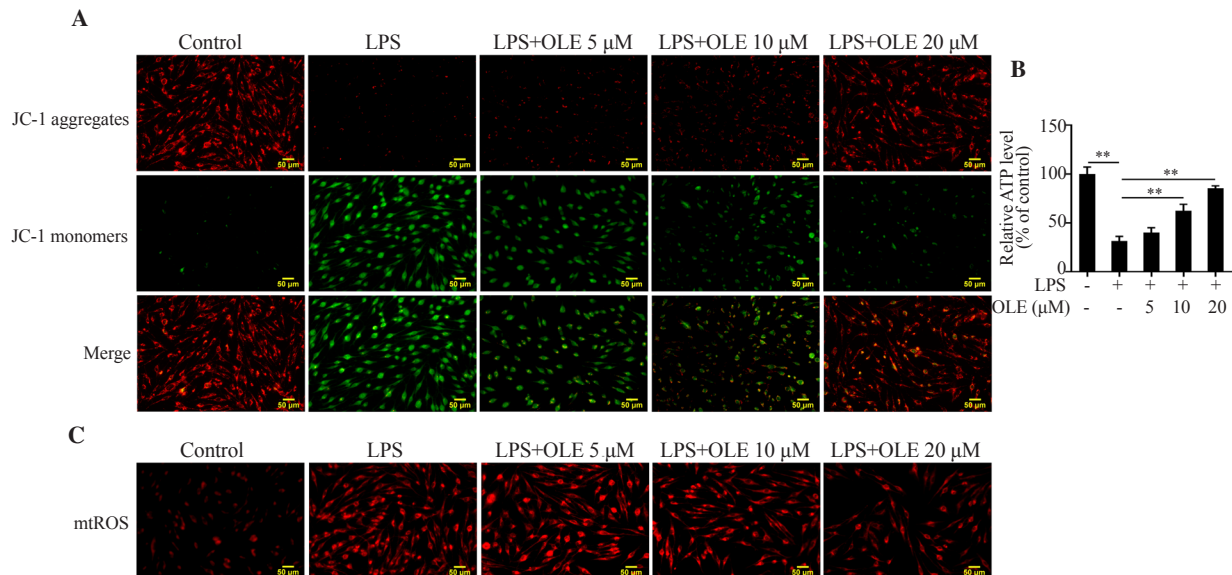


Figure 3. Oleuropein ameliorates mitochondrial dysfunction in LPS-induced BEAS-2B cells. (A) JC-1 staining was used to evaluate mitochondrial membrane potential (MMP) (Magnification: $\times 200$). (B) Adenosine triphosphate (ATP) production was detected using corresponding assay kits. (C) MitoSOX Red assay detected mitochondrial ROS (mtROS) formation (Magnification: $\times 200$). The data are presented as mean $\pm$ SD of triplicate experiments and analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. ** $P < 0.01$.

cells, cleaved caspase 3 and Bax expressions were increased and Bcl-2 expression was lowered by LPS, which were all reversed after treatment with oleuropein (Figure 2B). All these results indicate that oleuropein could inhibit apoptosis in an LPS-mediated cell model of sepsis-induced ALI.

3.3. Oleuropein ameliorates mitochondrial dysfunction in LPS-induced BEAS-2B cells

Mitochondrial dysfunction is widely known as a common event during the process of septic ALI. As illustrated by JC-1 staining (Figure 3A), LPS exposure resulted in an increase in green fluorescence and a decrease in red fluorescence. After oleuropein treatment, green fluorescence was markedly decreased while red fluorescence was intensified, suggesting that oleuropein alleviated the collapse of MMP. Also, ATP production was inhibited in LPS-challenged BEAS-2B cells and was enhanced in oleuropein-treated BEAS-2B cells exposed to LPS (Figure 3B). Besides, the LPS-induced elevated mitochondrial ROS level in BEAS-2B cells was dose-dependently downregulated by oleuropein (Figure 3C).

3.4. Oleuropein upregulates AMPK/Nrf-2/HO-1 signaling

Nuclear Nrf2, Nrf2, HO-1, and p-AMPK expressions were all

increased following the induction of LPS, which were all promoted by oleuropein treatment (Figure 4A-C). In view of the prominent effect in the LPS + oleuropein 20 μ M group, oleuropein at a concentration of 20 μ M was selected for follow-up experiments.

3.5. Oleuropein protects against LPS-elicited inflammation, oxidative stress, apoptosis, and mitochondrial dysfunction in BEAS-2B cells by activating the AMPK/Nrf-2/HO-1 signaling

To confirm whether oleuropein alleviates septic ALI by the AMPK/Nrf-2/HO-1 signaling, the AMPK inhibitor compound C was applied. As expected, after the addition of compound C, the distinct upregulation of nuclear Nrf2, Nrf2, HO-1, and p-AMPK in oleuropein-treated BEAS-2B cells were all depleted (Figure 5A-C). Meanwhile, in LPS-insulted BEAS-2B cells, oleuropein treatment prominently decreased TNF- α , IL-6, IL-1 β , and IL-8 levels, which were all upregulated by compound C (Figure 5D). Compound C also restored the descending MDA level and ascending SOD and GSH activities induced by oleuropein in LPS-treated BEAS-2B cells (Figure 5E). In addition, oleuropein decreased apoptosis and raised Bcl-2 expression while lowering Bax and cleaved caspase 3 expression in BEAS-2B cells upon exposure to LPS, which were all abrogated by compound C (Figure 6A-B). Moreover, in LPS-

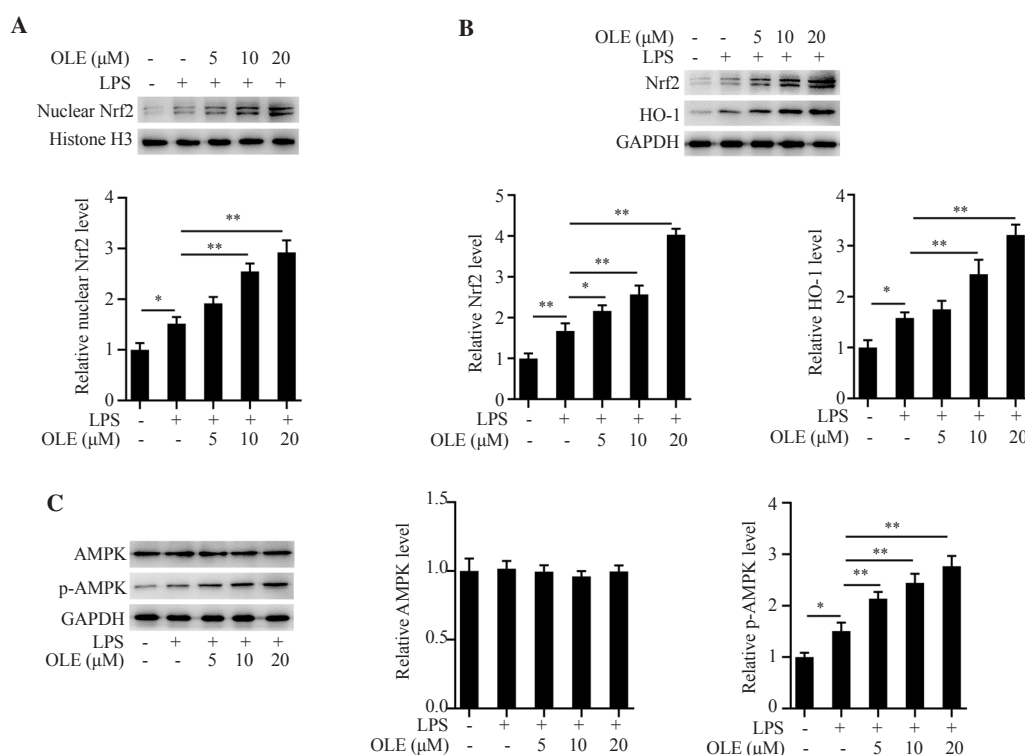


Figure 4. Oleuropein upregulates the AMPK/Nrf-2/HO-1 signaling. Western blot was used to detect (A) nuclear Nrf2, (B) Nrf2, HO-1, (C) p-AMPK, and AMPK expression. The data are presented as mean $\pm$ SD of triplicate experiments and analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. * P <0.05 and ** P <0.01.

challenged BEAS-2B cells, the mitochondrial damage and ROS generation were reduced while ATP content was increased following treatment with oleuropein, which were all partially counteracted by compound C (Figure 6C-E).

3.6. Oleuropein alleviates pathological lung injury, inflammatory reaction, oxidative damage, and apoptosis in a CLP-induced mouse model of septic ALI

To further corroborate the role of oleuropein in septic ALI, animal experiments were performed. Through H&E staining, it was noticed that the severe pathological damage in the CLP group was ameliorated by oleuropein at the concentration of 50 mg/kg, which

was also reflected by decreased lung injury score after oleuropein administration (Figure 7A-B). Besides, in CLP-induced mice of septic ALI, oleuropein significantly reduced lung wet-to-dry ratio, MPO activity as well as total protein concentration in BALF (Figure 7C-E), implying that oleuropein decreased pulmonary edema and injury. Consistent with the results of *in vitro* experiments, oleuropein downregulated MDA, TNF- α , IL-6, IL-1 β , and IL-8 levels while upregulating SOD and GSH activities in the lung tissues of CLP-induced septic mice (Figure 7F-G), highlighting that oleuropein also mitigated inflammatory reaction and oxidative damage in septic ALI *in vivo*. Furthermore, compared with the CLP group, Bcl-2 expression was increased, while Bax and cleaved caspase 3 expression were both decreased in the CLP + oleuropein 50 mg/kg group (Figure 7H). In

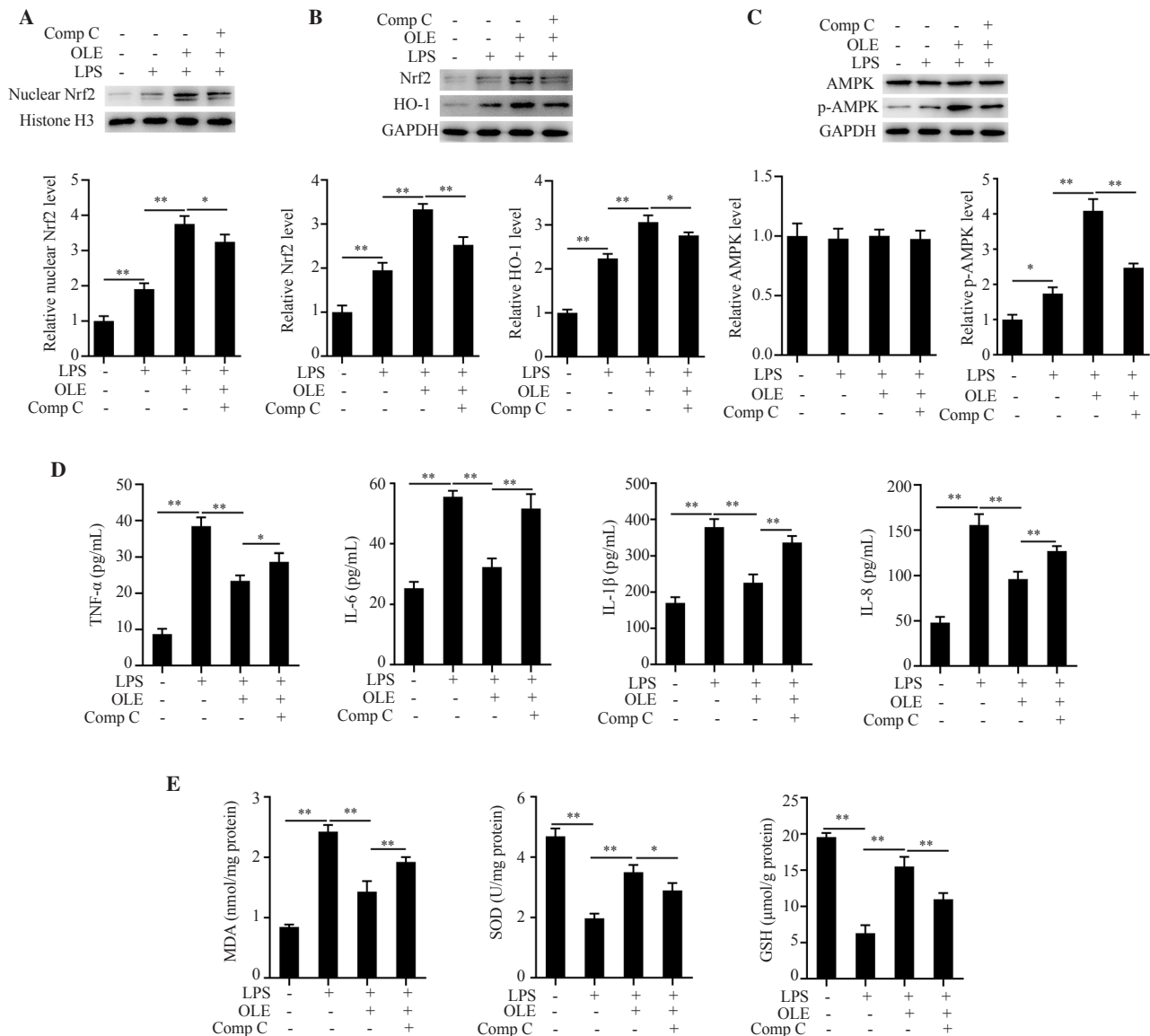


Figure 5. Oleuropein protects against LPS-induced inflammation and oxidative stress in BEAS-2B cells by activating the AMPK/Nrf-2/HO-1 signaling. BEAS-2B cells were exposed to 10 μ g/mL LPS and then treated with 20 μ M oleuropein and 10 μ M compound C. Western blot was used to detect (A) nuclear Nrf2, (B) Nrf2, HO-1, (C) p-AMPK, and AMPK expression. (D) ELISA assay was used to measure the inflammatory levels. (E) The activities of oxidative stress markers were detected using corresponding assay kits. The data are presented as mean $\pm$ SD of triplicate experiments and analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. * P <0.05 and ** P <0.01. Comp C: compound C.

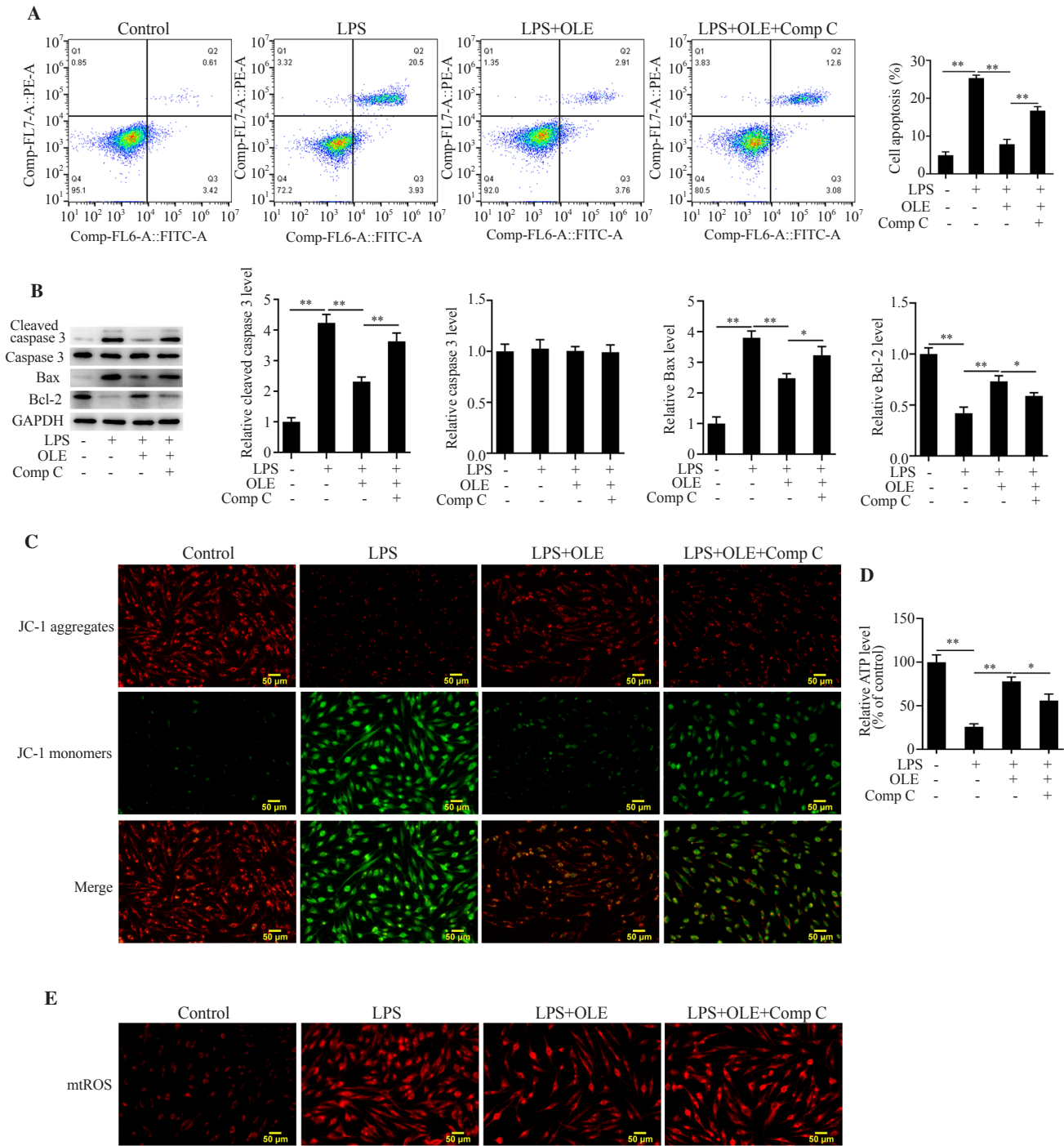


Figure 6. Oleuropein protects against LPS-induced apoptosis and mitochondrial dysfunction in BEAS-2B cells by activating the AMPK/Nrf-2/HO-1 signaling. (A) Flow cytometry analysis was used to detect cell apoptosis. (B) Western blot was applied to detect the expression of proteins associated with apoptosis. (C) JC-1 staining was used to detect MMP (Magnification: $\times 200$). (D) ATP production was detected using corresponding assay kits. (E) MitoSOX Red assay detected mtROS formation (Magnification: $\times 200$). The data are presented as mean $\pm$ SD of triplicate experiments and analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. * $P < 0.05$ and ** $P < 0.01$.

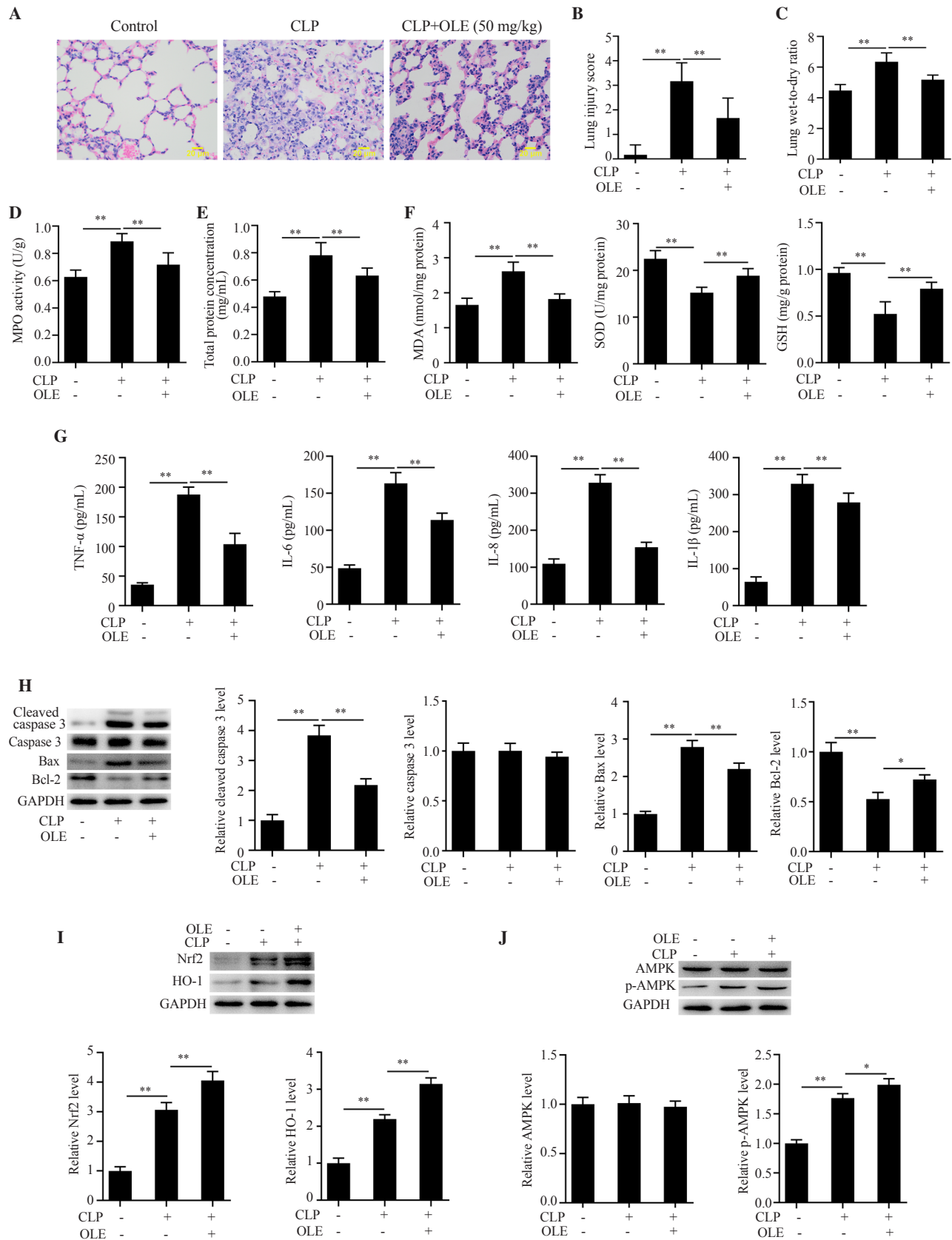


Figure 7. Oleuropein alleviates pathological lung injury, inflammatory response, oxidative stress as well as apoptosis in a CLP-induced mouse model of septic ALI. (A) H&E staining was used to detect the histological alternations of lung tissues (Magnification: $\times 400$). (B) Lung injury score. (C) Lung wet-to-dry ratio. (D) Myeloperoxidase activity was detected using corresponding assay kits. (E) Quantification of total protein concentration in bronchoalveolar lavage fluid. (F) The activities of oxidative stress markers were detected using corresponding assay kits. (G) ELISA assay was used to detect the inflammatory levels. (H-J) Cleaved caspase 3, caspase 3, Bax, Bcl-2, Nrf2, HO-1, p-AMPK and AMPK expressions were determined by Western blot. The data are presented as mean $\pm$ SD of six experiments and analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. * $P < 0.05$ and ** $P < 0.01$. CLP: cecal ligation and puncture.

addition, in the lung tissues of septic mice, oleuropein remarkably increased Nrf-2, HO-1, and p-AMPK expression (Figure 7I-J).

4. Discussion

Septic ALI remains a devastating disease characterized by excess and uncontrolled inflammatory response[22]. LPS, a component of the outer membrane of Gram-negative bacteria, is well documented as the primary driver of ALI by provoking the production of proinflammatory cytokines and initiating acute inflammation[23,24]. The CLP model that has been identified to ideally mimic the *in vivo* changes in patients with clinical sepsis is viewed as the gold standard in studying sepsis[25]. In the present work, an LPS-mediated cell model of sepsis-induced ALI and a CLP-induced mouse model of septic ALI were developed. It was noticed that oleuropein relieved inflammatory reactions, oxidative injury, apoptosis as well as mitochondrial dysfunction *in vitro*. The anti-inflammatory, anti-oxidant, and anti-apoptotic effects of oleuropein were also validated *in vivo*. Additionally, the protective role of oleuropein in LPS-induced cells might partly be attributed to the activation of AMPK/Nrf-2/HO-1 signaling.

Lung edema is a common pathophysiological sign of acute lung damage[26]. The present experimental data demonstrated that oleuropein significantly reduced lung damage following CLP surgery, as evidenced by reduced histological damage of lung tissues, and declined lung injury score, lung wet-to-dry ratio, MPO activity, and total protein concentration in BALF. It is well-accepted that inflammation is aberrantly activated during sepsis-induced ALI dependent on recruiting inflammatory cells and generating inflammatory cytokines such as TNF- α , IL-6, IL-1 β , and IL-8, and anti-inflammatory therapy is also considered a crucial and effective treatment strategy[27,28]. Previous evidence has shown the potent anti-inflammatory role of oleuropein[29]. In particular, oleuropein has been reported to decrease TNF- α , IL-1 β , and IL-6 expressions to mitigate inflammatory reactions in the hepatic and renal tissues of LPS-treated septic mice[30]. Moreover, oleuropein inhibited the production of inflammatory factors in lung tissues[8–10]. In line with these researches, our findings uncovered that increasing concentrations of oleuropein inhibited the levels of pro-inflammatory factors in LPS-insulted BEAS-2B cells and in the lung tissues of mice with CLP surgery. Oxidative stress remains a key pathological basis of sepsis-stimulated lung damage[27]. As a chain-breaking antioxidant, oleuropein downregulated pro-oxidant marker MDA level and upregulated anti-oxidant enzyme GSH level in the liver and kidney tissues of LPS-treated mice[30] and serum of LPS-treated rats[9]. Our investigation also showed that oleuropein administration concentration-dependently lowered MDA activity while elevating SOD and GSH activities in an LPS-induced septic ALI cell

model and mice with CLP-induced septic ALI. Also, apoptosis, a programmed cell death mode, is responsible for the maintenance of cellular homeostasis, which may be linked with sepsis-associated ALI[31,32]. In this study, the anti-apoptotic role of oleuropein was also confirmed in LPS-induced cells and septic mice, as manifested by the ascending anti-apoptotic Bcl-2 expression and the descending pro-apoptotic Bax and cleaved caspase 3 expression.

Mitochondrial dysfunction emerges as a major hallmark of sepsis-related ALI[33]. Mitochondria are organelles where energy is produced that are central to the whole life activities of the human body[34]. ATP is the common energy currency of cells[35]. Moreover, ROS formation in the mitochondria impairs organelle function[36]. MMP is a prerequisite for oxidative phosphorylation and ATP synthesis, the decline of which may represent damaged mitochondrial function[37]. Existing research has elaborated that oleuropein may improve mitochondrial function in spontaneously hypertensive rats[38] and neurodegenerative diseases[39]. This study revealed that oleuropein boosted MMP, and ATP production and decreased mitochondrial ROS level, indicating its suppressive effects in mitochondrial dysfunction in an LPS-induced septic ALI cell model.

AMPK, a heterotrimeric protein kinase, mainly works as an energy sensor in the modulation of metabolic homeostasis[13]. Upon AMPK activation, Nrf-2 is activated and accumulated in the nucleus, initiating the transcription of HO-1 which is a crucial antioxidant enzyme downstream of Nrf-2[11,13]. More intriguingly, numerous studies have unraveled that oleuropein may serve as an activator of the AMPK signaling and Nrf-2/HO-1 signaling in gestational diabetes mellitus[17], arthritis[18], and lupus nephritis[19]. In this study, it is proved that oleuropein raised nuclear Nrf2, Nrf2, HO-1, and p-AMPK expression in septic ALI both *in vitro* and *in vivo*. It is recognized that the AMPK/Nrf2/HO-1 signaling participates in the process of sepsis or ALI and may play a positive role in ameliorating inflammation and oxidative stress[14,40]. In this study, after the addition of an AMPK inhibitor compound C, the effects of oleuropein on the AMPK/Nrf2/HO-1 signaling, inflammatory reaction, oxidative injury, apoptosis as well as mitochondrial dysfunction were abrogated in LPS-induced BEAS-2B cells.

In summary, oleuropein demonstrated significant protective effects in septic ALI by attenuating inflammation, oxidative stress, apoptosis, and mitochondrial damage by activating the AMPK/Nrf-2/HO-1 signaling. Therefore, it might be a promising therapeutic agent for sepsis-induced ALI. However, the limitations of this paper cannot be negligible. For instance, the experiment was limited to BEAS-2B cells and the findings need to be verified in other cell lines. Additionally, the role of oleuropein in clinical samples also needs to be explored in the future to comprehensively confirm the potential of oleuropein in sepsis-associated ALI.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

SHW wrote the manuscript and analyzed the data. SHW and YYW performed the experiments and revised the manuscript for important intellectual content. XJX conceived and supervised the study, and confirmed the authenticity of all the raw data.

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