Neferine Enhances Autophagy to Alleviate Cytotoxicity and Apoptosis Induced by CSE in AEC-II through AMPK/mTOR Signaling Pathway

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Abstract
COPD is a clinical common chronic respiratory disease, its incidence case fatality rate is higher, there is currently no cure drugs and methods. In this study, in order to make clear its role in the development of autophagy in COPD, COPD cell model is established. To further explore whether regulating autophagy could have a protective effect to fight against CSE-induced cytotoxicity and apoptosis, we choose neferine as an autophagy inducer. Neferine activated cell autophagy in an vitro CSE-induced COPD cell model and gradually attenuated CSE-induced cell apoptosis. Furthermore, this process happens largely through the AMPK/mTOR signaling pathway. As a autophagic flux inhibitor, chloroquine abolished the prosurvival autophagy effect, and AMPK inhibitor Compound C blocked neferine-mediated autophagy and then neferine failed to protect COPD cell model from CSE-induced apoptosis. Overall, our findings suggested that neferine possibly has a potentially protective effect in cell damage mechanisms caused by CSE. It hints that neferine has the prospect of turning into a potential therapeutics to cure CSE-induced cytotoxicity and apoptosis and even COPD patients.

Key words: CSE; Neferine; Autophagy; AEC-II; AMPK/mTOR
1. Introduction

Chronic obstructive pulmonary disease (COPD) is a clinical common chronic respiratory disease characterized by persistent airflow limitation\cite{1-3}. Smoking is the most common risk factor for COPD. Smoking inhalation can cause airway pathophysiological changes and then result in airflow limitation. Nevertheless, the mechanism by which smoking provokes the airflow limitation in COPD patients has not been clearly determined, although inflammation\cite{4}, oxidative stress\cite{5}, and protease-antiprotease imbalance\cite{6} have all been proposed to contribute to the development of COPD\cite{7}. Several studies have proved that autophagy and apoptosis play roles in pathophysiological changes of airflow limitation\cite{8}.

Autophagy, a highly conserved autodigestion cellular program, aim to deliver cytoplasmic materials to the lysosome and then maintain cellular homeostasis\cite{9}. Many studies have shown autophagy has an essential role in the regulation of intracellular homeostasis and even in the pathogenesis of some chronic diseases\cite{10}. Initiation of autophagy and its important functions have sparked heated discussion in respiratory diseases\cite{11}. Cigarette smoke extract (CSE) was usually used to make COPD cell model\cite{12,13}. Alveolar epithelial type-II cells (AEC-II) of the rat is a stem cell of alveolar epithelium, whose functions include proliferation, damage repair, synthesis and secretion of pulmonary surfactant (PS), maintenance of fluid balance inside and outside the alveoli, and involvement in immune regulation. The impact of CSE on AEC-II is worth exploring further\cite{14,15}.

Neferine is a major bisbenzylisoquinoline alkaloid extracted from the green embryos of Nelumbo nucifera Gaertn (Fig. 4.). Considering its non-toxic nature\cite{16}, Nelumbo nucifera Gaertn is often used as a traditional Chinese medicine and even used to make soup or tea. It not only is a strong inducer of autophagy \cite{16-19} but also has anti-apoptosis properties\cite{20}. Increasing researches begin to focus on the possibly protective role of neferine in disease pathology, such as ROS-mediated oxidative stress and tissue injury \cite{10,20}. Furthermore, we hypothesized that neferine may have a protective role in reducing apoptosis in the pathogenesis of COPD. However, the protective mechanisms remain unclear. With these findings and hypotheses, we intended to explore the cytoprotective effect of neferine on CSE-induced cytotoxicity and apoptosis in an in-vitro model.

In this study, by establishing an in-vitro CSE-induced COPD cell model, we aim to confirm that neferine can enhance autophagy and reduce apoptosis without affecting the normal function of cells. Mechanistically, we showed that neferine plays an important role in inducing autophagy through AMPK/mTOR signaling pathway. Taken together, our results report that neferine may alleviate cell damage caused by CSE by enhancing autophagy, which can provide research basis for the future therapeutic application of COPD.
2. Materials and Methods

2.1. Chemicals and antibodies

Neferine (98.2% by HPLC with MW624.77), obtained from Chengdu purechem-standard co., LTD, diluted to concentration 3 mM with DMSO as stock solution. Chloroquine (autologously lysosomal degradation blocker) and Compound C (AMPK inhibitor) were obtained from Sigma-Aldrich. Primary monoclonal antibodies for Beclin-1, p62, LC3, mTOR, p-mTOR, phospho-AMPK, and GAPDH were all purchased from Abcam. The secondary antibodies both for anti-mouse and anti-rabbit were purchased from Cell Signaling Technology. Chemicals and antibodies for testing were prepared freshly.

2.2. Cell culture and treatment

Alveolar epithelial type-II cells of the rat (AEC-II) was cultured in 10% fetal bovine serum (FBS) and DMEM, Ham’s F-12 nutrient medium mixture (DMEM/F-12; Invitrogen-Gibco) at 37°C under 5% (vol/vol) CO2. According to each experiment, AEC-II were plated at an appropriate confluence. AEC-II stimulated with CSE was used to make COPD cell model. For stimulation, cells were exposed to the gradient concentrations of neferine, and then pretreated with chloroquine and Compound C. At last, cells were collected for analysis.

2.3. Preparation of cigarette smoke extract

Cigarette smoke extract (CSE) was made by a waterpipe three-tube smoking system and prepared from fifteen filtered cigarettes (CHIENMEN, each cigarette containing 11 mg tar and 0.8 mg nicotine, Daqianmen Group, Inc., China Hong Kong) as described previously[12,21,22]. Briefly, smoke was continuously collected from 2 cigarette and babbled through 20 mL prewarmed serum-free DMEM/F-12 containing 10% PBS into a bottle connected to the tee. Each cigarette needed to be fully burned within 2 minute. The pH of the CSE was adjusted to 7.4. The CSE was then identified as 100% CSE, sterilized through a 0.22μM filter (Sartorius Stedim Biotech, Goettingen, Germany), and stored at -80°C. CSE diluted to different concentrations in cell culture medium for each experiment.

2.4. Cell counting kit-8 (CCK8)

Cell proliferation and viability were measured with CCK8 (Tongren Company, Japan). AEC-II was seeded at a suitable density in a 96-well plate and incubated until the cells confluent to 60%. Then add different concentrations of CSE or neferine to test its toxic effect on AEC-II. After stimulation for 24h, 10ml of CCK8 and 100 ml of
DMEM/F-12 containing 10% PBS were added to each well, and after incubation at 37°C for 1h, 2h, 3h, and 4h, the absorbance values were measured by CCK8 assay after incubation to calculate the cell viability, and the color change was observed.

2.5. Western blot analysis

After treatment, cell protein was lysed in RIPA buffer (Beyotime, China) and supplemented with protease and phosphoric acid inhibitors. BCA protein assay kit (Beyotime, China) was used to detect the protein concentration. Different proteins were separated by SDS-PAGE and then transferred to membranes (Millipore, Bedford, MA), which were blocked in 5% nonfat milk for 2h and were incubated at 4°C overnight with specific antibodies against GAPDH (Cell Signaling), actin (Cell Signaling), LC3B, p62, mTOR, p-mTOR, AMPK and p-AMPK at a concentration of 1:1000 as recommended previously at 4°C overnight. Membranes were incubated for 2h at room temperature with secondary antibodies and then washed three times with TBST. The blots result were scanned by ChemiDocTM MP Imaging System [17].

2.6. Cellular immunofluorescence assay

AEC-II was inoculated onto a sterilized coverslip. After cultivation, AEC-II cells were washed 3 times using phosphate buffered saline, and then fixed with 4% paraformaldehyde for 20 minutes. Cells needed to be ruptured with 0.1% Triton X-100 (Biosharp), and washed with PBS 3 times, and with 3% bovine serum albumin. Block for 30 minutes and then incubate with the antibody (LC3II, Servicebio, GB13124) overnight at 4°C. Block for 30 minutes and then incubate with the primary antibody Servicebio, GB25303) for 50 minutes. DAPI was used for nuclear staining (blue) at room temperature for 10 minutes. Images of AEC-II were snapped using an upright fluorescence microscope (NIKON ECLIPSE C1, Japan) using a NIKON DS-U3 imaging system.

2.7. Electron microscopy

AEC-II were seeded into a 6-well plate and incubated until the cells confluent to 60% and then incubated with CSE for 24h and/or neferine for 2h. After fixed with 2.5% glutaraldehyde, AEC-II cells were analyzed by transmission electron microscope (TEM). Next, the cells were sequentially fixed and stained. Last, cells’ photos were recorded under an electron microscope (PhilipsCM20) to observing magnetic domain structures.

2.8. Flow Cytometric Analysis of Apoptosis

Following the Annexin V/PI method, flow cytometric analysis of apoptosis was
performed according to the instructions of Annexin V-FITC/PI Apoptosis Detection Kit (BD Pharmingen, CA, USA). AEC-II were incubated in 6-well plates overnight. After CSE/neferine/chloroquine/Compound C treatment and digested with trypsin, the cells were recovered and washed 3 times with PBS\[^{23,24}\]. Then the cells were incubated at 37°C for 15 minutes in the dark and stained with a mixture of Annexin V-FITC and PI. After addition of FITC-conjugated Annexin V, the specific fluorescence of AEC-II cells were immediately analyzed by FACS Calibur (BD Biosciences, CA, USA) within 1 hour.

2.9. Statistics

All Statistics were performed using SPSS version 18.0 at least in triplicate. The results are counted as the mean(SD) with P value of less than 0.05 as statistically significant. Groups containing multiple comparisons were analyzed by ANOVA with t-test, one-way analysis and Tukey’s multiple-comparison test. All experiments were repeated 3 times.

3. Results

3.1. Effect of CSE on cell viability and autophagy in AEC-II
Alveolar epithelial type-II cells (AEC-II) of the rat were firstly stimulated by low-to-high concentration gradient of 0%-18% CSE for 24 h[25,26]. CCK-8 assay was used to examine the effect of CSE on the vitality of AEC-II cells (Fig. 1 A). We recorded the cell viability of the CSE group with different concentrations in the form of a percentage expressed as the control group, when the cell viability of the control group was defined as 100%. We observed that the half-inhibitory concentration (IC50) of CSE was 6.8%. The results showed that 2% CSE had little effect on cell viability of AEC-II, and in the range of 2%-10% CSE cell activity of AEC-II gradually decreased. When the CSE concentration was greater than 10%, the cell viability changed into a platform trend. And then we tested the autophagy of AEC-II caused by in vitro CSE treatment. Western blot analysis confirmed that there was a significant dose- and time-dependent change in the expression levels of P62, ATG5 and LC3II in AEC-II induced by CSE (Fig. 1 B-E). After 24 h CSE treatment in AEC-II with different concentrations, the results showed that 2%-8% CSE treatment significantly increased the protein expression of Atg5 and LC3-II, while the protein expression of p62 decreased significantly, which is consistent with previous reports[27-29]; however, compared with the 8% CSE group, p62 increased significantly and Atg5 and LC3II decreased obviously when treated with 10% CSE (Fig. 1 B and C). After exposure to 10%CSE for different stimulation times, the expression of Atg5 and LC3II were highest at 24 h, but then decreased for 48 h; the expression of P62 was lowest at 24 h, but then increased for 48 h (Fig. 1D and E). Therefore, we believed that autophagy is induced during the action of CSE before AEC-II apoptosis or death. It is considered that AEC-II are damaged by the autophagy system under smoke-stimulated conditions at a concentration of 10%CSE. It was regarded as the best intervention condition that using 10% CSE to induce 24h, and was employed to the subsequent experiments.
3.2. Role of neferine on cell viability and autophagy in AEC-II

![Neferine Chemical Structure](image1)

**Fig. 2.** AEC-II was treated with neferine. (A) The chemical structure of neferine. (B) CCK8 showing various concentrations of neferine-induced changes after 24h in cell viability. Survival curve displayed IC50 value (30 μM). (C) GAPDH level is shown as a protein loading control. After 24h for different concentrations (2, 4, and 8 μM), LC3 protein expression in AEC-II cells were determined by Western blotting analysis. (E) Treated with neferine, IF images of AEC-II induced LC3II expression changes (green). Nuclei was labeled with DAPI (blue). (D,F) Results are presented as the means ± SD of at least 3 independent experiments. * p < 0.05 compared to control; # p < 0.01 compared to control.
CCK-8 assay was used to examine the role of neferine on the vitality of AEC-II. The viability of AEC-II at other concentrations neferine was expressed as a percentage compared to the control group, under the premise that cell viability of the control group was set as 100%. Result revealed IC50 value was 30 μM after 24h exposure to neferine (Fig. 2 B). Next, after 24h for different concentrations (2, 4, and 8 μM), LC3 protein in AEC-II was induced by neferine (Fig. 2 C and D). Moreover, to visualize LC3II protein, the results of immunofluorescence (IF) staining showed that LC3II protein expression also significantly increased as the concentration of neferine changes. The data further confirmed autophagy induction under the stimulation of neferine (Fig. 2 E and F).

3.3. Effect of neferine on AEC-II survival in CSE by autophagy

![Fig. 3. Effect of neferine on AEC-II survival in CSE by autophagy. (A,B) GAPDH level is shown as a protein loading control. After 2h for different concentrations neferine (0, 2, 4, and 8 μM) with/without 10%CSE for 24h, LC3 and P62 protein expression in AEC-II cells were determined by Western blotting analysis. (C) After 2h for neferine at a dose of 8 μM with/without 10%CSE for 24h, electron microscope images presented representative autophagosomes or autolysosomes (marked by black arrows; Bar, 500 nm). Results are presented as the means ± SD of at least 3 independent experiments. * p < 0.05 compared to control; # p < 0.05 compared to the CSE group without neferine.](image-url)
Some literature pointed out that autophagy may be a major mechanism of airway diseases\cite{11}, we assumed that neferine can protect cells by autophagy under certain conditions\cite{30}. To confirm this conjecture, we examined the protein expression of LC3 and P62, which are closely involved in the autophagy-related ubiquitination reaction mechanism through LC3\cite{31}. Neferine resulted in increased expression of LC3II and decreased expression of p62 at 8μM. This change shows that AEC-II treated with 8μM neferine had stronger autophagy ability. We further made clear that autophagy caused by neferine was found in AEC-II under electron microscope. After 2h for neferine at 8μM with/without 10%CSE for 24h, the representative electron microscope images presented more autophagosomes or autolysosomes were identified in AEC-II treated with neferine. In summary, we found that neferine could effectively enhance autophagy activation in AEC-II. Then we chose 8μM as the therapeutic concentration of neferine in the following experiments.

3.4. Autophagy inhibitor attenuated neferine's effect in protecting AEC-II against CSE

![Fig. 4. Autophagy inhibitor attenuated neferine's effect in protecting AEC-II against CSE. AEC-II were pretreated](image-url)
for 2h with neferine(8 μM), or neferine(8 μM) together with chloroquine(20 μM), followed by treatment with 10%CSE for 24h. (A) Phase-contrast microscopy images presented cells viability (Bar, 100 μm). (B) AEC-II was analyzed by flow cytometric analysis of apoptosis. (C,D) GAPDH level is shown as a protein loading control. LC3 and P62 protein expression in AEC-II were determined by Western blotting analysis. Results are presented as the means ± SD of at least 3 independent experiments. * p < 0.05 compared to control; # p < 0.05 compared to the CSE group; & p < 0.05 compared to the CSE and neferine group.

To further investigate whether neferine can alleviate cytotoxicity and apoptosis of AEC-II induced by CSE via enhance autophagy, chloroquine was used to confirm whether upstream autophagy is activated after stimulation by blocking autochromosomal degradation[8,17,32,33]. As we have observed, both morphological observation and flow cytometry analysis showed that chloroquine had no significant effect on cell death and apoptosis, but it could significantly prevent neferine from reducing the protective effect of CSE-induced cell death and apoptosis(Fig. 4A and B). Moreover, western blotting further pointed that the expression of LC3II induced by CSE combined with neferine was higher than CSE alone. However, a decrease in LC3II expression was found in cells treated with CSE, neferine and chloroquine. This indicates that neferine mediated anti-apoptotic protection through autophagy(Fig. 4C and D). The data suggested that neferine significantly induced autophagy. At the same time we detected the protein expression level of another autophagy marker, P62. The expression of P62 induced by CSE combined with neferine was lower than CSE alone. Simultaneously, the increased expression level of P62 was found in cells treated with CSE, neferine, and chloroquine. Collectively, the results indicate that neferine have a cytoprotective effect on AEC-II against CSE-induced death and apoptosis by enhancing autophagy.

3.5. Neferine protected AEC-II against CSE via the AMPK/mTOR pathway
AMPK / mTOR signaling is the iconic upstream autophagy regulation pathway, and was observed to study neferine by regulating autophagy when AEC-II was stimulated by CSE\textsuperscript{[30]}. Optical microscope observation and flow cytometry analysis statistics (Fig. 5A and 5B) showed that treatment with Compound C alone had no significant effect on cell death and apoptosis, and Compound C prevented neferine from protecting AEC-II from CSE. To further explore the potential mechanism, as shown in Fig. 5C and D, we explored the effect of AMPK / mTOR and analyzed the role of Compound C, which was used as a AMPK inhibitor to confirm whether upstream pathway is activated in neferine-induced autophagy\textsuperscript{[34]}. Decreased LC3II and p-AMPK expression and increased p-mTOR expression revealed that compound C significantly inhibited autophagy and lost the role of neferine to protect AEC-II from CSE. In summary, these data indicated that neferine induced autophagy by activating AMPK, thereby acting as a protective mechanism against CSE in AEC-II from the apoptosis and death.

4. Discussion

In this study, we verified that neferine alleviate CSE-induced AEC-II apoptosis and death by inducting autophagy. We further discovered that the occurrence of this process is closely related to the AMPK / mTOR signaling pathway.

Smoking is one of the most common risk factors for chronic obstructive pulmonary disease. Cigarette smoke contains more than 4,000 kinds of harmful substances, including tar, carbon monoxide, nicotine, benzopyrene, nicotine, nitrogen oxides,
hydrogen cyanide, acrolein, etc. These substances induce oxidative stress, cytotoxicity, and activate corresponding changes in various cells in the lung. It is common that exposure to CSE is used to explore the mechanism and pathogenesis of COPD in vivo or in vitro. CSE can cause autophagy enhancement. CSE can cause apoptosis. We also found that CSE induces autophagy in a time- and dose-dependent manner (0-24h, 0-8%). However, when the concentration of CSE is too large, cellular activity is clearly at a low level and autophagy is weakened. We considered that AEC-II are damaged by the autophagy system under smoke-stimulated conditions at an excessive CSE concentration. And CSE does cause apoptosis in AEC-II cells in our experiment. Inhibition of autophagy maybe the pathogenesis of COPD.

Neferine, the major bisbenzylisoquinoline alkaloids from lotus embryos, possesses a variety of pharmacological activities. Previous reports have indicated that neferine has an anti-apoptotic effect. Baskaran et al reported that neferine can block the ROS / Akt / NF-kB pathway to inhibit high glucose-induced apoptosis in endothelial cells. In our research, we have explored that neferine could indeed induce enhanced autophagy and neferine pretreatment obviously attenuated CSE-triggered apoptosis. Autophagy process is a dynamic equilibrium process, including initiation, extension, maturation and degradation. The autophagy-associated protein Atg5 is involved in the formation of Atg5 related complex and some ubiquitination protein processing modification processes, which plays a crucial impact on binding to Atg12 and LC3 modification.

In addition to being a marker of autophagy activation via linking LC3 to ubiquitinated proteins, P62 protein can also be an important regulator of apoptosis. Neferine treatment enhanced autophagy process, while chloroquine as an autophagy flux inhibitor significantly weakens the protective effect of neferine on CSE-triggered apoptosis and death, suggesting that neferine induces protection from CSE-induced apoptosis by autophagy.

mTOR, as a conserved serine/threonine protein kinase, is the confluence of upstream pathways including AMPK / mTOR that regulate cell growth, reproduction, movement, survival and autophagy. Studies have confirmed that AMPK regulated mTOR signaling pathway is closely related to intracellular energy balance. Under glucose starvation conditions, AMPK was shown to activate autophagy by directly stimulating the mammalian autophagy promoter kinase Ulk1. Neferine has been shown to have the ability to protect cells from apoptosis due to hypoxia through the Akt / mTOR signaling pathway. In the Huntington's disease model, neferine can induce mTOR/AMPK-mediated autophagy. Our study also pointed out that neferine mediates autophagy through the AMPK/mTOR signaling pathway. These results indicated to one point: neferine induces autophagy by activating the AMPK / mTOR signaling pathway to prevent CSE-induced AEC-II cytotoxicity and apoptosis.

In conclusion, neferine-induced autophagy may play a cytoprotection role in COPD.
caused by smoking. This may be a promising approach to reduce CSE-induced cytotoxicity and apoptosis and suggested targeting AMPK/mTOR pathway-mediated autophagy as a new treatment strategy for COPD; however, the association between autophagy activity and lung protective mechanisms must be illustrated by further researches.
Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All study participants provided informed consent.

Availability of data and material
The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests
All authors declared that they have no competing interests. They scanned and approved the final version of manuscript.

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Authors' contributions
F.H. was corresponding author and supervised the entire research. M.W. was first author and designed the research, interpreted results of experiments, analyzed the data, and drafted the manuscript. M.W., H.Y. and F.H. revised manuscript. Y.S., P.C., and Q.W. involved in performing the cell experiments.

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