Protective effect of luteolin on cigarette smoke extract-induced cellular toxicity and apoptosis in normal human bronchial epithelial cells via the Nrf2 pathway

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Abstract. Luteolin, one of the most common abundant flavonoids in vegetables and herbs, has antitumor effects on various tumors by inducing apoptosis, antioxidant effects and inhibition of angiogenesis. However, the potential chemoprevention of luteolin on lung cell damage and its related mechanism(s) are not fully known. The present study evaluated the protective effects of luteolin on cigarette smoke extract (CSE)-induced toxicity and apoptosis in normal human bronchial epithelial (NHBE) cells and explored its underlying mechanism(s). MTT assay showed that pretreatment with luteolin increased CSE-decreased cell viability (p<0.05). Luteolin increased cellular glutathione (GSH) levels but decreased reactive oxygen species (ROS) generation (p<0.05). Cytometry assay and western blot analysis showed that luteolin attenuated CSE-induced apoptosis and apoptosis-related protein activation, including caspase-3, -8 and -9 (p<0.05). The expression of CSE-induced NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) were decreased significantly by luteolin (p<0.05). Furthermore, luteolin attenuated CSE-induced apoptosis, noticeably reduced CSE-induced expression of NF-E2-related factor 2 (Nrf2), NQO1 and HO-1 using a small interfering RNA (siRNA) transfection assay. The data demonstrated that CSE-induced oxidative damage and apoptosis through the Nrf2 pathway was inhibited by luteolin and it may serve as a chemopreventive agent for the prevention and treatment of lung cancer.

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Introduction

Lung cancer is one of the most commonly diagnosed types of cancer and the leading cause of cancer-related morbidity and mortality in the world (1). It is estimated that there will be approximately 228,000 new cases of lung cancer and 159,500 deaths in the United States in 2013 (2). Smoking, particularly smoking cigarette, is the most significant risk factor resulting in lung cancer (1). Cigarette smoke contains several types of chemical constituents, including tar, nicotine, polycyclic aromatic hydrocarbons, quinines and carbon monoxide. The lungs are the most important target organs for cigarette smoke-induced diseases. It has been shown that cigarette smoke extract (CSE) may produce a clear cytotoxic effect. It is well established that tentatively assigned to semiquinones present in aqueous extracts of CS are cytotoxic and cause protein and DNA damage and, therefore, cause lung cell damage or apoptosis (3-6). The smoking-induced pathological damage of epithelial cells was associated with the oxidative damage and transcription factor NF-E2-related factor 2 (Nrf2) (7,8). Activated-Nrf2 regulated the activity and expression of multiple genes and topoisomerase, including encoding xenobiotic metabolizing enzymes NAD(P)H: quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione S-transferase (GST) and glutathione (GSH) (9-11). Therefore, preventing CSE-induced damage may be an effective method to reduce the high morbidity and mortality of lung cancer.

Luteolin, shown in Fig. 1, is one of the most common abundant flavonoids in vegetables and herbs, and has antitumor effects on various tumors by inducing apoptosis, antioxidant effects and anti-angiogenesis (12-17). Notably, in non-small cell lung cancer A549 cells, which possess constitutively active Nrf2, luteolin elicited a marked reduction in Nrf2 at both the mRNA and the protein levels, leading to decreasing Nrf2 binding to AREs, downregulation of ARE-driven genes, and depletion of reduced GSH (18).

It has been shown that luteolin has potent curative properties against N-nitrosodiethylamine-induced hepatocellular carcinoma in albino rats and has potentiality in chemoprevention (19). However, the potential protection of luteolin

on CSE-induced cell damage and apoptosis and its related mechanism has not yet been reported. In the present study, a normal human bronchial epithelial cell (NHBE) model was used to observe the influence of CSE on cell survival, apoptosis, DNA damage and intracellular stress response and luteolin intervention in CSE-induced cytotoxicity. Our findings showed that luteolin may be a promising chemopreventive agent for smoking-induced lung cancer.

Materials and methods

Reagents. RPMI-1640 medium was purchased from KeyGen (Nanjing, China). Luteolin was provided by Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). Fetal bovine serum (FBS) was purchased from Gibco (USA). Nrf2, HO-1 and NQO1 antibodies were obtained from Santa Cruz Biotechnology, Inc. (USA). Cleaved caspase-3, -8 and -9, Bcl-2 and Bax antibodies were purchased from ImmunoWay Biotechnology Company (USA). The other chemicals and reagents used were of analytical grade.

Cell culture. NHBE cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The NHBE cells were cultured with RPMI-1640 medium containing 10% FBS, penicillin (80 U/ml) and streptomycin (0.08 mg/ml). Cells were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The medium was replaced every day. For CSE treatment, NHBE cells were grown to 90% confluence and restored in fresh medium without FBS.

Preparation of CSE. CSE was prepared according to a method previously described (20,21). In order to obtain 100% CSE, the filters were cut from the cigarettes before the experiment. One commercial cigarette (Nanjing, China) containing 11 mg tar and 1.1 mg nicotine was burning and drawing the smoke by vacuum into 10 ml of cell growth medium at a rate of 5 min. CSE was adjusted to pH 7.4, filtered through a 0.22-μm pore acaroids syringe filter (Pall, USA) and subsequently adjusted with medium to an absorbance of 0.15 at 320 nm. The prepared medium containing CSE was used within 30 min of preparation.

MTT assay for cell viability. The effect of luteolin on NHBE cell viability was detected by the MTT assay. Cell suspension ($1x10^4$ cells/ml) was seeded into wells of 96-well plates. After incubation for 24 h, the NHBE cells were pretreated with luteolin (0.3125, 0.625, 1.25, 2.5, 5, 10, 20 and 40 μM) and then treated with 10% CSE for another 24 h, 10% CSE was used to induce the NHBE damage model, while medium served as the negative control. At the end of the incubation, these cells were incubated with 0.5 mg/ml MTT for 4 h. The purple crystals were dissolved by 100 μl DMSO and were then shaken in a shaker for 10 min. Finally, the absorbance of samples was measured in a microplate reader at 570 and 490 nm. All the experiments were repeated 3 times.

$$\text{Cell viability ratio} = \frac{A_{570\text{treated}} - A_{490\text{treated}}}{A_{570\text{control}} - A_{490\text{control}}} \times 100\%$$

Reactive oxygen species (ROS) assay. The pre-fluorescent probe DCFH-DA was used to determine the intracellular
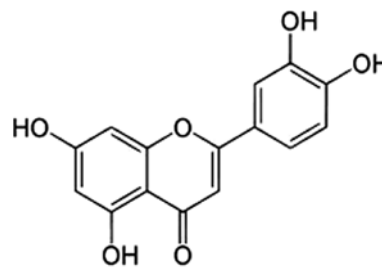

Figure 1. The chemical structure of luteolin.

ROS overgeneration induced by CSE according to the manufacturer's protocols (KeyGen). Briefly, after treatment with luteolin or (luteolin + 10% CSE), the cells were incubated with DCFH-DA (10 μmol/l) for 20 min in an incubator in the dark at 37˚C. The fluorescence intensity was measured by a FACScan flow cytometer (BD, USA) at an excitation maximum of 488 nm and an emission maximum of 525 nm.

Determination of GSH. Cells were collected from 6-well plates, washed with 4˚C PBS twice and lysed in 200 μl cell lysis buffer (Biyuntian Biotech. Co. Ltd., Nantong, China) with PMSF at 4˚C for 30 min, then centrifuged for 5 min (4˚C, 13,000 rpm), and the supernatant was used to detect cellular GSH level according to the manufacturer's protocols (NJJC-BIO, Nanjing, China). The final absorbance was detected at 405 nm in a microplate reader.

Apoptosis analysis by flow cytometry. NHBE cells ($1x10^6$ cells/ml) were incubated with luteolin + 10% CSE for 24 h. Then, the washed cells for apoptosis analysis were resuspended in Annexin V binding buffer and stained simultaneously with FITC-conjugated Annexin V-APC and PI at room temperature for 15 min in the dark, prior to the addition of binding buffer. The apoptotic cells were measured using a FACScan flow cytometer at an excitation maximum of 650 nm and an emission maximum of 660 nm.

Western blot analysis. Cells were collected from 6-well plates and washed with PBS twice and then lysed in 200 μl cell lysis buffer (Biyuntian Biotech. Co. Ltd.) with PMSF at 4˚C for 30 min. The cell lysis buffer was centrifuged for 5 min (4˚C, 13,000 rpm). The supernatant was separated by 12% of SDS-PAGE. The SDS-separated proteins were equilibrated in transfer buffer (50 mM Tris-HCl, pH 6.8, 40 mM glycine, 10% SDS and 20% methanol) and electrotransferred to Immobilon-P transfer membranes (Millipore Corporation, Bedford, MA, USA). The blot was blocked with 5% non-fat dry milk and washed with Tris-buffered saline 10 mM Tris, 150 mM NaCl (Sigma Chemical). Subsequently, membranes were probed with the primary antibodies against Bcl-2, BAX, cleaved caspase-3, -8 and -9, Nrf2, NQO1, HO-1 and β-actin. Membranes were washed twice with TBST, the horseradish peroxidase-bound secondary antibodies were added to membranes for 1 h at room temperature. Membranes were visualized with chemiluminescence reagents. Image Pro Plus (IPP) software for densitometry analysis was applied for the quantification of protein expressions.
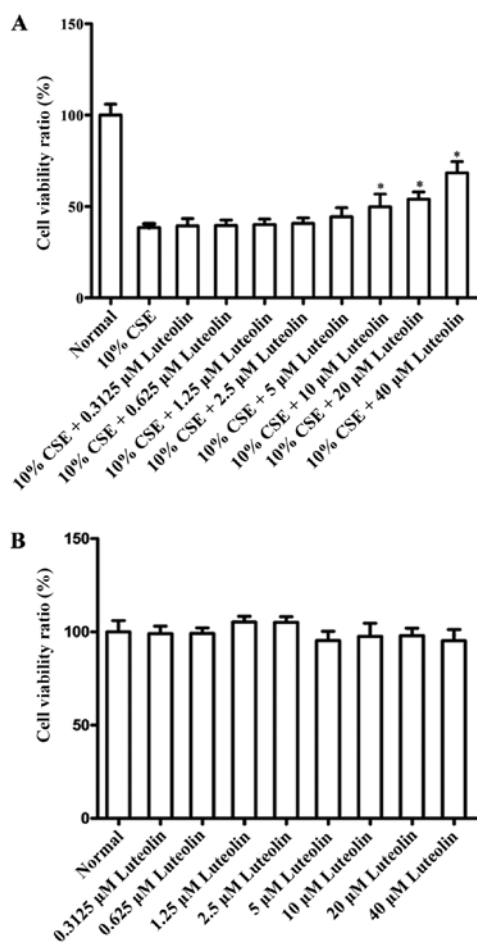
Figure 2. Effect of luteolin on the cell viability in NHBE cells. (A) Cells were treated with 10% CSE + luteolin for 24 h. (B) Cells were treated with single luteolin for 24 h. The cell viability was determined by MTT assay. Values are means ± SD of 3 independent experiments. *p<0.05, (10% CSE + luteolin) vs. CSE. NHBE, normal human bronchial epithelial; CSE, cigarette smoke extract.
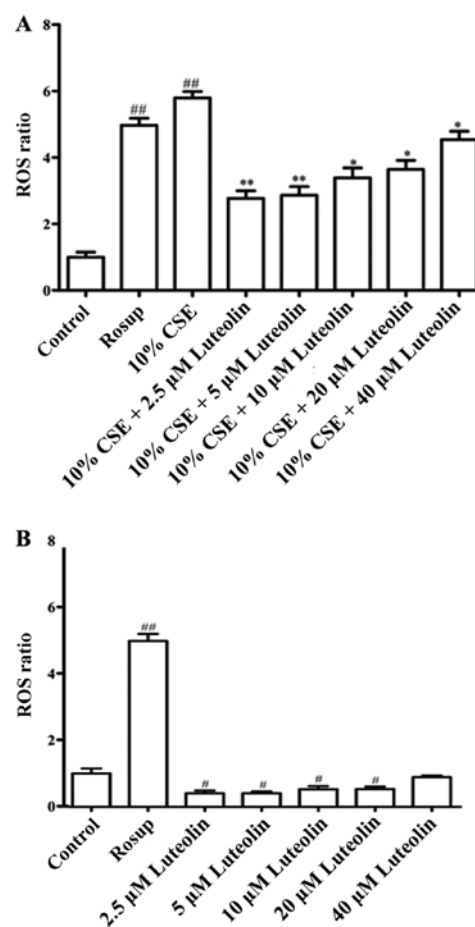

Figure 3. The reduction of luteolin on CSE-induced ROS overgeneration. (A) Intracellular ROS by 10% CSE + luteolin. (B) Intracellular ROS by luteolin; *p<0.05, **p<0.01 10% CSE vs. 10% CSE + luteolin; #p<0.05, ##p<0.01 control vs. 10% CSE or luteolin. CSE, cigarette smoke extract; ROS, reactive oxygen species.

Small interfering RNA (siRNA) transfection assay. For the transfections, NHBE cells were seeded ($1x10^5$ cells/ml) in 6-well plates with antibiotic-free normal growth medium supplemented with FBS. For each transfection, 0.8 ml siRNA transfection medium (sc-36868; Santa Cruz Biotechnology, Inc.) were added to each tube containing the siRNA transfection reagent mixture (solution A + solution B). The directed against human Nrf2 (sc-37049) and control-siRNA (a non-targeting siRNA; sc-37049) (both from Santa Cruz Biotechnology, Inc.) were transfected into NHBE cells using the Lipofectamine™ 2000 reagent according to the manufacturer's instructions. NHBE cells were incubated with the transfection complexes for 24 h and subsequently analyzed by western blotting for protein expression. The effects of Nrf2 siRNA were compared with those of corresponding control siRNA using the same transfection method. This approach was used to verify the direct link between luteolin, Nrf2 and CSE.

Statistical analysis. The data from three individual experiments are presented as means ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS 16.0 software. p<0.05 was considered to indicate a statistically significant difference.

Results

Effect of luteolin on CSE-induced NHBE cell viability. Several studies reported that the toxicity induced by CSE may significantly inhibit the viability of NHBE cells in light of a potentially pathogenic event, such as oxidative stress (4,22). Herein, we observed that the exposure to CSE may markedly reduce NHBE cell viability to 38.55±2.13% (p<0.05, CSE vs. normal). Pretreatment with luteolin at the dose of 0.3125-40 µM significantly improved the cell viability (Fig. 2A). However, the single luteolin at the same range of concentrations had no effect on the cell viability after being treated for 24 h (Fig. 2B). The data indicated that luteolin attenuated CSE-induced cellular damage and significantly improved the cell viability in NHBE cells.

Luteolin reduces CSE-induced ROS overgeneration. As shown in Fig. 3A, 10% CSE triggered oxidative stress and significantly increased intracellular ROS overgeneration as compared to the normal control (p<0.05). Markedly, CSE-induced overgeneration of ROS was inhibited significantly by the treatment with single luteolin or luteolin + 10% CSE in a concentration-dependent manner (Fig. 3A and B). The results showed that luteolin reduced the oxidative damage in NHBE cells.
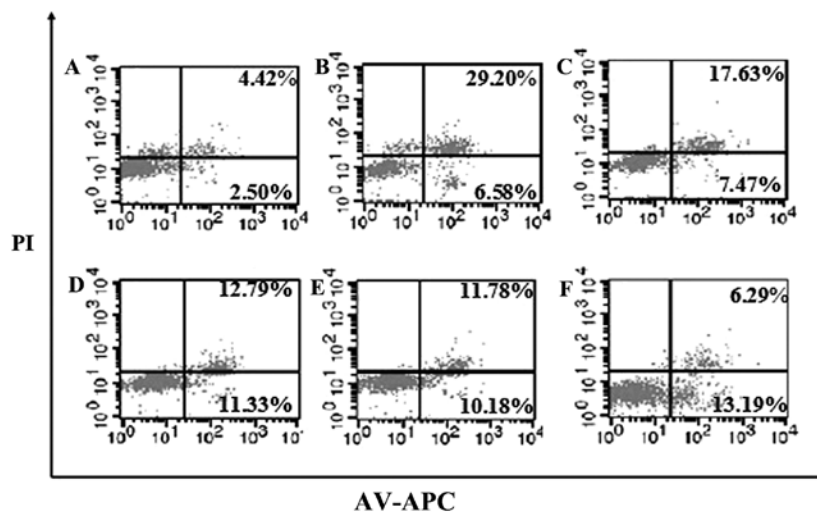
Figure 4. Luteolin prevents CSE-induced NHBE cell apoptosis. NHBE cells were pretreated with luteolin + 10% CSE for 24 h and stained with Annexin V-PI kit. (A) Control, (B) 10% CSE, (C) 10% CSE + 5 μM luteolin, (D) 10% CSE + 10 μM luteolin, (E) 10% CSE + 20 μM luteolin, (F) 10% CSE + 40 μM luteolin. CSE, cigarette smoke extract; NHBE, normal human bronchial epithelial.
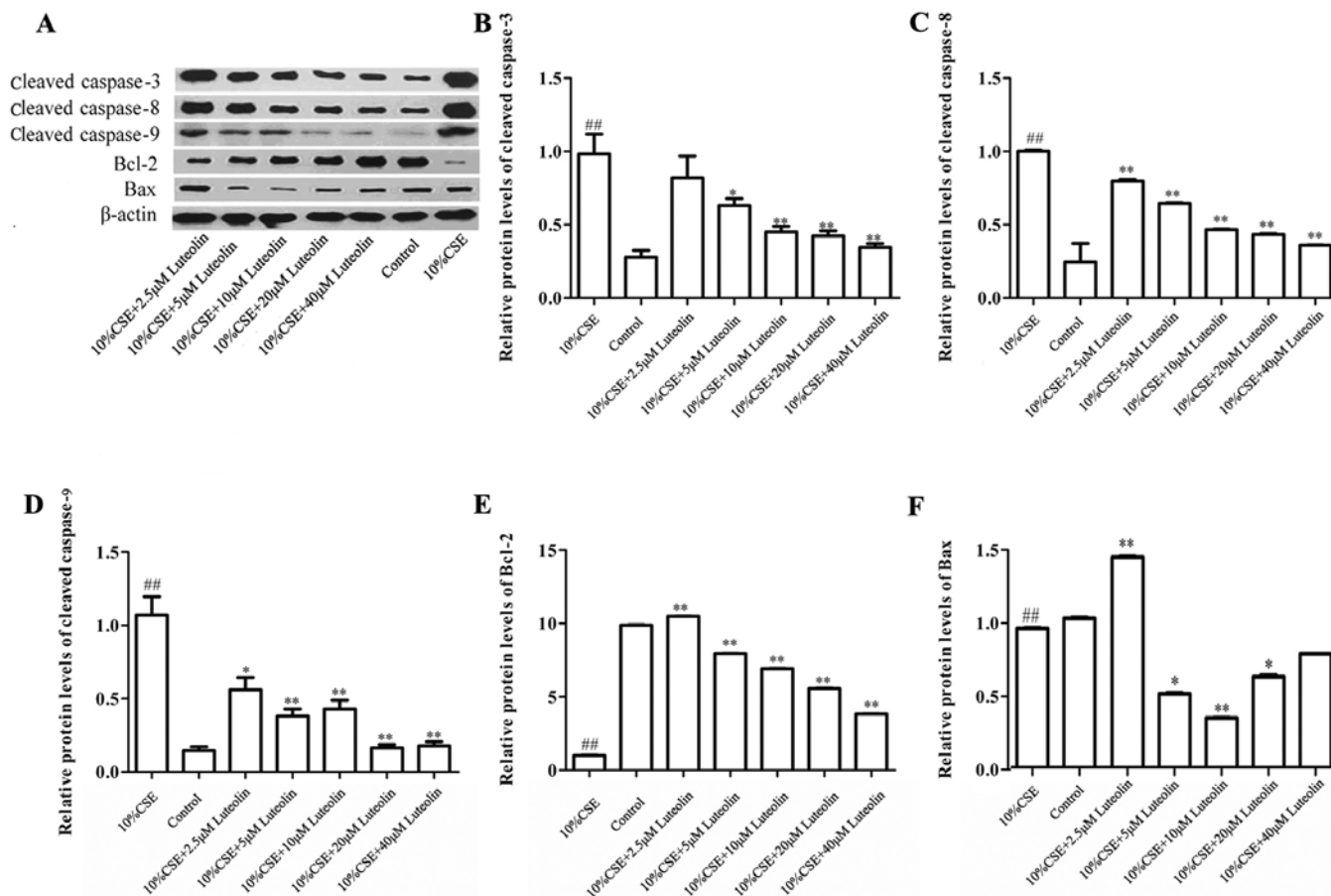

Figure 5. The effects of luteolin on CSE-induced expressions of apoptotic proteins. (A) NHBE cells were treated with 10% CSE + luteolin for 24 h and cleaved caspase-3, -8 and -9, Bax and Bcl-2 were analyzed by western blotting; (B) cleaved caspase-3 expression; (C) cleaved caspase-8 expression; (D) cleaved caspase-9 expression; (E) Bcl-2 expression; (F) Bax expression. $^{\#\#}$p<0.01 control vs. CSE; *p<0.05, **p<0.01 CSE vs. 10% CSE + luteolin. CSE, cigarette smoke extract; NHBE, normal human bronchial epithelial.

Luteolin reduces CSE-induced NHBE cell apoptosis. To examine whether luteolin prevents CSE-induced NHBE cell apoptosis, the Annexin V-APC^{+}/PI^{-} method was performed by flow cytometry in the present study. As shown in Fig. 4, after being treated with 10% CSE, the apoptosis ratio of NHBE cells was 35.78%. The statistical results showed that there
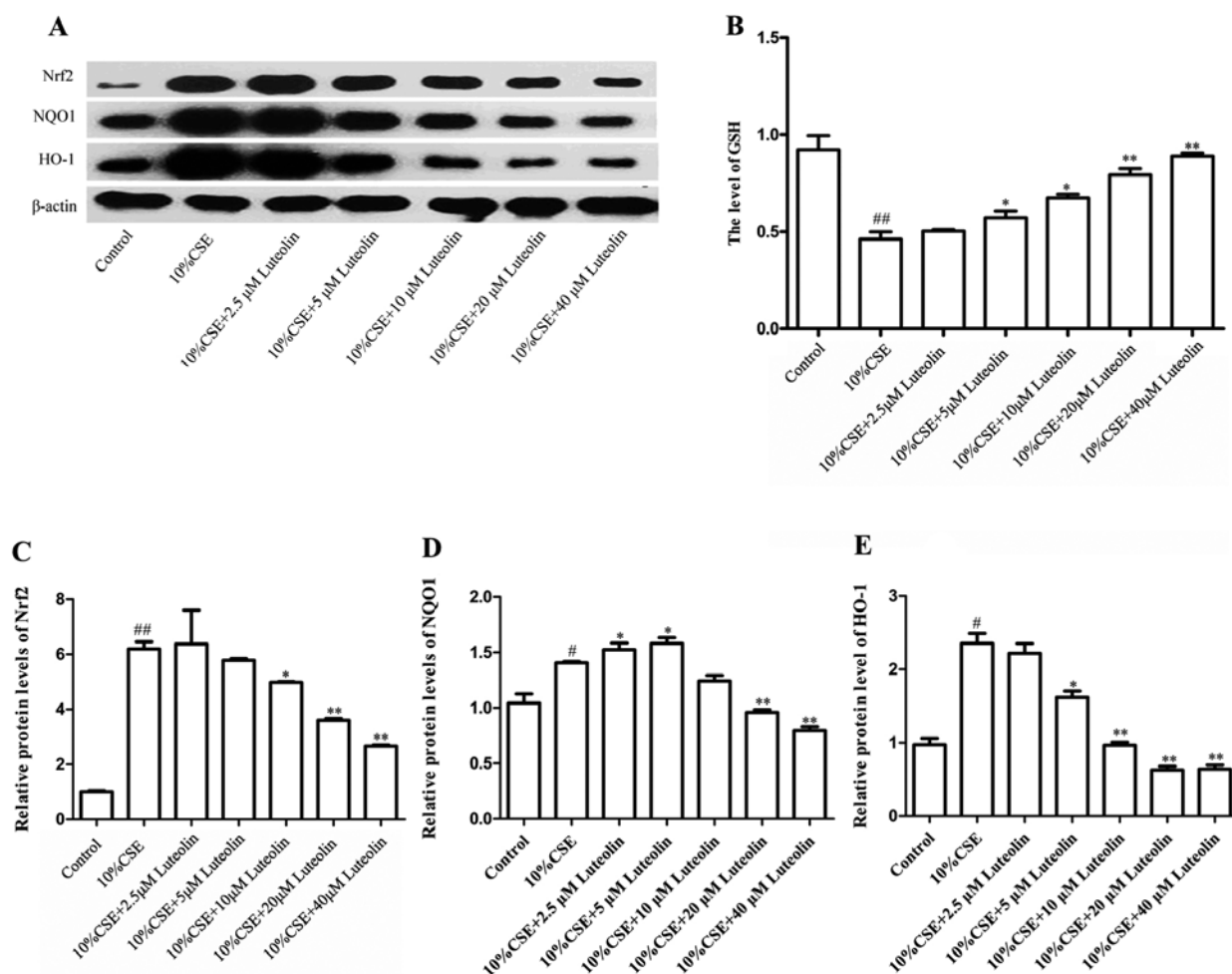
Figure 6. The effects of luteolin on the Nrf2 signaling pathway in NHBE cells. (A) Cells were treated with 10% CSE + luteolin, and Nrf2, NQO1 and HO-1 expressions were reduced when compared with 10% CSE; (B) luteolin recovered intracellular GSH level; (C) the protein level of Nrf2 expression was semi-quantified by densitometry analysis; (D) the protein level of NQO1 expression; (E) the protein level of HO-1 expression. $^{\#}p<0.05$, $^{\#\#}p<0.01$ control vs. CSE; $^{*}p<0.05$, $^{**}p<0.01$ CSE vs. 10% CSE + luteolin. Nrf2, NF-E2-related factor 2; NHBE, normal human bronchial epithelial; CSE, cigarette smoke extract; NQO1, NAD(P)H: quinone oxidoreductase 1; HO-1, heme oxygenase-1; GSH, glutathione.

was a significant difference between 10% CSE groups and normal control (6.92%). Notably, the apoptosis ratio of cells treated with luteolin (5-40 μM) + 10% CSE was lower than that of 10% CSE (35.78%); the apoptosis ratios of NHBE cells were 19.48, 21.96, 24.02 and 25.10%, respectively. The results showed that luteolin prevented CSE-induced NHBE cell apoptosis in a concentration-dependent manner.

Luteolin downregulates CSE-induced activation of caspases and Bax/Bcl-2 expression. Caspase-3, -8 and -9, Bax and Bcl-2 were the major effector proteins and played a critical role in the characteristic apoptotic events, including chromatin condensation, DNA fragmentation and the formation of apoptotic bodies. Therefore, the effect of luteolin on CSE-induced activation of caspase-3, -8 and -9 was investigated. As shown in Fig. 5, CSE exposure markedly increased the activation of caspase-3, -8 and -9, and upregulated the ratio of Bax/Bcl-2 in NHBE cells. These data strongly indicated that the protective effect of luteolin against CSE-induced apoptosis in NHBE cells was through modulating the activation of caspase-3, -8 and -9 and reducing the ratio of Bax/Bcl-2.

Luteolin attenuates CSE-induced Nrf2, NQO1 and HO-1 protein expressions and improves GSH level. In order to evaluate whether luteolin attenuates CSE-induced oxidative stress or electrophilic attack by activating Nrf2-mediated signaling pathway, Nrf2, NQO1 and HO-1 protein expressions were analyzed by western blot analysis while antioxidant GSH was detected by kits. Following treatment with 10% CSE in the presence of luteolin (2.5, 5, 10, 20 and 40 μM) for 24 h, the Nrf2, NQO1 and HO-1 protein expressions in NHBE cells were enhanced by the exposure of 10% CSE, when compared with the normal group ($p<0.01$) (Fig. 6A). The treatment with luteolin (2.5-40 μM) may significantly attenuate the protein expressions of Nrf2 ($p<0.01$). Additionally, the expressions of downstream signaling NQO1 and HO-1 proteins associated with Nrf2 activation were also markedly reduced by the treatment with luteolin ($p<0.01$). The results showed that the attenuation of luteolin on 10% CSE-induced oxidative damage may be associated with the blockade on Nrf2-mediated signaling pathway. GSH played an important role in the Nrf2-mediated antioxidant defense system in organism. To examine the effect of luteolin on 10% CSE-induced oxidative damage,
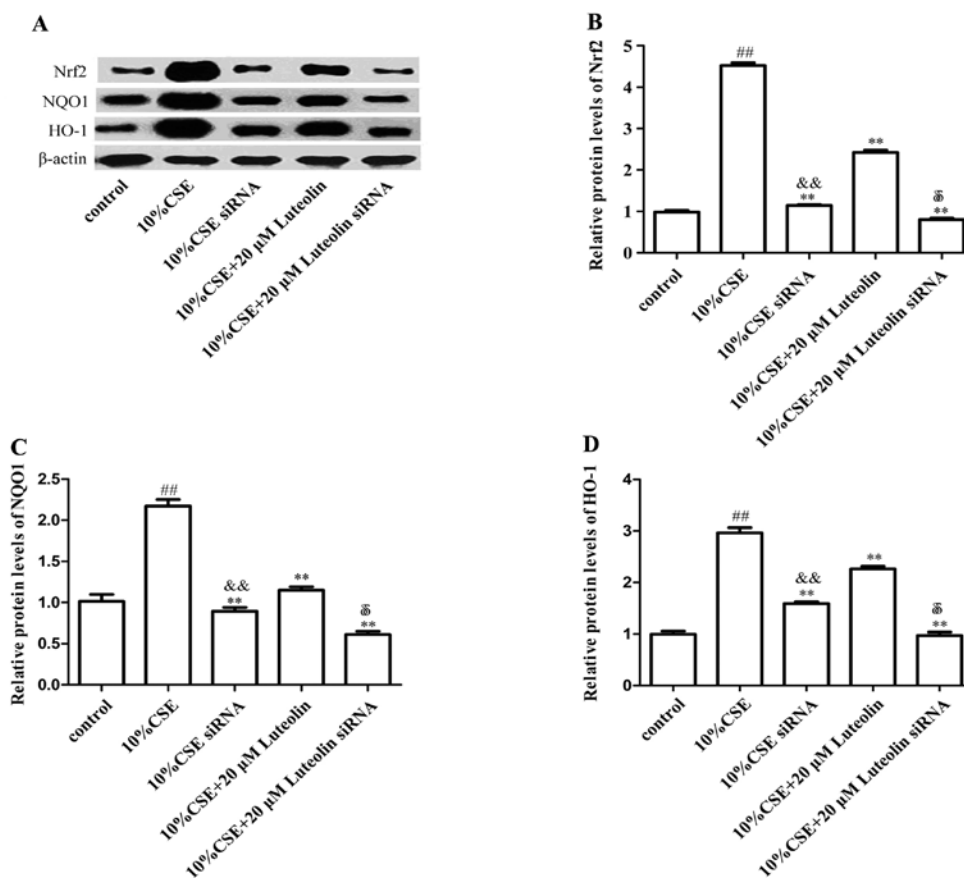
Figure 7. The Nrf2 pathway is specifically responsible for luteolin-induced cytoprotection. (A) Nrf2 siRNA is effective in silencing Nrf2 translocation and activation of target genes. After transfection, cells were exposed to 10% CSE or 10% CSE + luteolin for 24 h, and the protein levels of Nrf2, NQO1 and HO-1 were confirmed by western blot analysis. (B-D) The protein levels of Nrf2, NQO1 and HO-1 expressions were semi-quantified by densitometry analysis. [##]p<0.01 control vs. 10% CSE; [&&]p<0.01 10% CSE vs. 10% CSE siRNA; [δδ]p<0.01 10% CSE + luteolin vs. 10% CSE + luteolin siRNA; [**]p<0.01 10% CSE vs. experimental group. Nrf2, NF-E2-related factor 2; siRNA, small interfering RNA; CSE, cigarette smoke extract; NQO1, NAD(P)H: quinone oxidoreductase 1; HO-1, heme oxygenase-1.
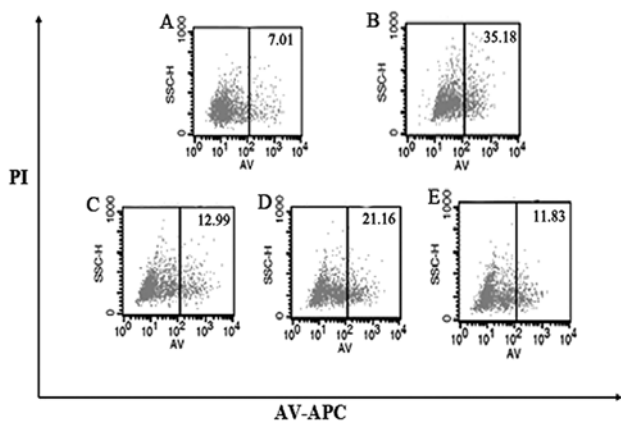

Figure 8. The Nrf2 pathway specifically responsible for luteolin prevented CSE-induced NHBE cell apoptosis. After transfection, cells were exposed to 10% CSE or 10% CSE + luteolin for 24 h and stained with Annexin V-PI kit. (A) Control, (B) 10% CSE, (C) 10% CSE + siRNA, (D) 10% CSE + 20 μM luteolin, and (E) 10% CSE + 20 μM luteolin + siRNA. Nrf2, NF-E2-related factor 2; CSE, cigarette smoke extract; NHBE, normal human bronchial epithelial; siRNA, small interfering RNA.

the level of intracellular GSH in NHBE cells was measured. Luteolin (2.5-40 μM) significantly reduced 10% CSE-induced intracellular GSH level from 0.461 to 0.889 (p<0.01, 10% CSE vs. luteolin + 10% CSE) (Fig. 6B). The results indicated that the reduction of luteolin on 10% CSE-induced GSH level may contribute to Nrf2-mediated oxidative damage.

Nrf2 knockdown diminishes the protective effect of luteolin in NHBE cells. To evaluate the role of the Nrf2 pathway in the protection of luteolin against CSE-induced toxicity, we developed an Nrf2 gene knockdown model in NHBE cells by using siRNA transfection. After transfection for 48 h, cells were treated with 10% CSE or 10% CSE + 20 μM luteolin for 4 h. Gene silencing against Nrf2 suppressed CSE-induced translocation of Nrf2 and the expression of NQO1, HO-1 and apoptosis in NHBE cells (Figs. 7 and 8). The protein levels of Nrf2, NQO1 and HO-1 were confirmed by western blot analysis. The Annexin V-APC+/PI- method was performed by flow cytometry in the present study. The transfection of siNrf2 led to the reduction in the Nrf2, NQO1 and HO-1 protein level and decreased the CSE-induced Nrf2 expression in NHBE cells (p<0.05). A significant difference was observed between the 10% CSE groups (35.18%) and 10% CSE + siRNA (12.99%). Of note, the apoptosis ratio of cells treated with 20 μM luteolin + 10% CSE + siRNA (11.83%) was lower than that of 10% CSE + 20 μM luteolin (21.16%). These results indicated that the Nrf2 pathway played a key role in the luteolin-induced cytoprotection against CSE.

Discussion

Cigarette smoke extract (CSE), a mixture of >4,000 chemicals, includes significant amounts of free radicals, particles, reactive chemicals and gases, producing an overwhelming oxidative burden on the lungs (23,24). Cigarette smoking is considered a major cause of morbidity and mortality of lung cancer (25,26). In China, there is a large number of smoking and passive smoking populations (27). The CSE-induced lung injuries on those people are mainly correlated with oxidative stress damage (28). The long-lived radicals presented in aqueous extracts of CSE were cytotoxic and caused protein and DNA damage resulting in the occurrence and development of lung cancer (29,30). Additionally, accumulating evidence has indicated that CSE may produce a clear cytotoxic effect on normal human bronchial epithelial (NHBE) cells (4,6,31-34). In our present study, the NHBE cell model was used to explore the protection of luteolin against the CSE-induced damage.

Chemoprevention, an effective prevention and treatment measure for lung and other types of cancer, has been defined as using natural, synthetic substances to prevent, inhibit or reverse the initiation and development of precancerous lesions through reducing the morbidity and mortality of cancer. Recent studies showed that chemoprevention is considered a potential approach to control lung cancer (35). In the present study, the chemoprevention of luteolin on CSE-induced NHBE cell damage was performed to explore the potential benefits and underlying mechanism.

The inhibition on apoptosis of normal cells plays a crucial role in the prevention process of lung cancer. Apoptosis, a highly controlled physiological process and a core signaling pathway, may be triggered by stimuli, such as CSE (36). In the present study, we detected the protein of Bax/Bcl-2 and caspase series caspase-3, -8 and -9 related mitochondrial pathways by western blot analysis. Our data indicated that luteolin downregulated CSE-induced activation of caspases and Bax/Bcl-2 expression.

Oxidative stress is caused by an imbalance of reactive oxygen species (ROS)/reactive nitrogen species (RNS) and the antioxidative stress defense systems in cells. ROS/RNS or carcinogen metabolites may attack intracellular proteins, lipids and nucleic acids, which may result in genetic mutations, carcinogenesis and other diseases. In the present study, 10% CSE was used to induce oxidative stress in NHBE cells. Notably, luteolin may attenuate 10% CSE-induced oxidative stress. In addition, we observed an intracellular GSH level increased by the treatment with luteolin. Since γ-glutamylcysteine synthetase, a phase II enzyme regulated by the Nrf2 pathway, is the rate-limiting enzyme in GSH biosynthesis, the GSH level increase may reflect the induction of γ-glutamylcysteine synthetase by luteolin (37,38).

Nrf2, a redox-sensitive transcription factor, has emerged as a novel target for chemoprevention. Experimental evidence shows that Nrf2 protects cells from oxidative stresses by activating the expression of a battery of cytoprotective genes via the antioxidant response element (39). Nrf2 plays a critical role in the regulation of many antioxidative stress/antioxidant and detoxification enzyme genes, such as xenobiotic metabolizing enzymes [NAD(P)H: quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione

S-transferase (GST), glutathione (GSH)] (9), directly via the antioxidant response element. Several studies have shown that dietary phytochemicals possess cancer chemopreventive potential through the induction of Nrf2-mediated antioxidant/detoxification enzymes to protect organisms against cellular damage caused by oxidative stress (40). The present study showed that luteolin protects cells from CSE-induced toxicity through activation of the Nrf2 pathway. Here, we confirmed that luteolin induced Nrf2 translocation to the nucleus and activated its downstream target genes encoding proteins in NHBE cells, such as phase II enzymes NQO1 and HO-1. Induction of NQO1 and HO-1 has been established as strong cellular defense against oxidative stress. This is consistent with the previous reports on the protection of the Nrf2 signaling pathway against chemical carcinogen-induced cytotoxicity (41-43).

Luteolin-induced antioxidative activities were deleted as Nrf2 was knocked down, indicating a specific role of the Nrf2 pathway in luteolin-induced cytoprotection against CSE. The discovery of the target pathway was significant as it provided a signal, which leads to identifying compounds with better efficacy. In the present study, we found that the luteolin-induced cytoprotection showed a clear dose-dependent manner (0-40 μM). Therefore, luteolin may have optimal Nrf2-mediated antioxidative protection effect. However, further preclinical and clinical studies on the protective activity of luteolin on CSE toxicity need to be performed.

In conclusion, in the present study, we found that luteolin has a cytoprotective effect on CSE-induced NHBE cells and it may protect cells from CSE-induced toxicity through the activation of the Nrf2 pathway. Luteolin may act as a chemopreventive agent for the prevention and treatment of lung cancer.

Acknowledgements

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