

# Medicinal foods YT and RH suppress cigarette smoke-induced inflammation and oxidative stress by inhibiting NF- $\kappa$ B/ERK signaling pathways

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## ABSTRACT

**Background:** Cigarette smoke is a risk factor for COPD. Given the lack of a COPD curative treatment, dietary management of COPD patients has become important. This study investigated whether the medicinal foods (YT and RH) could suppress cigarette smoke exposure-induced inflammation and oxidative stress.

**Methods:** Chronic pulmonary inflammation in male C57 mice were induced by 4 weeks cigarette smoke (CS) exposure. The medicinal foods YT and RH were orally administered to mice 1 week prior to CS exposure. The protective effects were assessed by measuring the pulmonary function and histopathological evaluations. Inflammatory cell numbers and cytokines levels in BALF and blood serum were analyzed by enzyme-linked immunosorbent assay (ELISA). MDA and SOD levels of lung were analyzed. Furthermore, The levels of phosphorylated ERK and NF- $\kappa$ B of mice lungs and RAW264.7 cells were also detected.

**Results:** YT and RH combination significantly improved pulmonary function, and suppressed the inflammation including cell number and cytokines in BALF relative to the CS group, histological examination revealed protective effect of YT and RH combination in the lungs of mice exposed to CS. Moreover, the MDA level of YT and RH combination group mice lung was lower and the SOD activity was higher, *in vitro* YT and RH combination attenuated ROS expression in mouse macrophage RAW264.7 cells stimulated with CSE. YT+RH combination significantly reduced the expression of pNF- $\kappa$ B and pERK in the lung tissues and macrophage stimulated with CSE.

**Conclusion:** YT and RH combination attenuates cigarette smoke induced inflammation and oxidative stress through inhibition of NF- $\kappa$ B/ERK signaling pathway activation.

**Key words:** YT; RH; cigarette smoke; inflammation; oxidative stress; NF- $\kappa$ B/ERK

Asian Journal of Complementary and Alternative Medicine. Volume 08 Issue 2

Published on: 16/11/2020

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Cite this article as: Li Y, Wang L, Gao Q, Luo J, Chen YQ, Xu J, et al. Medicinal foods YT and RH suppress cigarette smoke-induced inflammation and oxidative stress by inhibiting NF- $\kappa$ B/ERK signaling pathways. Asian Journal of Complementary and Alternative Medicine, Vol 8 (2), 25-37:2020.

## INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized with progressive airflow limitation, chronic pulmonary inflammation and emphysema. The disease global prevalence

in adults aged 40 years is estimated to be 9–10% [1]. In addition, COPD is predicted to become the third leading cause of morbidity and mortality worldwide [2-4]. The current therapeutic options, including corticosteroids, bronchodilators, phosphodiesterase-4 inhibitors and  $\beta_2$ -agonists, can slow COPD

progression, they are not curative [5]. Therefore, developing new methods to prevent and treat COPD is essential.

Cigarette smoke (CS) is a critical risk factor for COPD [6,7]. Besides the induction of consistent airway inflammatory responses, CS induces the production of enormous amounts of ROS, which are mainly released from activated cells, including macrophages, neutrophils or structural cells like epithelial cells in COPD [8,9]. Oxidative stress occurs when ROS are produced beyond the antioxidant capacity, and damages the cellular components such as DNA, lipids, and proteins. Such damage could result in lung cell death, degradation of extracellular matrix, and loss of alveolar unit [10,11]. Oxidative stress also induces inflammatory response in the airway causing bronchial wall remodeling, mucosal thickening and mucus hypersecretion [10]. Discovery of new prevention and therapeutic methods capable of attenuating inflammation and oxidative stress has been suggested as a viable therapeutic strategy for the treatment of COPD. Accumulating evidence suggest that antioxidant foods could decrease oxidative damage by preventing oxidative deterioration [12]. Based on other previous studies, consumption of certain fruits, vegetables, wholegrains, and fish may prevent COPD, while consumption of processed foods may increase the prevalence of COPD [13,14].

YT and RH are Chinese medicinal foods, YT consists of *Poriacocos*, *dictyophoraindusia*, *lentinusedodes*, *tremellafuciformis*, *Astragalusmembranaceus*, the ingredients in which has been reported to exert anti-oxidative stress *in vivo* and *in vitro* [13,14]. RH is made of honey, *SaccharumsinenseRoxb*, *Ficusarica*, *imperata cylindrical rhizomes*, *eleocharistuberosa*, and *lily bulb*, according to Chinese traditional medicine theory the ingredients are helpful to the respiratory system. However, whether YT or RH can reduce COPD pulmonary inflammation and oxidative stress is still unclear.

In this study, we hypothesised that YT or RH elicits its protective effects by suppressing the pulmonary inflammation and oxidative stress. To test the hypothesis, we administrated the YT and/or RH to CS exposure mice and examined the protection effect by investigating the mice pulmonary function, histological changes in lung and oxidative stress levels, moreover we explored the mechanism involved *in vivo* and *in vitro*.

## MATERIALS AND METHODS

YT and RH were sourced from Infinitus (China) Co., Ltd (Guangzhou, China). The cigarettes (YeShuLabel: tar 11 mg/cigarette, nicotine 1 mg/cigarette, CO yield 13 mg/cigarette) were purchased from China tobacco Guangdong industrial Co.,Ltd (Guangzhou, China). SOD and MDA assay kits were purchased from Jiancheng Bioengineering Institute (Nanjing,

China). KC and MCP1 ELISA kits were purchased from R&D (Minneapolis, USA). Antibodies against pNF- $\kappa$ B p65, HO-1 were purchased from Abcam Biotechnology (Cambridge, USA); antibodies against p-ERK, ERK and GAPDH were purchased from Cell Signaling Technology (MA, USA); H2DCFDA were purchased from Thermo Fisher Scientific (MA, USA); the HRP-labeled Goat Anti-Rabbit/Mouse IgG (H+L) were purchased from Abcam Biotechnology (Cambridge, USA). The polyvinylidene fluoride (PVDF) membranes were obtained from Millipore Corporation (Billerica, USA). ECL-Plus detection kit probe was purchased from Tanon Science & Technology Co., Ltd (Shanghai, China). All cell culture reagents were purchased from Gibco (Carlsbad, CA), and the rest from GBCBIO Technologies Inc. (Guangzhou, China) unless stated otherwise.

## Animals and treatment

Male C57/BL/6 mice (6-7 weeks) were sourced from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China), and housed in a specific pathogen free (SPF) room at a controlled temperature of 25°C with a 12 h photo-period. Animal experimental protocol was approved by the Ethics Committee of Huamiao Biotechnology Co., Ltd. The mice were randomly divided into six groups namely: 1) room air exposure plus saline gavage administration (Blank group); 2) CS exposure plus saline administration (CS group); 3) CS exposure plus YT administration (YT group); 4) CS exposure plus RH administration (RH group); 5) CS exposure plus YT and RH combination administration (YT+RH group); 6) CS exposure plus Carbocysteine administration (S-CMC group). All protocols were reviewed and approved by the Huamiao Biotechnology Co., Ltd animal care committee.

Air or cigarette smoke exposure experiments were carried out for six consecutive days for 4 weeks. The cigarette exposure was conducted twice a day with a at least 4 h interval between the sessions. The number of cigarettes and exposure time were gradually increased to the target dose as follows: on the 1<sup>st</sup> day, mice were exposed to CS of 6 cigarettes for 30 min per session; on the 2<sup>nd</sup> day, the exposure time with 6 cigarettes increased to 1 h per session; on the 3<sup>rd</sup> and 4<sup>th</sup> days, the mice were exposed to CS with 9 cigarettes for 45minutes per session; on the 5<sup>th</sup> day, the time of exposure to CS from 9 cigarettes increased to 1 h per session, and continued for the rest of the experiment period. Each CS exposure lasted for 2 h with a 20 mins break. Mice in the Blank group were restrained for a similar duration with exposure to room air. RH (4g/kg/day) and YT (0.25g/kg/day) were orally administered once a day which began 7 days before CS exposure until the modeling ended. The mice were monitored throughout the smoke exposure procedure, and euthanized 24 h after completion of the last exposure.

## Pulmonary function

Pulmonary function of mice was evaluated with the Forced Pulmonary Maneuver System (DSI, CA, USA) according to the manufacturer's protocol. Briefly, mice were anesthetized with 1% Pentobarbital sodium (3ml/kg), then mice were tracheostomized, intubated, and put in the body chamber of the system. The average breathing frequency of anesthetized animals was forcibly set at 120 breaths/min. Functional residual capacity (FRC), and total lung capacity (TLC) were respectively recorded.

## BALF collection

Lungs were lavaged with 0.6ml sterile saline solution 6 times via the tracheal tube, the bronchoalveolar lavage fluid (BALF) was centrifuged at 1000rpm for 5 min followed by supernatant was stored in  $-80^{\circ}\text{C}$ , then cell differentials in BALF were evaluated based on morphology with diff-quick staining. At least 200 cells per mouse were counted on the slides in a blinded fashion.

## Lung histology

The mice left lung was fixed overnight in 10% formalin after inflated by intratracheal instillation 10% formalin with a 20 cmH<sub>2</sub>O pressure, then the tissue was embedded in paraffin, and 4 $\mu\text{m}$  thick sections were made and deparaffinized with xylene and graded ethanol (100%, 95%, 85%, 75%), the tissue was stained with haematoxylin and eosin (H&E) followed by two successive 5 min washes in phosphate buffered saline (PBS). Each slide was examined under a light microscope for alveolar destruction.

## Enzyme-Linked Immunosorbent Assay

MCP1 and KC levels in BALF and blood serum were detected with commercial ELISA kits according to the respective manufacturer's instructions.

## Measurement of MDA levels and SOD activities

The lung tissues of mice were homogenized in cold physiological saline solution, centrifuged at 3,500 rpm for 10 min at  $4^{\circ}\text{C}$ , then the supernatant was collected. Protein contents were determined using a BCA protein assay kit. SOD and MDA levels were measured with the respective kits according to the manufacturer's instructions.

## Western blotting

Mouse lung tissues and RAW 264.7 cells were homogenized in RIPA lysis buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, MO, USA), and 30  $\mu\text{g}$  protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, CA,

USA). The proteins were transferred to PVDF membranes, then incubated with a blocking solution (5% skim milk in TBST) for 1 h at room temperature, after washed three times with TBST, the membranes were incubated overnight with specific antibodies against NF- $\kappa\text{B}$  p65 (1:1000), HO1(1:5000), p-ERK, ERK and GAPDH (1:3000) respectively at  $4^{\circ}\text{C}$ . The membranes were thoroughly washed three times with TBST before incubation with horseradish peroxidase (HRP)-conjugated antibodies for 1 h at room temperature. After another TBST wash, the membranes were exposed with chemiluminescence (ECL) detection. Western blot image was obtained from the Tanon 5200 Chemiluminescence Imaging System (Shanghai Tanon Science & Technology, Shanghai, China).

## Immunohistochemistry (IHC) Assay

Paraffin-embedded mouse lung section was dewaxed as described in the "Lung histology" section. Antigen retrieval was performed in 0.01M sodium citrate buffer (pH 6.0), after microwaved at  $100^{\circ}\text{C}$  for 15 min, the slides were washed in PBS for three times, subsequently the sections were incubated at  $37^{\circ}\text{C}$  for 30 min in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity followed by washed in PBS for 3 times, the slides were blocked at  $37^{\circ}\text{C}$  for 60 min with 5% bovine serum albumin (BSA), then stained with specific antibodies for p-NF- $\kappa\text{B}$  (1:200) at  $4^{\circ}\text{C}$  in a humidified chamber overnight. The slides were washed in PBS 3 times before incubated with a second antibody at room temperature for 60 mins, then the slides were stained with diaminobenzidine (DAB). All sections were counterstained with hematoxylin, and examined under an Olympus light microscope. Dark brown color signified positive immunostaining for a particular antigen expression. Negative controls were prepared by IgG for the primary antibodies.

## immunofluorescence (IF) Assay

the cells were fixed with 4% paraformaldehyde, then permeabilized in 0.1% Triton X-100 and 5% BSA for 30 min. Primary antibodies diluted in PBS incubated overnight at  $4^{\circ}\text{C}$ , the cells were washed three times with PBS, and stained with a secondary antibody for 1 h at room temperature. After washing, the samples were covered with PBS containing 50% glycerol. Images were obtained using microscopes.

## Preparation of CSE

To prepare CSE stock solution, smoke from 2 cigarettes was passed through 10ml serum-free Dulbecco's modified Eagle's medium and sterilized via filtration through a 0.22- $\mu\text{m}$  filter. The CSE stock solution (100%) was diluted to the desired concentrations in the experiment.

## Cell culture

RAW264.7 cells sourced from the Cell Bank of the Chinese Academy of Science (Shanghai, China) were cultured in DMEM (10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin) in a humidified incubator with 5% CO<sub>2</sub> at 37°C. RAW264.7 cells were seeded in 6-well plates with a 60~70% concentration, then treated with 3% CSE plus YT, RH and YT+RH respectively. Cells were washed three times with cold PBS 24 h after the treatments, and cell lysates harvested for Western blot analysis.

## Measurement of ROS

Total ROS production in RAW264.7 cells was determined by the DCFDA fluorescence microscopy method as follows, RAW264.7 (approximately 70% concentration in 6 wells plates) cells were incubated with 10mM DCFDA for 30 minutes at 37°C. Fluorescence of oxidized DCFDA and the index of

ROS formation was measured with a fluorescence microscope excitation and emission set at 490 and 530 nm respectively. CSE-induced ROS formation in cells was quantified with IPP.

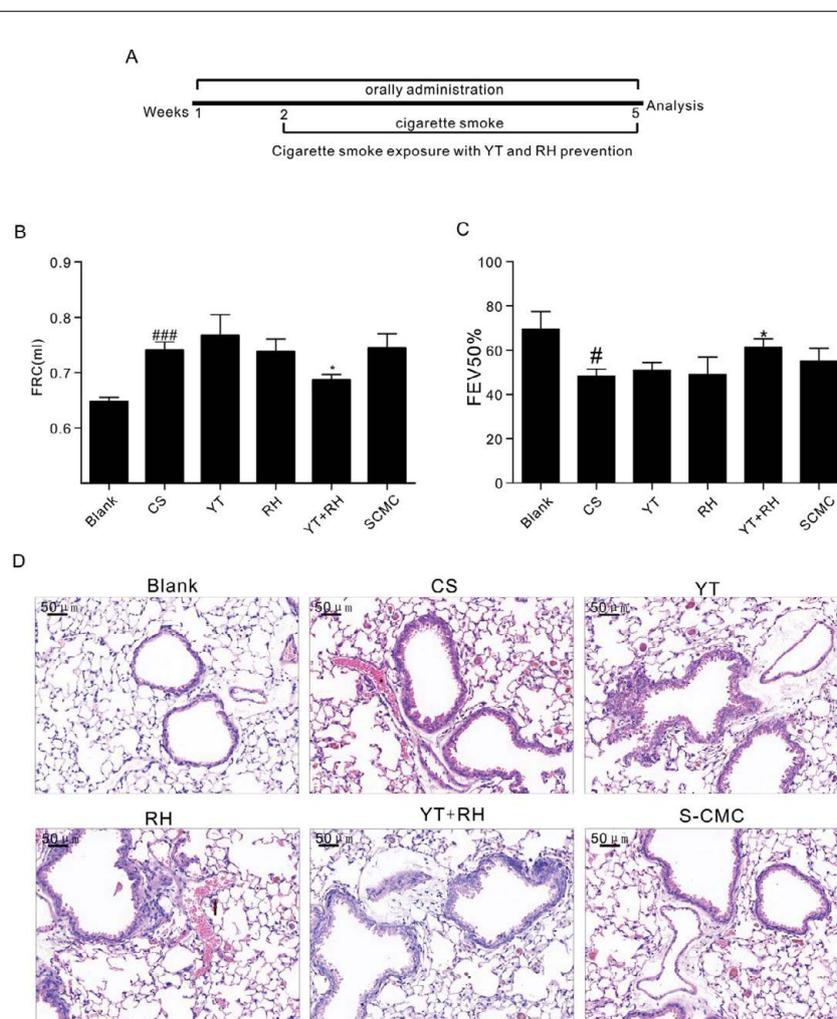
## Statistical analysis

All results are described as the means ± standard error of the means (SEM). Statistical difference among the experimental groups was detected by the one-way ANOVA analysis. Statistical significance was set at  $P < 0.05$ .

## RESULT

### YT+ RH improves pulmonary function and alleviates emphysema in CS-exposed mice

Mice were exposure to CS for 4 weeks (Figure 1A). Compared with mice of Blank group, CS exposure mice presented declined pulmonary function with increased FRC and decreased

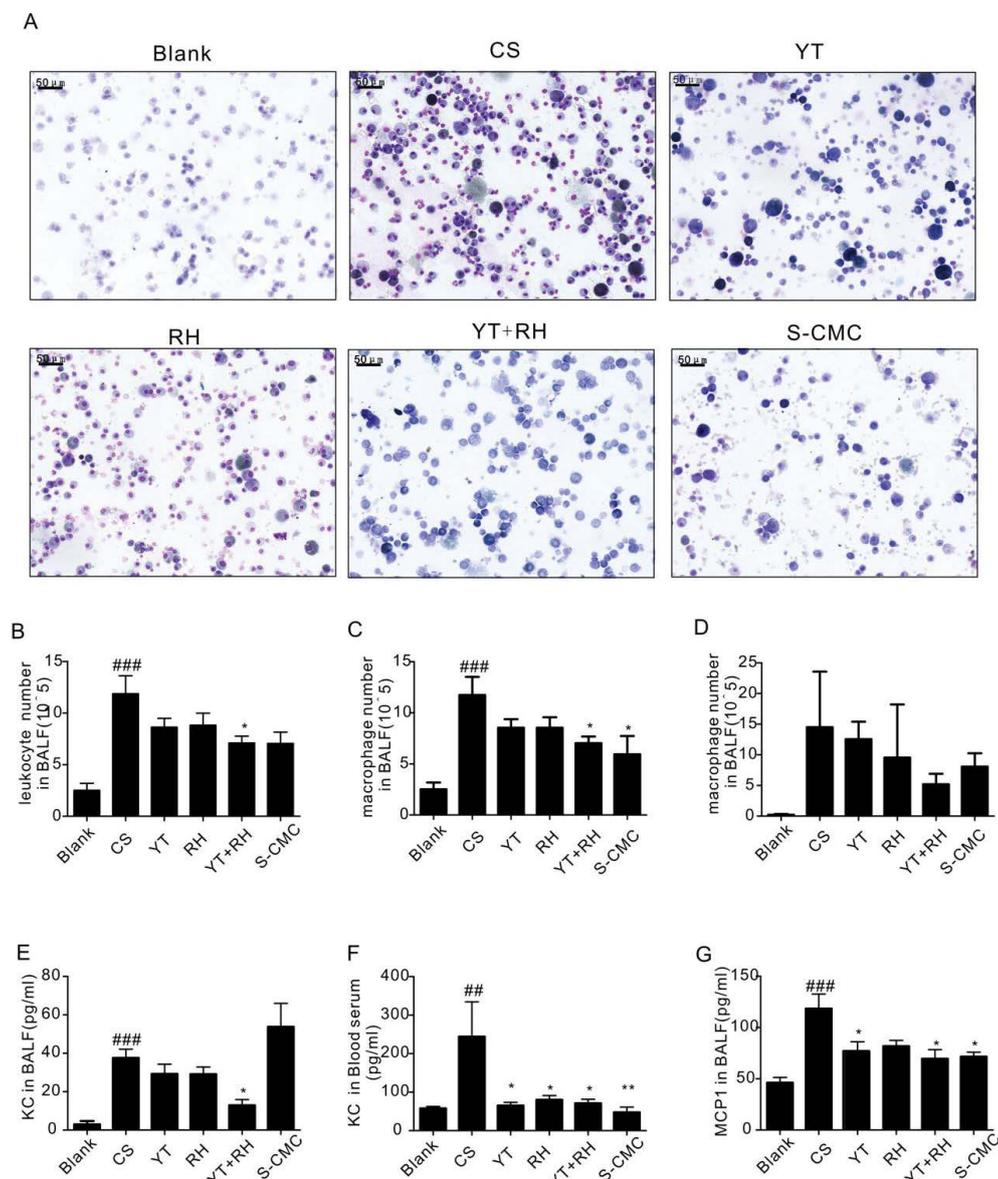


**Figure 1** YT+ RH improves pulmonary function and emphysema in CS-exposed mice. A) mice were exposed to CS for 4 weeks and pretreated. (B,C)The pulmonary function of mice were measured. (D)Pathological changes in the lungs of mice (H&E stained). Scale bar=50µm, n=6 in each group. The values are presented as the mean± SEM. vs Blank group. # $P < 0.05$ , ### $P < 0.001$ .vs CS group, \* $P < 0.05$ .

FEV50%, the pulmonary function of YT+RH group mice was significantly improved, as demonstrated by decreased FRC and increased FEV50% (Figure 1B, C). Pathologic alterations were examined after H&E staining, as shown in Figure 1D, the mean linear intercept (MLI) in the lungs of mice exposed to CS for four weeks were much larger than those exposed to normal room air. This implied that emphysema was successfully induced after four weeks of CS stimulation. YT+RH treatment significantly decreased MLI in the lungs.

### YT+RH reduces CS-induced inflammation in mice

Mice exposed to CS for 4 weeks exhibited typical inflammation, which was consistent with previous findings<sup>15,16</sup>. We measured the cell counts and differentiation in BALF to assess the effects of YT and RH on the CS-induced pulmonary inflammation (Figure 2A). CS significantly increased the number of inflammatory cells, including the cell counts of total cells, macrophages and neutrophils. YT+RH reduced inflammatory cell recruitment in the lung (Figure 2B, C, D). Evaluation of



**Figure 2** YT+RH reduces CS-induced inflammation in mice. (A) the inflammation cells differentials in BALF were stained with diff-quick staining. (B-D) The total counts of cells, macrophages and neutrophils from the BALF were counted. (E-J) inflammatory cytokines were detected by ELISA. n=7 in blank group, CS group, YT group and YT+RH group, n=6 in RH group, n=5 in SCMC group. The values are presented as the mean± SEM. vs Blank group. ##P<0.01, ###P<0.001 vs CS group, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

the effects of YT+RH on cytokine secretion in CS-treated mice revealed that YT+RH reduced CS-induced increase of KC and MCP-1 in the BALF (Figure 2E,2G) and KC in the blood serum (Figure 2F). These results suggest that the administration of YT + RH could attenuate CS-induced airway inflammation.

### YT+RH attenuates CS-induced oxidative stress in mice

CS causes oxidative stress in the mouse lungs as demonstrated by the increased and decreased MDA levels and SOD activities respectively. YT+RH treatment significantly attenuated MDA levels and the decreased SOD activities (Figure 3A, B). Moreover, Western blotting analysis showed that the protein levels of HO-1 were significantly enhanced in the lungs of CS-exposed mice, treatment with YT+RH reduced protein levels of HO-1 indicating the inhibitory effect of YT+RH on oxidative stress.

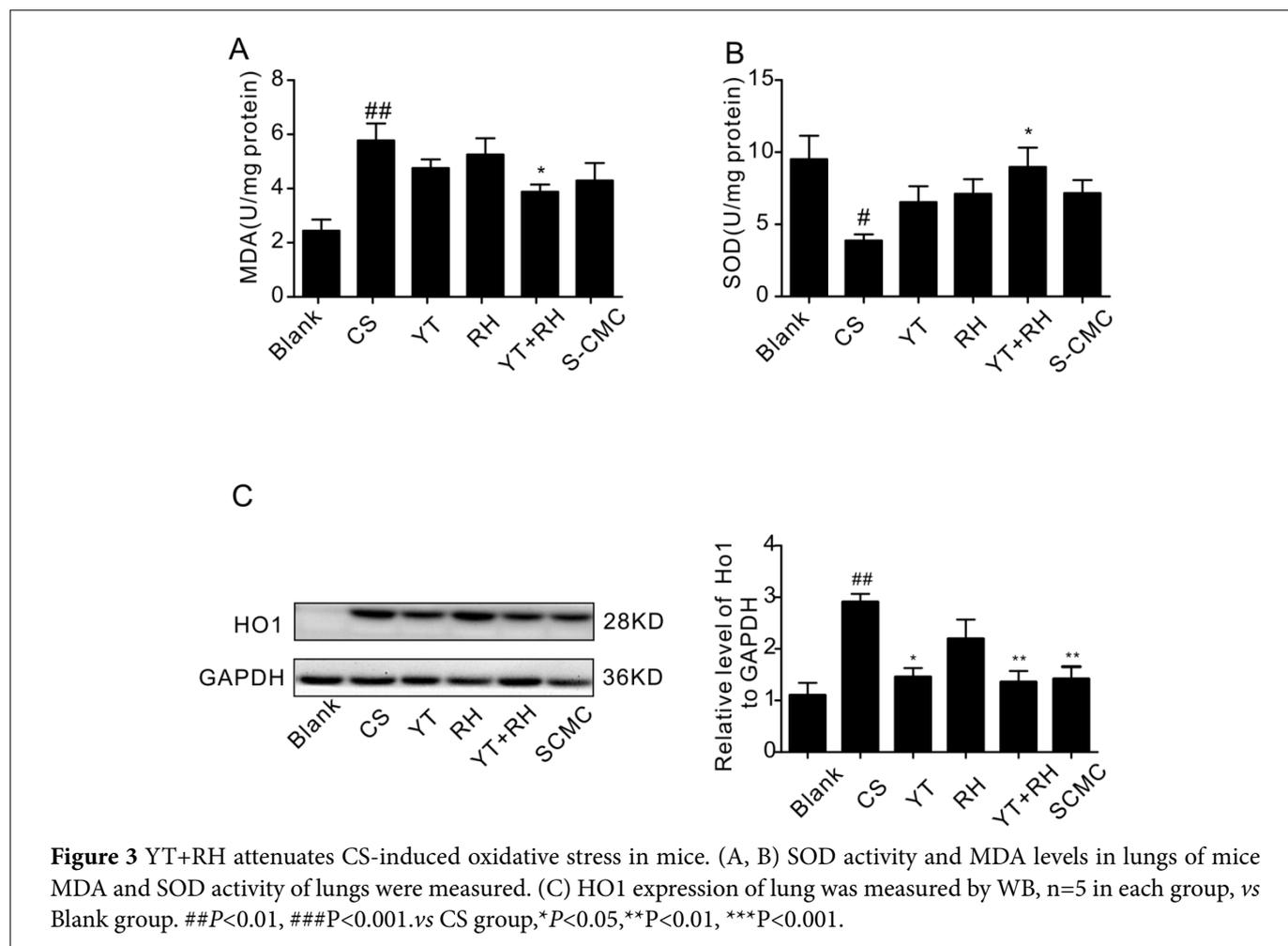
### YT+RH treatment attenuates CSE-induced oxidative stress in macrophages

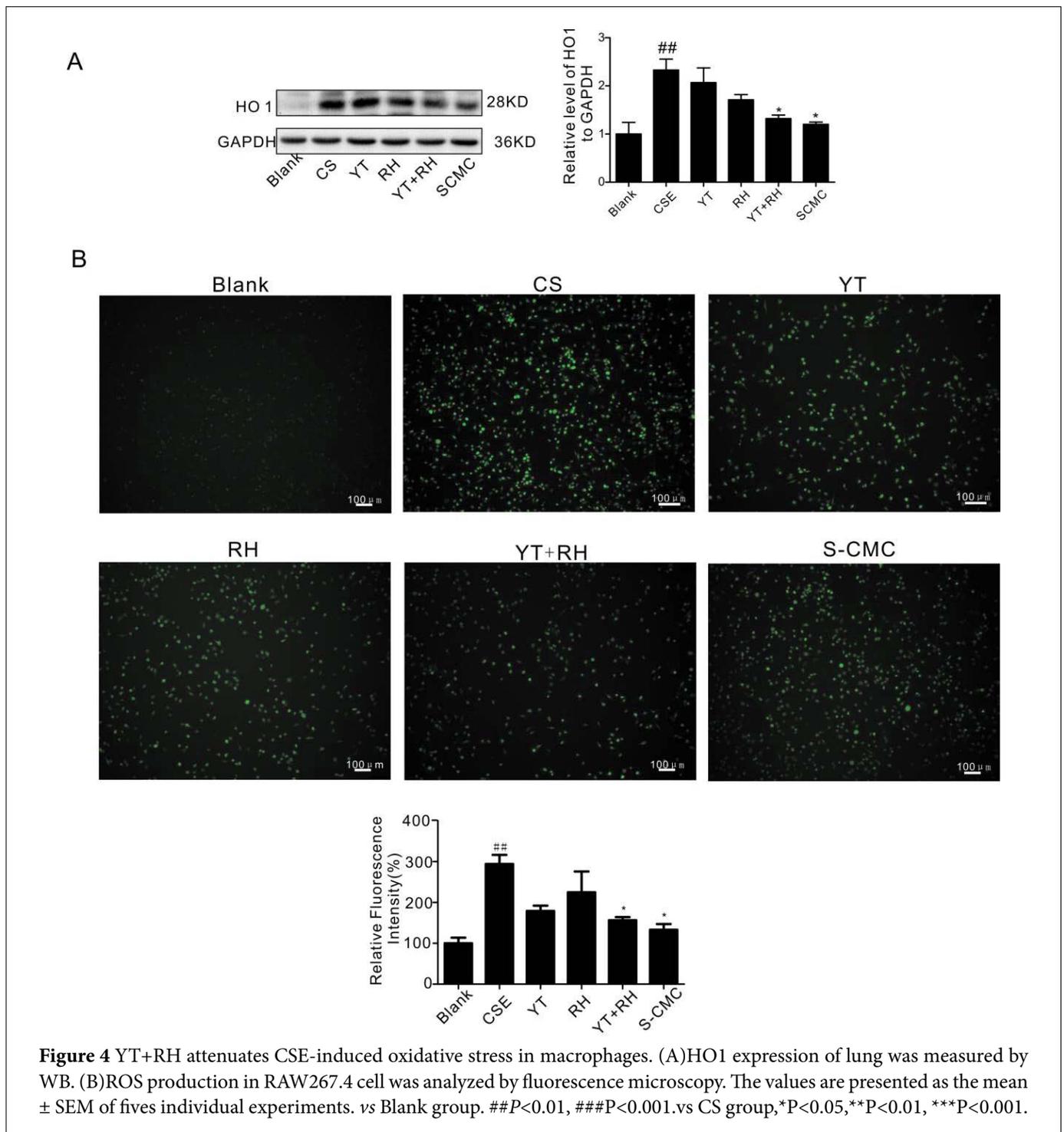
CSE induces oxidative stress in macrophages [17]. We also investigated the effects of YT+RH treatment on CS-induced

oxidative stress in macrophages. Western blotting analysis revealed a significant increase in the expression level of HO-1 in the CSE-induced macrophage, YT+RH significantly reduced the HO-1 expression levels in the CS-stimulated mice (Figure 4A). Given that oxidation reactions induced by ROS are regarded as a trigger of the oxidative stress, we examined the effects of YT+RH on intracellular ROS production in RAW 264.7 cells. As shown in Figure 4A, CSE significantly increased ROS levels of macrophages, which were significantly inhibited by YT+RH (Figure 4B).

### YT+RH treatment inhibits CS-induced activation of pERK and pNF- $\kappa$ B *in vivo*

Given that MAPKs and NF- $\kappa$ B signaling pathways play important roles in the regulation of CS-induced inflammation and oxidative stress [18-20], we investigated the effects of YT+RH on the phosphorylation of ERK and NF- $\kappa$ B. As demonstrated by the Western blot (Figure 5B) and immunohistochemical (Figure 5A) analyses, the pERK and pNF- $\kappa$ B were remarkably increased in the CS-exposed mouse lungs. YT+RH treatment significantly reduced these levels.

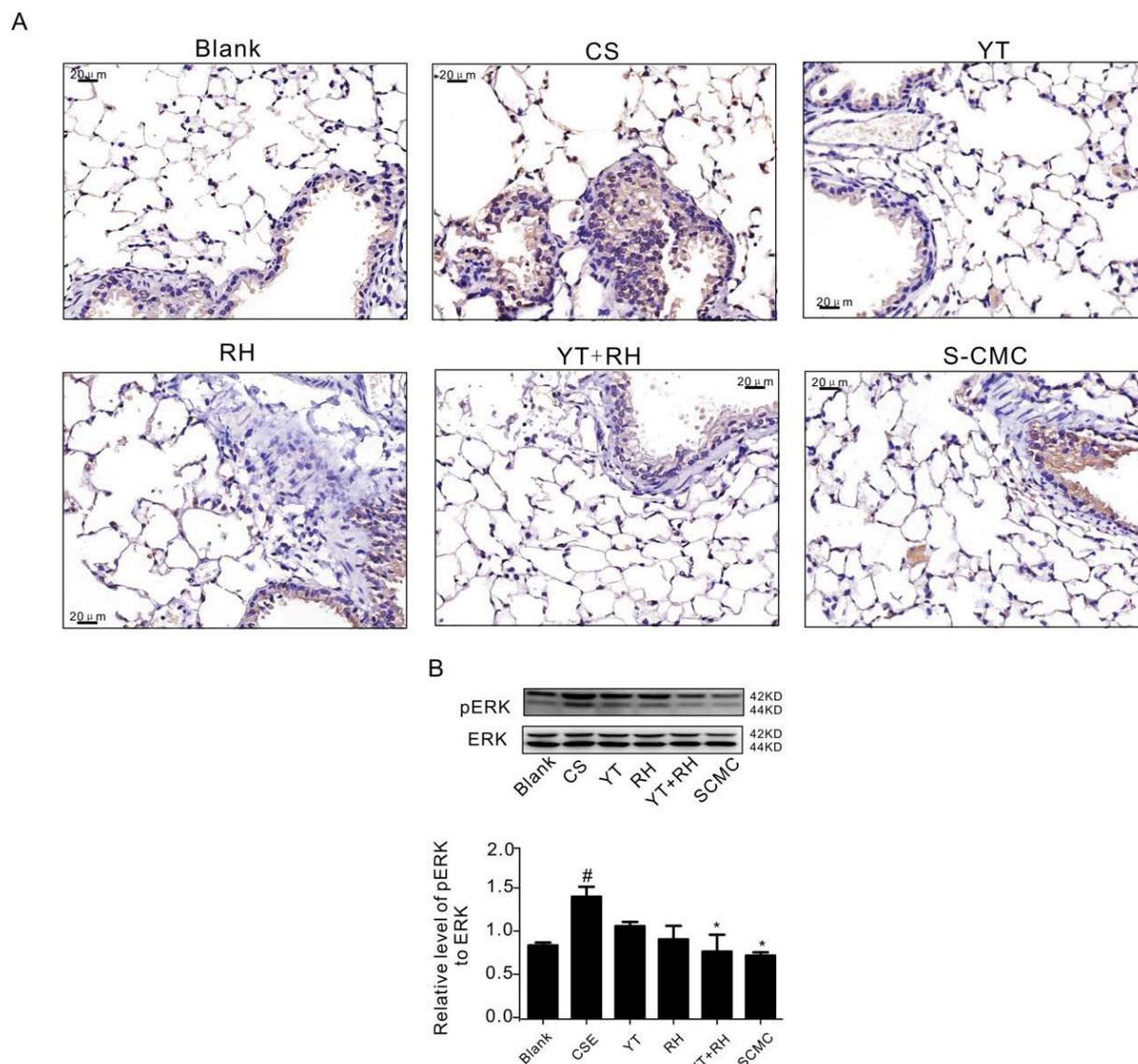




#### YT+RH treatment inhibits CS-induced activation of NF- $\kappa$ B *in vitro*

The suppression effects of YT+RH on p-NF- $\kappa$ B signaling pathways were validated in RAW 264.7 cells. Western blot analysis showed that CSE stimulation significantly increased the phosphorylation of NF- $\kappa$ B(Figure 6A).

Immunofluorescence of RAW 264.7 cells stimulated with CSE showed increased p-NF- $\kappa$ B levels (Figure 6B). Collectively, these data suggest that the inactivation of NF- $\kappa$ B signaling pathways contribute to the protective effects of YT+RH on the CS-induced inflammation and oxidative stress.



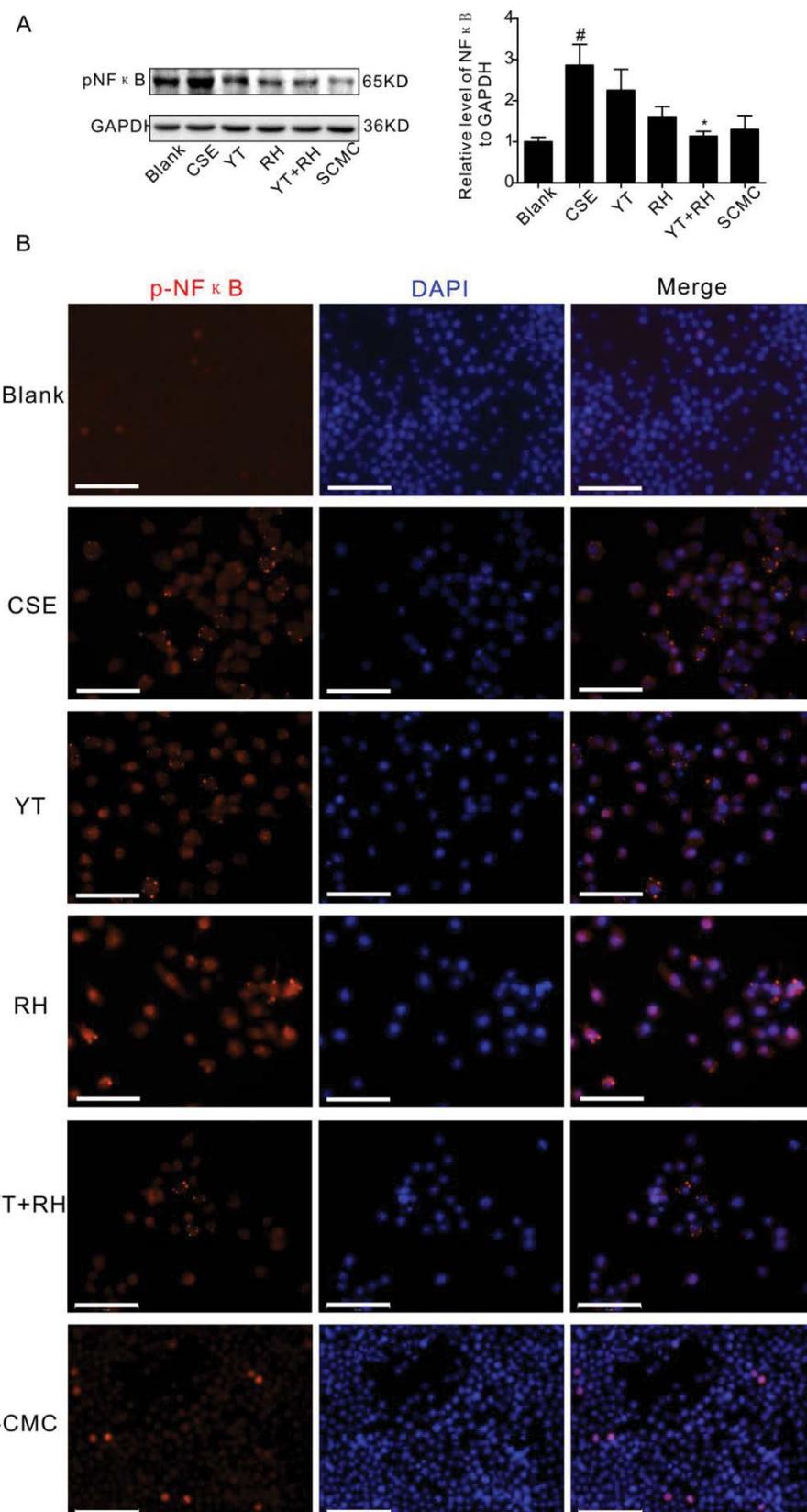
**Figure 5** YT+RH treatment inhibits CS-induced activation of pERK1/2 and p-NF- $\kappa$ B *in vivo*. (A) the expression of pNF- $\kappa$ B in lungs was detected by immunohistochemical experiments. (B) the expression of phosphorylation ERK of the lung was detected by WB. The values are presented as mean $\pm$ SEM of five individual experiments. vs Blank group. ## $P$ <0.01, ### $P$ <0.001. vs CS group, \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

### YT+RH treatment inhibits CS-induced activation of pERK *in vitro*

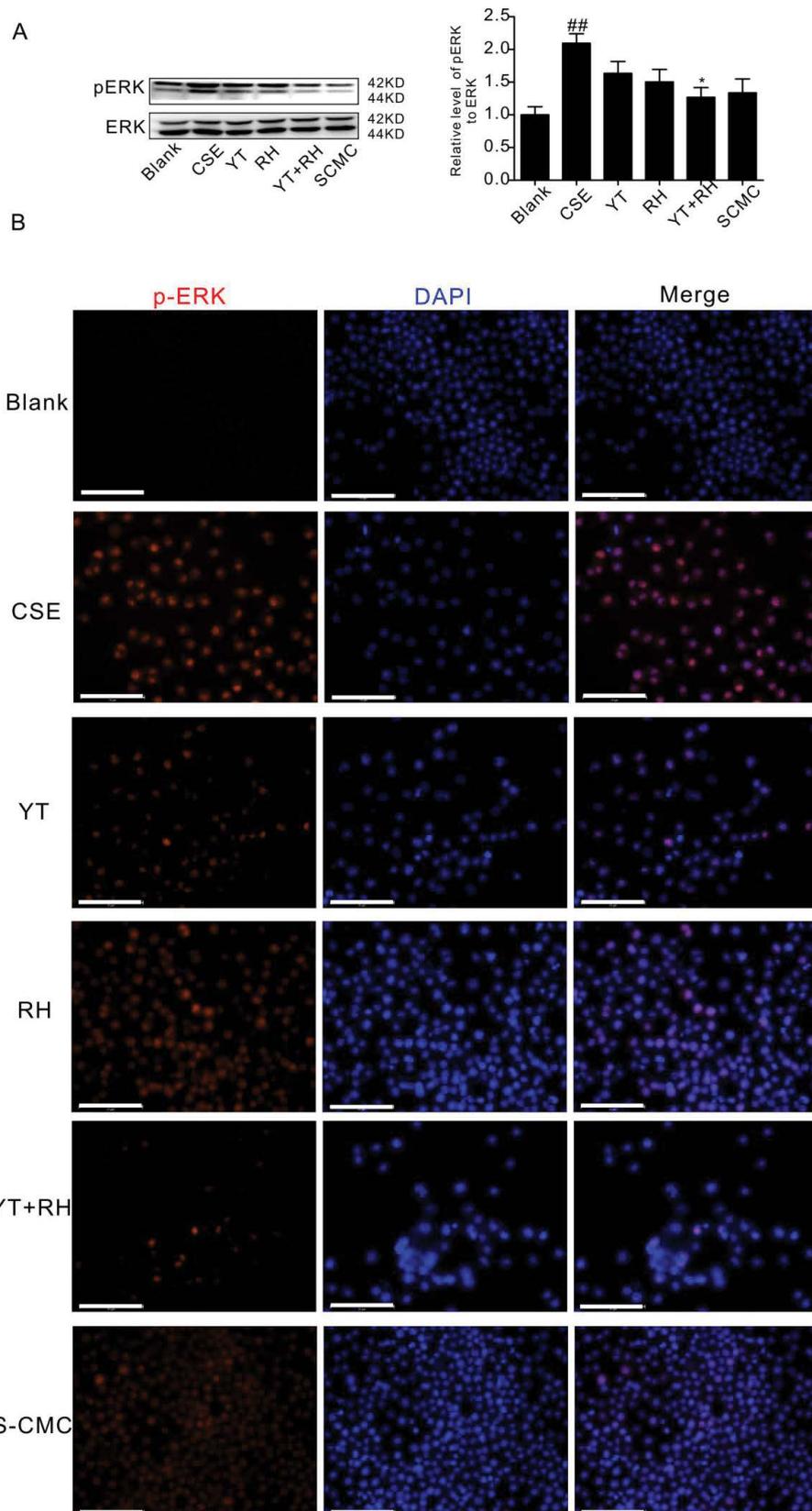
As shown in figure 7A, ERK1/2 phosphorylation signaling were increased in RAW264.7 cells stimulated with CSE. However the treatment with YT+RH attenuated these protein levels. Immunofluorescence of RAW 264.7 cells stimulated with CSE showed increased ERK levels (Figure 7B). Altogether, these results suggested that YT+RH protects lung from oxidative stress-induced activation of MAPK signal pathways.

### DISCUSSION

In this study, we demonstrate that the combination of YT and RH intervention (YT+RH) can alleviate CS-induced inflammation and oxidative stress, further YT+RH successfully inhibited cigarette smoke extract (CSE)-induced oxidative stress in macrophages. Finally, we demonstrate the protective effects of YT+RH are associated with the inhibition of CS-induced NF- $\kappa$ B/ERK expression *in vitro* and *in vivo*. These results provide a rationale for the pretreatment of COPD.



**Figure 6** YT+RH treatment inhibits CS-induced activation of pNF-κB in vitro. (A) the phosphorylation NF-κB expression of cell was detected by WB. (B) the phosphorylation NF-κB expression of cell was detected by IF. The values are presented as the mean ± SEM of five individual experiments. vs Blank group. #*P*<0.05. vs CS group, \**P*<0.05.



**Figure 7** YT+RH treatment inhibits CS-induced activation of pERK in vitro. (A) the phosphorylation ERK expression of cell was detected by WB. (B) the phosphorylation ERK expression of cell was detected by IF. The values are presented as the mean  $\pm$  SEM of five individual experiments. *vs* Blank group. ## $P < 0.01$ . *vs* CS group, \* $P < 0.05$ .

COPD is characterized by high incidence mortality and disability rate thereby posing a serious threat to human health. Presently symptomatic strategies such as anti-inflammatory, antispasmodic and anti-asthmatic are widely used in the treatment of COPD. However, given that there are no effective treatments to inhibit chronic inflammation in COPD, prevention treatment may be a viable strategy. Diet may contribute to antioxidant/oxidant and inflammatory status in COPD patients. Compared with healthy individuals, COPD subjects have diets with lower fruit, vegetable intake and poorer antioxidant content [14]. These correlate with impaired lung function and risk of developing COPD [21,22]. YT and RH are medicinal foods which have anti-inflammation and anti-oxidative properties, therefore we investigated the potential of these properties. This is the first study to report that YT and RH combination can inhibit cigarette smoke induced lung inflammation and oxidative stress through a mechanism that involves the downregulation inflammatory cytokines NF- $\kappa$ B.

Previous studies demonstrated Pachymic acid, a lanostane-type triterpenoid from *Poriacocos*, has been reported to reduce apoptosis by activating ROS-dependent JNK and ER stress pathways in lung cancer cells [23]. *Dictyophora indusial*, *lentinusedodes* and *tremellafuciformis* belong to different species of edible and/or medicinal mushrooms, which possess antioxidant activity due to their bioactive compounds, such as polyphenols, polysaccharides, vitamins, carotenoids and minerals<sup>12</sup>. In addition, they have antiobesity, antidiabetes, anticancer and antibiotic properties [24], and *Dictyophora indusial* polysaccharides have an immunestimulatory effect [25]. For instance, cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ), NO synthase and NF- $\kappa$ B of the RAW264.7 cells were upregulated upon treatment with *Dictyophora indusial* polysaccharides [26]. The B-Glucan from *Pleurotus Ostreatus*, which is another species of mushroom, decreased the incidence and duration of bacterial exacerbations in patients with COPD [27]. Further, *Astragalus membranaceus* extract reduced the inflammatory response induced by lipopolysaccharide from *E. coli* (LPS), reduced ROS release, and increased nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation [28,29].

Honey has a protective effect against oxidative stress and inflammatory response [30]. *Ficus caricad Fruits* manifested its anti-cancer properties via inhibiting proliferation, apoptosis, and necrosis of Huh7it Cells [31]. *Ficus carica* has emerged as a good source of traditional medicine and food for the treatment of various ailments such as anemia, cancer, diabetes, leprosy, liver diseases, paralysis, skin diseases, and ulcers [32-34]. Flavonoid compounds from *eleocharistuberosa* peel have also shown anti-tumor, antioxidant, and nitrite scavenging effects

[35]. Polysaccharides of lily bulb ameliorated menopause-like behavior in ovariectomized mice [36].

There is evidence that increased oxidant stress and inflammation with cigarette smoke injury lung, oxidative stress marks, such as increased MDA and decreased SOD activities were observed in this study, YT and RH combination significantly inhibited CS induced increased MDA and decreased SOD suggesting YT and RH combination could attenuate CS induced oxidative stress, Nuclear factor NF- $\kappa$ B (pro-oxidative) is one of the redox-sensitive transcription factors that coordinate the inflammatory response to cigarette smoke. The activation of these transcription factors is often evident in both the lung and extra-pulmonary tissues [37]. In this study we detected whether YT and RH protected against CS induced oxidative stress and inflammation by activating NF- $\kappa$ B, the results showed YT and RH combination could downregulate phosphorylation NF- $\kappa$ B and ERK signaling way.

Chronic CS exposure is associated with emphysema and airway remodeling in COPD patients. In this study, we used a CS-induced mouse model to investigate the effect of YT and RH on lung inflammation. Four weeks of cigarette smoke exposure was enough to induce inflammation in this model which was consistent with previous studies [38]. The mouse model of cigarette smoke exposure was designed to evaluate the degree of inhibition of cigarette smoke induced lung inflammation, but not the emphysema observed in chronic mouse cigarette smoke exposure models [38,39]. Human bronchial epithelium cell lines exposure to cigarette smoke augmented the release of neutrophil chemoattractant IL-8, and monocyte chemoattractant protein 1(MCP-1) [40,41]. Cigarette smoke induced the production and accumulation of burden oxidants in the respiratory tract [17,42]. Additionally, oxidative stress in the lung can trigger inflammation induced by CS [16,43]. Long-term use of carbocysteine has been documented to reduce exacerbation [44], hence may be considered as an anti-inflammation and anti-oxidation drug [45,46]. This informed our choice of carbocysteine as a positive control in this study.

Further, we analyzed the mechanisms employed by YT+RH treatment in attenuating cigarette smoke-induced inflammation and oxidative stress. Several studies show NF- $\kappa$ B to play a pivotal role in COPD inflammation [43]. Moreover, ERK1/2 contributes to CS-induced inflammation by modulating NF- $\kappa$ B DNA binding activity in A549 cells [47]. We found that YT+RH combination reduced activation of pERK1/2 and pNF- $\kappa$ B levels *in vivo* and *in vitro*, indicating that pERK1/2 and pNF- $\kappa$ B signaling may be involved in CS-induced oxidative stress and pathology damage. However, we did not elucidate the mechanism of ERK1/2 activation and NF- $\kappa$ B signaling.

In conclusion, YT+RH abrogated CS-induced inflammation and oxidative stress. This protective action may be attributable to the anti-inflammatory and anti-oxidant effects of YT+RH via ERK1/2 and NF- $\kappa$ B signaling pathways. This study provides a foundation for further investigation on the protective effects of YT+RH combination on COPD. Although we demonstrate the beneficial effects of YT+RH combination on cigarette smoke exposure in a mouse model, its efficacy in humans should be first assessed in clinical trials.

## ACKNOWLEDGMENTS

This work was supported by grants from State Key Laboratory of Respiratory Disease (SKLRD-QN-201917).

## CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

## REFERENCES

- Chung KF, Adcock IM. Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. *European Respiratory Journal*. 2008;31(6):1334-1356.
- Stanaway JD, Afshin A, Gakidou E, et al. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks for 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet*. 2018;392(10159):1923-1994.
- Collaborators GBDRF, Forouzanfar MH, Alexander L, et al. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*. 2015;386(10010):2287-2323.
- Wang C, Xu J, Yang L, et al. Prevalence and risk factors of chronic obstructive pulmonary disease in China (the China Pulmonary Health [CPH] study): a national cross-sectional study. *The Lancet*. 2018;391(10131):1706-1717.
- Tian Y, Li J, Li Y, et al. Effects of Bufei Yishen Granules Combined with Acupoint Sticking Therapy on Pulmonary Surfactant Proteins in Chronic Obstructive Pulmonary Disease Rats. *Biomed Res Int*. 2016;2016:8786235.
- Mannino DM, Buist AS. Global burden of COPD: risk factors, prevalence, and future trends. *Lancet*. 2007;370(9589):765-773.
- Zhong N, Wang C, Yao W, et al. Prevalence of chronic obstructive pulmonary disease in China: a large, population-based survey. *Am J Respir Crit Care Med*. 2007;176(8):753-760.
- <The Role of Oxidative Stress in the Pathogenesis of COPD.pdf>.
- MacNee W. Oxidants and COPD. *Curr Drug Targets Inflamm Allergy*. 2005;4(6):627-641.
- Barnes PJ, Shapiro SD, Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur Respir J*. 2003;22(4):672-688.
- <ajrcmb.22.2.3809.pdf>.
- Kozarski M, Klaus A, Jakovljevic D, et al. Antioxidants of Edible Mushrooms. *Molecules*. 2015;20(10):19489-19525.
- Annesi-Maesano I, Roche N. Healthy behaviours and COPD. *Eur Respir Rev*. 2014;23(134):410-415.
- Zheng PF, Shu L, Si CJ, Zhang XY, Yu XL, Gao W. Dietary Patterns and Chronic Obstructive Pulmonary Disease: A Meta-analysis. *COPD*. 2016;13(4):515-522.
- Lu W, Li D, Hu J, et al. Hydrogen gas inhalation protects against cigarette smoke-induced COPD development in mice. *J Thorac Dis*. 2018;10(6):3232-3243.
- Barnes PJ. Inflammatory mechanisms in patients with chronic obstructive pulmonary disease. *J Allergy Clin Immunol*. 2016;138(1):16-27.
- Fischer BM, Voynow JA, Ghio AJ. COPD: balancing oxidants and antioxidants. *Int J Chron Obstruct Pulmon Dis*. 2015;10:261-276.
- Sun SC. The non-canonical NF-kappaB pathway in immunity and inflammation. *Nat Rev Immunol*. 2017;17(9):545-558.
- Hellermann GR, Nagy SB, Kong X, Lockey RF, Mohapatra SS. Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells. *Respir Res*. 2002;3:22.
- Lee SY, Miller M, Cho JY, Song DJ, Karin M, Broide DH. Inactivation of I kappaB-kinase-beta dependent genes in airway epithelium reduces tobacco smoke induced acute airway inflammation. *Int Immunopharmacol*. 2010;10(8):906-912.
- Rodriguez-Rodriguez E, Ortega RM, Andres P, et al. Antioxidant status in a group of institutionalised elderly people with chronic obstructive pulmonary disease. *Br J Nutr*. 2016;115(10):1740-1747.
- Ochs-Balcom HM, Grant BJ, Muti P, et al. Antioxidants, oxidative stress, and pulmonary function in individuals diagnosed with asthma or COPD. *Eur J Clin Nutr*. 2006;60(8):991-999.
- Ma J, Liu J, Lu C, Cai D. Pachymic acid induces apoptosis via activating ROS-dependent JNK and ER stress pathways in lung cancer cells. *Cancer Cell Int*. 2015;15:78.
- Friedman M. Mushroom Polysaccharides: Chemistry and Antiobesity, Antidiabetes, Anticancer, and Antibiotic Properties in Cells, Rodents, and Humans. *Foods*. 2016;5(4).
- Habtemariam S. The Chemistry, Pharmacology and Therapeutic Potential of the Edible Mushroom *Dictyophora indusiata* (Vent ex. Pers.) Fischer (Synn. *Phallus indusiatus*). *Biomedicines*. 2019;7(4).
- Deng C, Shang J, Fu H, Chen J, Liu H, Chen J. Mechanism of the immunostimulatory activity by a polysaccharide from *Dictyophora indusiata*. *Int J Biol Macromol*. 2016;91:752-759.
- Minov J, Bislimovska-Karadzinska J, Petrova T, Vasilevska K, Stoleski S, Mijakoski D. Effects of Pleuran (Beta-Glucan from *Pleurotus Ostreatus*) Supplementation on Incidence and Duration of COPD Exacerbations. *Open Access Maced J Med Sci*. 2017;5(7):893-898.
- Adesso S, Russo R, Quaroni A, Autore G, Marzocco S. Astragalus membranaceus Extract Attenuates Inflammation and Oxidative Stress in Intestinal Epithelial Cells via NF-kappaB Activation and Nrf2 Response. *Int J Mol Sci*. 2018;19(3).

29. Bratkov VM, Shkondrov AM, Zdraveva PK, Krasteva IN. Flavonoids from the Genus Astragalus: Phytochemistry and Biological Activity. *Pharmacogn Rev.* 2016;10(19):11-32.
30. Mohd Sairazi NS, K NSS, Asari MA, Mummady S, Muzaimi M, Sulaiman SA. Effect of tualang honey against KA-induced oxidative stress and neurodegeneration in the cortex of rats. *BMC Complement Altern Med.* 2017;17(1):31.
31. Church DE, Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect.* 1985;64:111-126.
32. Badgujar SB, Patel VV, Bandivdekar AH, Mahajan RT. Traditional uses, phytochemistry and pharmacology of *Ficus carica*: a review. *Pharm Biol.* 2014;52(11):1487-1503.
33. Barolo MI, Ruiz Mostacero N, López SN. *Ficus carica* L. (Moraceae): An ancient source of food and health. *Food Chemistry.* 2014;164:119-127.
34. Ma J, Sun H, Liu H, et al. Hepatoprotective glycosides from the rhizomes of *Imperata cylindrical*. *J Asian Nat Prod Res.* 2018;20(5):451-459.
35. Zhan G, Pan L, Tu K, Jiao S. Antitumor, Antioxidant, and Nitrite Scavenging Effects of Chinese Water Chestnut (*Eleocharis dulcis*) Peel Flavonoids. *Journal of Food Science.* 2016;81(10):H2578-H2586.
36. Zhou XD, Zheng Y, Sharma R, Zhang ZJ. Total Polysaccharides of Lily Bulb Ameliorate Menopause-Like Behavior in Ovariectomized Mice: Multiple Mechanisms Distinct from Estrogen Therapy. *Oxid Med Cell Longev.* 2019;2019:6869350.
37. Khanna A, Guo M, Mehra M, Royal W, 3rd. Inflammation and oxidative stress induced by cigarette smoke in Lewis rat brains. *J Neuroimmunol.* 2013;254(1-2):69-75.
38. Cai B, Gan X, He J, et al. Morin attenuates cigarette smoke-induced lung inflammation through inhibition of PI3K/AKT/NF-kappaB signaling pathway. *Int Immunopharmacol.* 2018;63:198-203.
39. Botelho FM, Gaschler GJ, Kianpour S, et al. Innate immune processes are sufficient for driving cigarette smoke-induced inflammation in mice. *Am J Respir Cell Mol Biol.* 2010;42(4):394-403.
40. de Boer WI, Sont JK, van Schadewijk A, Stolk J, van Krieken JH, Hiemstra PS. Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *J Pathol.* 2000;190(5):619-626.
41. Damia Ade D, Gimeno JC, Ferrer MJ, Fabregas ML, Folch PA, Paya JM. A study of the effect of proinflammatory cytokines on the epithelial cells of smokers, with or without COPD. *Arch Bronconeumol.* 2011;47(9):447-453.
42. Lambros ML, Plafker SM. Oxidative Stress and the Nrf2 Anti-Oxidant Transcription Factor in Age-Related Macular Degeneration. *Adv Exp Med Biol.* 2016;854:67-72.
43. Yoshida T, Tuder RM. Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. *Physiol Rev.* 2007;87(3):1047-1082.
44. Zeng Z, Yang D, Huang X, Xiao Z. Effect of carbocysteine on patients with COPD: a systematic review and meta-analysis. *Int J Chron Obstruct Pulmon Dis.* 2017;12:2277-2283.
45. Pace E, Ferraro M, Siena L, et al. Carbocysteine regulates innate immune responses and senescence processes in cigarette smoke stimulated bronchial epithelial cells. *Toxicol Lett.* 2013;223(2):198-204.
46. Wang W, Guan WJ, Huang RQ, et al. Carbocysteine attenuates TNF-alpha-induced inflammation in human alveolar epithelial cells *in vitro* through suppressing NF-kappaB and ERK1/2 MAPK signaling pathways. *Acta Pharmacol Sin.* 2016;37(5):629-636.
47. Zhao J, Harper R, Barchowsky A, Di YP. Identification of multiple MAPK-mediated transcription factors regulated by tobacco smoke in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol.* 2007;293(2):L480-490.