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Original Research Article

Vitexin attenuates smoke inhalation-induced acute lung injury in rats by inhibiting oxidative stress via PKC β/p66Shc signaling pathway

Xuxin Chen[1,2], Huming Li[1], Hao Cheng[1], Meng Liu[1], Fan Wang[1], Jiguang Meng[1,2], Zhihai Han[1,2]*

[1]Department of Pulmonary and Critical Care Medicine, The Sixth Medical Center of Chinese PLA General Hospital, Beijing 100037, [2]School of Medicine, South China University of Technology, Guangzhou 510006, PR China

For correspondence:* ***Email:* *zhihaihandoctor@163.com;* ***Tel:*** *+86-010-66957581*

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Abstract

Purpose: *To investigate the protective effect of vitexin on smoke inhalation-induced acute lung injury (SI-ALI), and the underlying mechanism of action.*
Methods: *The ALI rat model was established by inhalation of smoke in a closed smoke chamber. Survival rate, arterial blood gas analysis, wet-to-dry weight ratio of lung tissues, bronchoalveolar lavage fluid protein concentration, lung tissue histology, and oxidative stress and inflammation level were evaluated. Expressions of protein kinase C β (PKC β), p66Shc, and phosphorylated p66Shc were determined by western blot or quantitative reverse transcription-polymerase chain reaction.*
Results: *Compared with smoke inhalation group, vitexin alleviated the decline in arterial partial pressure of oxygen ($p < 0.05$), reduced lung tissue exudation and pathological lung tissue damage, inhibited the expression of PKC β/p66Shc signaling pathway proteins, downregulated the level of oxidative stress and inflammation, and ultimately improved the survival rate in SI-ALI rats ($p < 0.05$).*
Conclusion: *Vitexin attenuates SI-ALI in rats by alleviating oxidative stress via inhibition of PKC β/p66Shc signaling pathway. Thus, this compound is a potential agent for the treatment of SI-ALI.*

Keywords: *Vitexin, Smoke inhalation, Acute lung injury, Oxidative stress, p66Shc*

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INTRODUCTION

Smoke inhalation-induced acute lung injury (SI-ALI) is a lung parenchymal or respiratory tract injury shown to be caused by smoke or toxic gas with or without heat [1]. It was found that 30 % of burn patients were complicated by SI-ALI and the mortality increased from 10 % to 60 – 80 % in SI-ALI patients [2]. Currently, there are no effective treatments for SI-ALI. Therefore, it is important to understand the pathogenesis of SI-ALI for novel and effective treatment strategies to be identified.

Oxidative stress refers to the disturbance of the balance between oxidants and antioxidants because of increased reactive oxygen species (ROS) and reactive nitrogen species levels (RNS) and/or decreased antioxidant levels.

Previous studies showed that inhibition of oxidative stress could attenuate SI-ALI [3]. P66Shc, a form of growth factor adaptor protein encoded by the proto-oncogene Shc, plays a key role in regulating the cell cycle and oxidative stress. Activation of protein kinase C β (PKC β) is necessary for the phosphorylation at Ser 36 of p66Shc [4]. Inhibition of the PKC β/p66Shc pathway was shown to reduce the levels of oxidative stress, whereas activation of this pathway increased oxidative stress [5]. However, it is unclear whether the PKC β/p66Shc signal participates in the regulation of oxidative stress in SI-ALI.

Vitexin, a flavonoid with anti-inflammatory and anti-oxidant effects, is extracted from hawthorn, mung bean, and polygonaceae plants [6]. Previous studies demonstrated that vitexin attenuated lipopolysaccharide (LPS)-induced acute lung injury in rats by inhibiting oxidative stress via the nuclear factor erythroid-2-related factor (Nrf2) signaling pathway [6]. In addition, vitexin attenuated chronic myocardial ischemia/reperfusion injury in rats by inhibiting oxidative stress [7] and protecting dopaminergic neurons through the phosphatidylinositol 3-kinase/protein kinase B signaling pathway [8]. Modern pharmacological studies show that vitexin has a variety of pharmacological activities and is safe. *In vitro*, vitexin (IC_{50} > 200 *μg*/mL) presented no cytotoxicity for the mouse macrophage cell line RAW264.7 [9]. Moreover, a high vitexin dose (10 mg/kg intraperitoneal injection) exerted no significant acute or sub-chronic toxicity, genotoxicity, or liver toxicity *in vivo* [10].

Collectively, these observations indicate that vitexin functions as an antioxidant in various disease states. Through negative regulation of oxidative stress, vitexin can attenuate LPS-induced ALI. Furthermore, the PKC β/p66Shc signaling pathway plays an important role in the regulation of oxidative stress. With this in mind, it is hypothesized that vitexin could attenuate SI-ALI in rats by inhibiting oxidative stress via the PKC β/p66Shc signaling pathway. Therefore, the present study investigated the effect of vitexin on PKC β/p66Shc pathway using a SI-ALI rat model.

EXPERIMENTAL

Smoke generator

The smoke generator used in this study was an improved version of that of Zhu *et al* [11]. The smoke generator consisted of an electric stove for generating smoke and a closed smoke chamber in which rats were exposed to the smoke. These two chambers were connected with pipes. A fan was installed in the chamber for generating smoke to adjust the smoke concentration in the smoke chamber. A glass plate was installed in the smoke chamber to observe the condition of the rats.

Study design

All animal procedures were approved by the Animal Care and Use Committee of the Chinese PLA General Hospital (approval no. SCXK-Army-2012-0012) and were conducted in accordance with the 'Animal Research: Reporting *in vivo* experiments guidelines 2.0'. The Guidelines for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committees of the United States (2011 edition) were followed [12]. In brief, a total of 42 Sprague Dawley rats (8 weeks, 180 – 220 g, Certificate number: SCXK-Army-2012-0004; Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing, China) were randomly assigned to seven groups as follows: control group (no treatment and 25 min air inhalation), smoke group (no treatment and 25 min smoke inhalation), smoke + vitexin group (treated with 3 mg/kg vitexin + 25 min smoke inhalation), smoke + LY333531 group (treated with 1 mg/kg LY333531 + 25 min smoke inhalation), smoke + PMA group (treated with 1 mg/kg PMA + 25 min smoke inhalation), smoke + vitexin + LY333531 group (treated with 3 mg/kg vitexin and 1 mg/kg LY333531 + 25 min smoke inhalation), and smoke + vitexin + PMA group (treated with 3 mg/kg vitexin and 1 mg/kg PMA + 25 min smoke inhalation) (all n = 6). Vitexin (Beijing Solarbio Science and Technology, Beijing, China), LY333531 (Medchem Express, Monmouth Junction, NJ, USA), and PMA (Medchem express) were intraperitoneally injected daily for 5 days in rats before smoke inhalation. The establishment of the model has been described in a previous study [13]. At 12 h after smoke inhalation, the rats in each group were sacrificed by a supraphysiological dose of pentobarbital sodium (100 mg/kg), and the lung tissue, blood, and BALF were collected. In addition, a further 84 rats were similarly assigned to the seven groups and treated the same way as described above. The survival rates after 72 h were observed.

Blood gas analysis

At 12 h after smoke or air inhalation, arterial blood samples were obtained from the animals in each group using a PE-50 catheter, which was inserted in the abdominal aorta after the rats were anesthetized with 30 mg/kg of pentobarbital

sodium. The arterial partial pressure of oxygen (PaO_2) was measured using a blood gas analyzer (Siemens, Munich, Germany) at room temperature. The oxygenation index was determined by the ratio of PaO_2 to the fraction of inspired oxygen (PaO_2/FiO_2). Because all rats were allowed free access to water and food and were housed at room temperature in a room that was continuously ventilated after the smoke assault or air inhalation, the FiO_2 was determined at 21 %.

Lung W/D weight ratio

The lung W/D weight ratio was calculated to evaluate lung vascular permeability. The right middle lobe of the lung was weighed immediately after the lung was removed. Subsequently, the right middle lobe of the lung was dried at 60 °C in an oven for 48 h and the weight was obtained.

BALF analysis

The total protein concentration of BALF is one indicator of pulmonary capillary permeability and edema. Therefore, the BALF was collected following the appropriate experiments. To collect BALF, an 18G sterile catheter (Carelife Co. Ltd., Shanghai, China) was inserted into the corresponding bronchopulmonary segment, the left lower lobe of the lung was lavaged three times with PBS (1 mL each time) and approximately 80 % of the instilled volume was retrieved. The BALFs were then centrifuged at 4 °C for 10 min at 1200*g* to pellet the cells. The clear supernatant was stored at − 80 °C until the total protein concentration was determined. The total BALF protein concentration was determined using a BCA Protein Assay kit (Thermo, Rockford, IL, USA).

Histopathological examination

Animals were sacrificed at 12 h after smoke inhalation. Lung tissue samples were collected for general observation. Then, the right lower lobe of the lung was collected and treated with 4 % paraformaldehyde. The lung lobe was dehydrated and embedded in paraffin, and sections of 5 μm thickness were cut. Subsequently, the sections were stained with hematoxylin-eosin and observed by standard light microscopy (Olympus Company, Tokyo, Japan) at x200 magnification.

A blinded observer evaluated the lung injury score according to the following standards: (1) alveolar hemorrhage and/or congestion, (2) inflammatory cell infiltration, (3) alveolar wall thickening, and (4) alveolar and/or interstitial edema. Each criterion was graded according to a five-point scale: 0 = 0 %, 1 = 0 - 25 %, 2 = 25 - 50 %, 3 = 50 - 75 %, and 4 = 75 - 100 %. The scores for criteria 0 to 4 were summed to represent the lung injury score (total score: 0 - 16) [14].

Detection of lung interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) levels

The left lower lobe of the lung was collected from each group to prepare lung tissue homogenates. In brief, lung tissues were homogenized in PBS (4 °C) with 0.05 % Tween 20 (Beyotime Institute of Biotechnology, Haimen, China) and the protein inhibitor cocktail (Beyotime) using a tissue homogenizer. Then, the homogenates were centrifuged at 10,000*g* for 15 min at 4 °C, and the clarified supernatants were collected and stored at − 70 °C until the detection of cytokine levels. The IL-1β (Cat no. ERC007), IL-6 (Cat no. ERC003), and TNF-α (Cat no. ERC102a) production in lung tissue homogenate were measured using enzyme-linked immunosorbent assay Kits (Neobioscience Technology, Shenzhen, China).

Determination of malondialdehyde (MDA), lipid peroxides (LPO), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) levels

Lung tissue homogenate was prepared as described above. The MDA (Cat no. A003-1), LPO (Cat no. A106-1), SOD (Cat no. A003-3), and GSH-PX (Cat no. A005) levels in the lung tissue homogenate and plasma were detected according to the assay kits' instructions (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China).

RT-qPCR of PKC β mRNA

Following smoke stimulation, as described above, the right upper lobe of the lung was collected. Total RNA was extracted from the lung tissue using TRIzol Reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Reverse transcription of the lung samples was performed using the Takara PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Polymerase chain reaction (PCR) was performed using SYBR Green qPCR Master Mix-SYBR Advantage (Takara Biotechnology Co., Ltd) according to the manufacturer's instructions. Rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as an internal standard. The primer sequences are listed in Table 1. The PCR conditions were as follows: 95 °C for 5 min,

then 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 sec. All reactions were performed in triplicate, and reports were generated by Rotor-gene Real-time Analysis Software 6.0 (Corbett Research, Sydney, Australia). The relative expression of the target gene was calculated by the $2^{-\Delta\Delta C}$t method.

Table 1: Primer sequences used for quantitative PCR

Gene		Primer sequence (5'-3')
PKC β	Forward	GGGGGCGACCTCATGTAT
	Reverse	GCAATTTCTGCAGCGTAAAA
GAPDH	Forward	TGGCCTTCCGTGTTCCTAC
	Reverse	GAGTTGCTGTTGAAGTCGCA

Western blot analysis

The right upper lobe of the lung was homogenized in a lysis buffer containing protease inhibitor and the supernatant protein concentrations were measured using the BCA Protein Assay kit. A total protein of 20 µg was separated on a 10 % sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with tris-buffered saline with 1/1,000 tween (TBST) containing 5 % skimmed milk for 1 h at room temperature and was incubated with anti-p66Shc antibody (EP332Y) (1:1,000 TBST) (Abcam, Cambridge, United Kingdom), anti-p-p66Shc (Phospho S36) antibody (6E10) (1:1,000 TBST) (Abcam), or anti-PKC β antibody (1:1,000 TBST) (Sigma, St. Louis, Missouri, USA) at 4 °C overnight.

The membranes were washed three times with TBST, incubated for 1 h at room temperature with horseradish peroxidase-labeled anti-rabbit secondary antibody, and washed again three times with TBST. Finally, the membranes were incubated with ECL PLUS high-sensitive developer (Solarbio Science and Technology). The membranes were photographed using a Gel Documentation and Analysis System (GBOX-HR, Syngene, Frederick, Md), and band intensities were measured using Adobe Photoshop Version 7.0.1 software (Adobe Systems, San Jose, CA). Experiments were repeated at least three times.

Statistical analysis

Statistical analysis was conducted using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as means ± standard deviation (SD). Comparison of means was performed by one-way analysis of variance, and the Student-Newman-Keuls test was used for comparisons between multiple groups. Statistical significance was determined at $p < 0.05$.

RESULTS

Vitexin treatment improved survival rate of SI-ALI rats

A significantly higher survival rate of 66.7 % was observed in the vitexin treatment (smoke + vitexin group) compared with 16.7 % in the smoke group ($P < 0.05$) (Figure 1). There was no significant difference in the survival rate between the smoke + vitexin, smoke + LY333531, or smoke + vitexin + LY333531 groups ($p > 0.05$, smoke + vitexin group vs. smoke + LY333531 and smoke + vitexin + LY333531 groups).

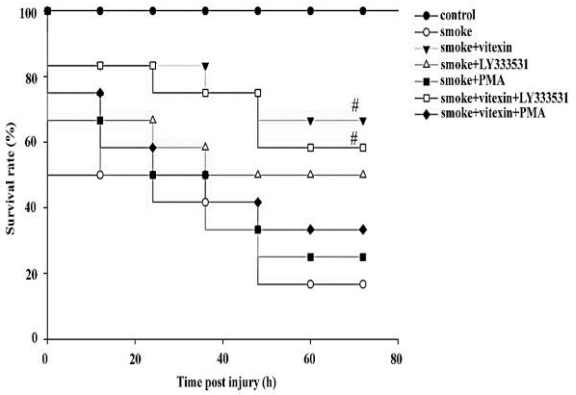

Figure 1: Effect of vitexin treatment on survival rates in rats with smoke inhalation. Rats were completely untreated (control), or untreated or treated with vitexin, LY333531, PMA, vitexin + LY333531, or vitexin + PMA before smoke inhalation ($n = 12$). The survival rates were observed for 72 h and were compared by log-rank test. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. smoke group

Vitexin treatment enhanced oxygenation in SI-ALI rats

The PaO_2 level and oxygenation index were significantly decreased after smoke inhalation ($P < 0.05$, smoke group vs. control group) (Figure 2). Both vitexin and vitexin + LY333531 treatment increased the PaO_2 level and oxygenation index compared with the smoke group ($P < 0.05$, smoke + vitexin and smoke + vitexin + LY333531 groups vs. smoke group).

Vitexin attenuated lung tissue exudation

The lung W/D weight ratio and the BALF protein concentration are common indices to evaluate the severity of lung tissue exudation. Therefore, the lung W/D ratio (Figure 3 A) and the BALF protein concentration (Figure 3 B) in each group were determined.

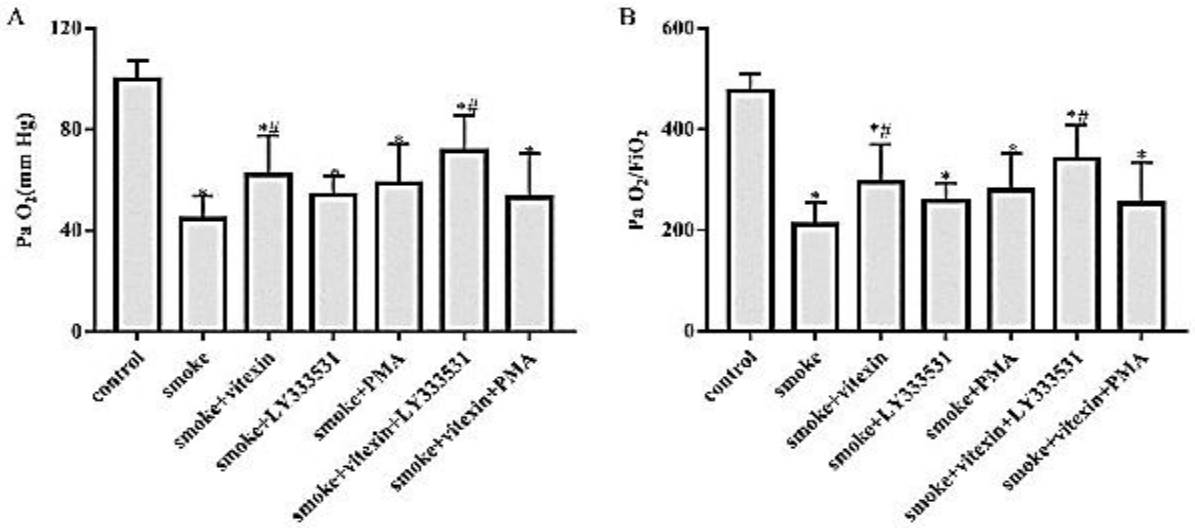

Figure 2: Effect of vitexin treatment on blood gas analysis in rats after smoke inhalation. Vitexin increased (A) the partial pressure of oxygen (PaO_2) and (B) the oxygenation index (PaO_2/FiO_2). All data are expressed as the means ± SD (n = 6). *P < 0.05 vs. control group, $^{\#}P$ < 0.05 vs. smoke group

Following smoke inhalation, the lung W/D ratio and BALF protein concentration was significantly increased (p < 0.05, smoke group vs. control group). Treatment with vitexin and/or LY333531 attenuated the- increase in the lung W/D ratio and BALF protein concentration induced by smoke inhalation (p < 0.05, smoke + vitexin, smoke + LY333531, and smoke + vitexin + LY333531 groups vs. smoke group).

Moreover, on treatment with vitexin and/or LY333531, the lung W/D ratio was not significantly different compared with the control group (p > 0.05, smoke + vitexin, smoke + LY333531, and smoke + vitexin + LY333531 groups vs. smoke group) (Figure 3).

Vitexin alleviated the degree of lung injury

Following smoke inhalation, a naked-eye observation and histological evaluation of lung tissue in each group were performed. The lung tissues of the control group appeared pink with a smooth and rosy surface; no blood, bleeding spots, congestion, or edema were observed (Figure 4 A). However, the rats of the smoke, smoke + PMA, and smoke + vitexin + PMA groups displayed clear lung tissue injury: lung tissues appeared as dark red; extensive exudation, diffuse hyperemia, and edema were visible.

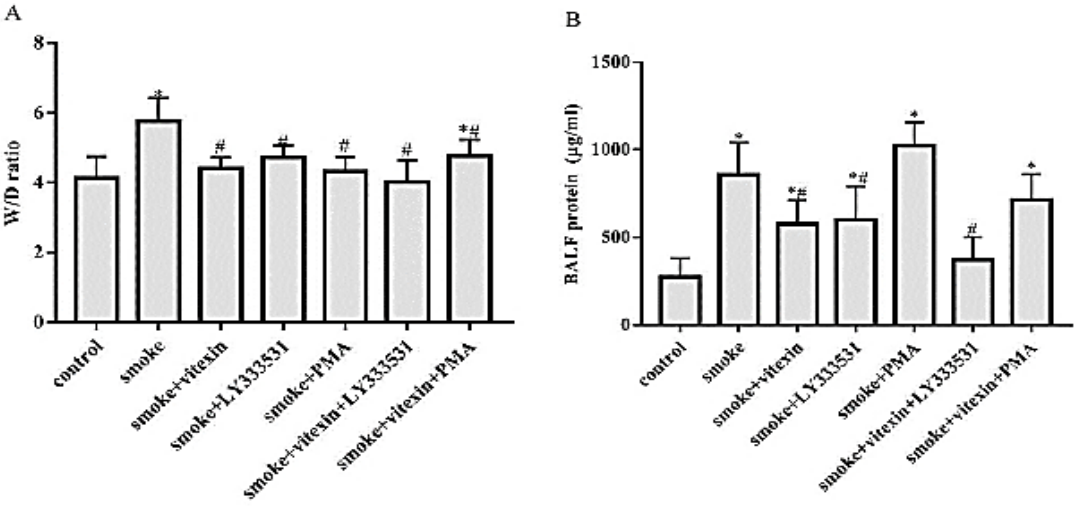

Figure 3: Effect of vitexin treatment on lung tissue exudation in rats after smoke inhalation. Vitexin decreased (A) the W/D weight ratio and (B) BALF protein concentration. All data are expressed as the means ± SD (n = 6). *P < 0.05 vs. control group, $^{\#}P$ < 0.05 vs. smoke group

By contrast, in smoke + vitexin, smoke + LY333531, and smoke + vitexin + LY333531 rats, the degree of exudation, hyperemia, and edema was attenuated (Figure 4 B - G).

Furthermore, typical histological features of ALI were observed in the smoke, smoke + PMA, and smoke + vitexin + PMA groups, including alveolar hemorrhage and congestion, inflammatory cell infiltration, alveolar wall thickening, and interstitial edema after smoke inhalation (Figure 4 B – G). Moreover, the lung injury scores of these rat groups were significantly higher than the control group rats ($P < 0.05$, smoke, smoke + PMA, and smoke + vitexin + PMA groups vs. control group) (Figure 4 H). Treatment with vitexin and/or LY333531 attenuated these smoke-induced histological changes and significantly reduced the lung injury score compared with the rats of the smoke group ($p < 0.05$, smoke + vitexin, smoke + LY333531, and smoke + vitexin + LY333531 groups vs. smoke group) (Figure 4).

Vitexin attenuated lung tissue inflammation levels

IL-1β, IL-6, and TNF-α are common pro-inflammatory factors in the early stage of ALI. Following smoke inhalation, the IL-1β, IL-6, and TNF-α levels in lung tissue were significantly increased, particularly in the smoke and smoke + PMA groups ($P < 0.05$, smoke, smoke + PMA, and smoke + vitexin + PMA groups vs. control group) (Figure 5). By contrast, vitexin or vitexin + LY333531 treatment reduced the IL-6 and TNF-α levels in the lung tissue of SI-ALI rats ($P < 0.05$, smoke + vitexin and smoke + vitexin + LY333531 groups vs. smoke group) (Figure 5).

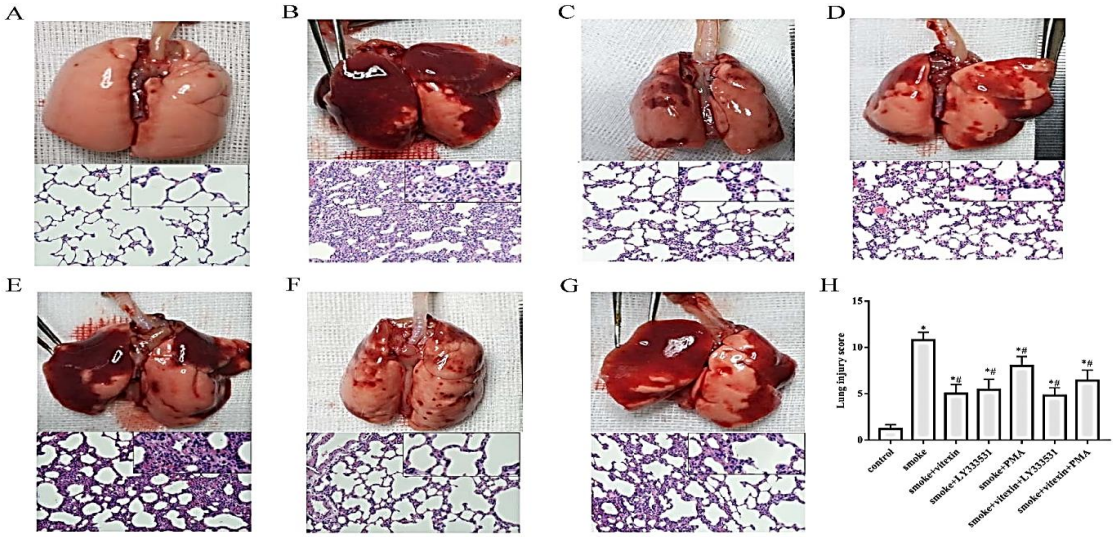

Figure 4: Effect of vitexin treatment on the degree of lung injury in rats after smoke inhalation. (A) control, (B) smoke, (C) smoke + vitexin, (D) smoke + LY333531, (E) smoke + PMA, (F) smoke + vitexin + LY333531, and (G) smoke + vitexin + PMA groups (Magnification, ×200). (H) Lung injury scores were assessed by a blinded pathologist. All data are expressed as the means ± SD ($n = 6$). *$P < 0.05$ vs. control group, #$P < 0.05$ vs. smoke group

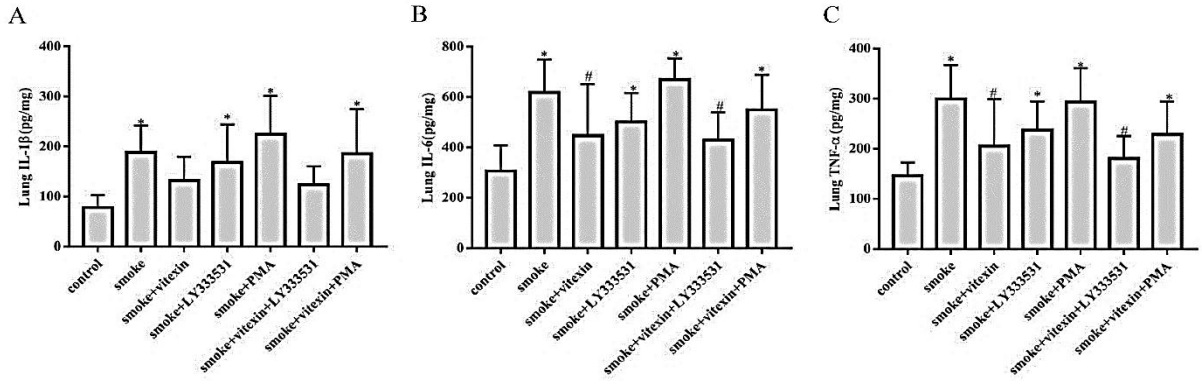

Figure 5: Effect of vitexin treatment on the lung tissue inflammation level in rats after smoke inhalation. Vitexin decreased the (A) IL-1β, (B) IL-6, and (C) TNF-α levels. All data are expressed as the means ± SD ($n = 6$). *$P < 0.05$ vs. control group, #$P < 0.05$ vs. smoke group

Vitexin attenuated oxidative stress in lung tissues and plasma

MDA and LPO are harmful products that reflect the level of ROS, whereas SOD and GSH-PX reflect antioxidant capacity in the body. Therefore, the MDA, LPO, SOD, and GSH-PX levels of lung tissue and plasma in each group were detected. Following smoke inhalation, the MDA and LPO levels in the lung tissue (Figure 6) and plasma (Figure 7) were significantly increased, particularly in the smoke, and smoke + PMA groups, whereas the SOD and GSH-PX levels in lung tissue (Figure 6) and plasma (Figure 7) were significantly decreased ($P < 0.05$, smoke and smoke + PMA groups vs. control group). The smoke-induced MDA and LPO levels were significantly decreased when the rats were treated with vitexin and/or LY333531, while the SOD and GSH-PX levels were increased by vitexin and/or LY333531 treatment compared with the smoke group ($P < 0.05$, smoke + vitexin, smoke + LY333531, and smoke + vitexin + LY333531 groups vs. smoke group) (Figure 6 and Figure 7).

Vitexin attenuated PKC β, p66Shc, and p-p66Shc expressions

The results of western blot analysis and qRT-PCR showed that the PKC β protein and mRNA expression were significantly increased after smoke inhalation ($p < 0.05$, smoke and smoke + PMA groups vs. control group) (Figure 8). Similarly, p66Shc and p-p66Shc protein expression were significantly increased after smoke inhalation ($p < 0.05$, smoke and smoke + PMA groups vs. control group) (Figure 9). Vitexin, and/or LY333531 treatment inhibited this increase by various degrees ($p < 0.05$, smoke + vitexin, smoke + LY333531, and smoke + vitexin + LY333531 groups vs. smoke group) (Figure 8 and Figure 9).

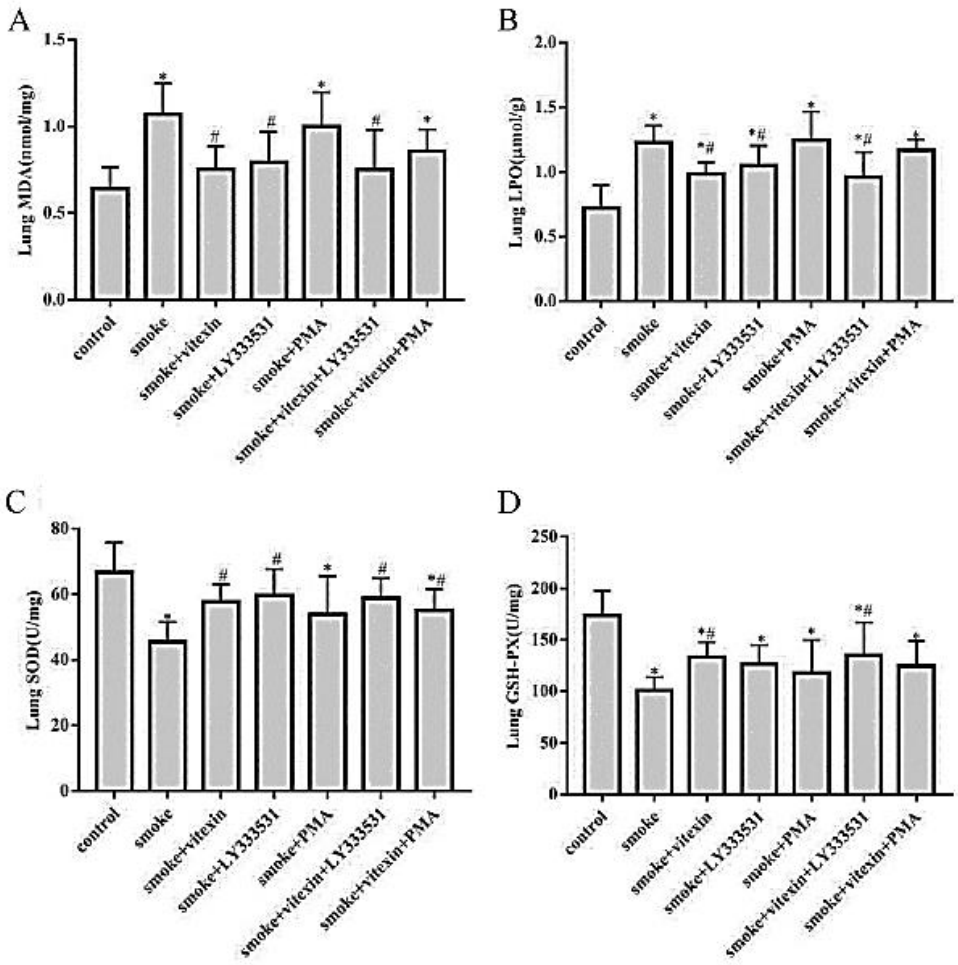

Figure 6: Effect of vitexin treatment on lung tissue oxidative stress level in rats after smoke inhalation. Vitexin decreased the (A) MDA and (B) LPO levels and increased the (C) SOD and (D) GSH-PX levels. All data are expressed as the means ± SD (n = 6). *$P < 0.05$ vs. control group, #$p < 0.05$ vs. smoke group

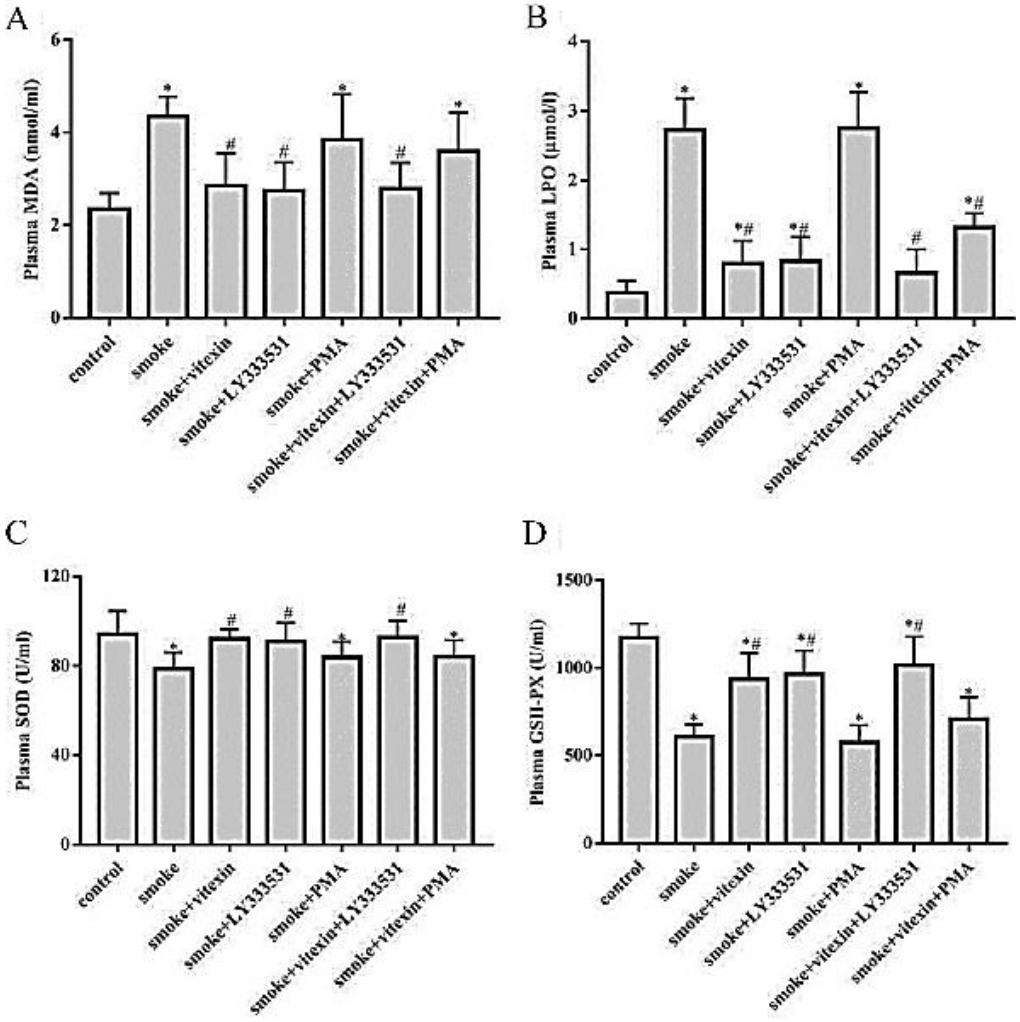

Figure 7: Effect of vitexin treatment on the plasma oxidative stress level in rats after smoke inhalation. Vitexin decreased the (A) MDA and (B) LPO levels and increased the (C) SOD and (D) GSH-PX levels. All data are expressed as the means ± SD (n = 6). *P < 0.05 vs. control group, #p < 0.05 vs. smoke group

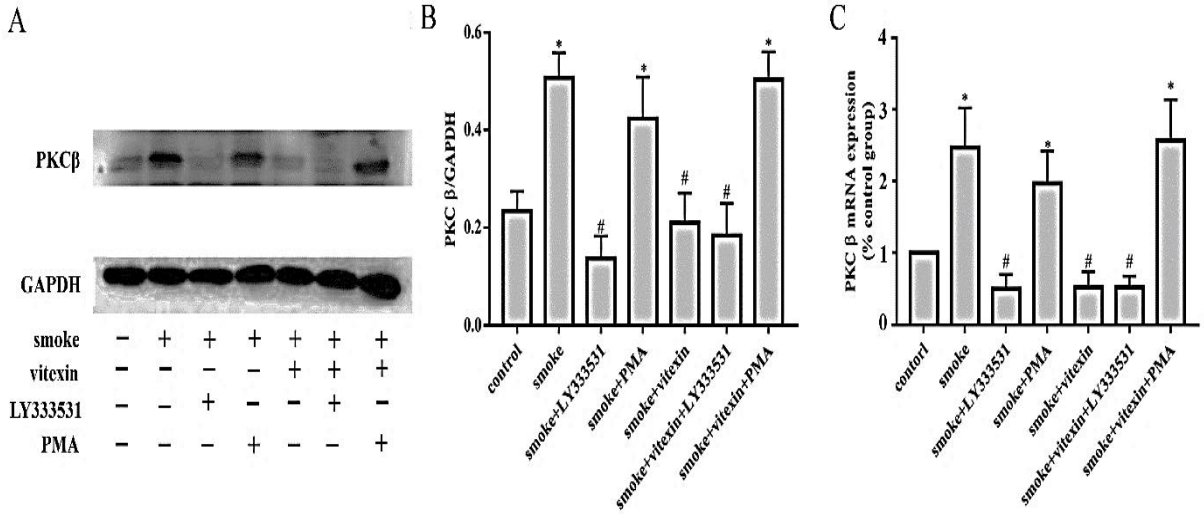

Figure 8: Effect of vitexin treatment on PKC β protein and mRNA expression after smoke inhalation. (A) PKC β protein expression was observed by western blot. (B) The grayscale values were determined using image J. (C) PKC β mRNA expression was detected by RT-qPCR. All data are expressed as the means ± SD (n = 6). *P < 0.05 vs. control group, #p < 0.05 vs. smoke group

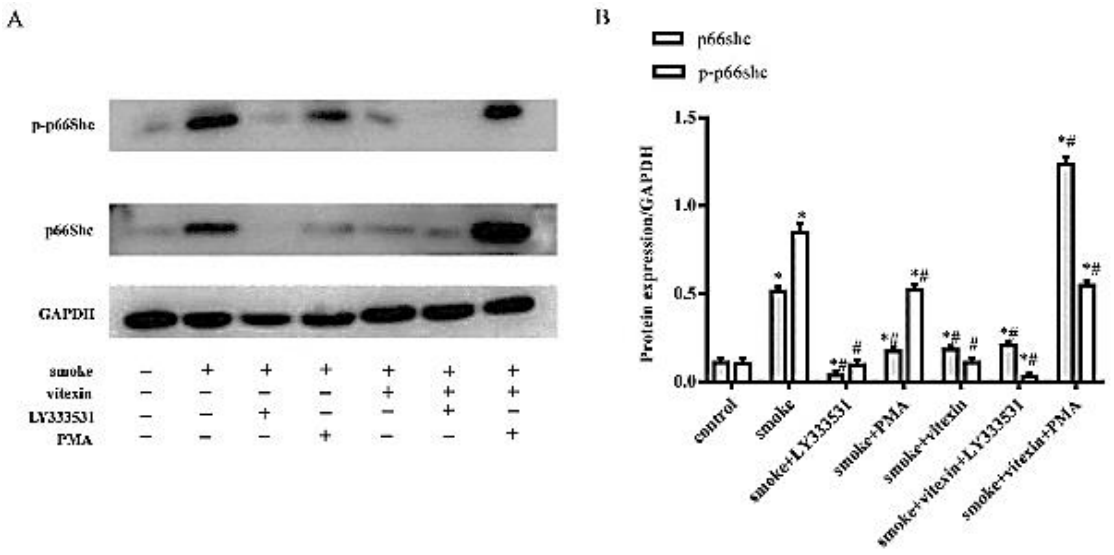

Figure 9: Effect of vitexin treatment on p66Shc and p-p66Shc expression after smoke inhalation. (A) p66Shc and p-p66Shc expression were observed by western blot. (B) The grayscale values were determined using image J. All data are expressed as the means ± SD (n = 6). *P < 0.05 vs. control group, #p < 0.05 vs. smoke group

DISCUSSION

An SI-ALI rat model was established using mixed materials to simulate a real fire scenario. According to an official standard reported by the ATS Workshop in 2022, in model animals, the standard model of ALI must display at least three of the following four main characteristics: first, the histological evidence of tissue injury; secondly, alteration of the alveolar-capillary barrier; third, the presence of an inflammatory response; and finally the physiologic dysfunction [15]. In the present study, the lung injury score increased after smoke inhalation as indicated by histological evidence, the W/D ratio and the BALF protein concentration reflected alteration of the alveolar-capillary barrier, the levels of IL-1β, IL-6, and TNF-α reflected an inflammatory response, and the PaO_2 reflected physiological dysfunction. Therefore, the SI-ALI rat model was established successfully.

For various etiologies, there are differences in the pathogenesis and pathophysiologic processes of ALI/acute respiratory distress syndrome. Both oxidative stress and excessive inflammation are very important in the development and progression of LPS-induced ALI and SI-ALI, but each model has its focus on two different etiologies. Regarding LPS-induced ALI, excessive inflammation predominated in its pathogenesis, and oxidative stress might tend to occur secondarily [16]. LPS can induce the production of pro-inflammatory mediators including TNF-α and IL-6, which was believed to facilitate ROS generation and promote oxidative stress [16]. Of course, oxidative stress in turn enhances inflammatory reactions [16]. However, in the pathogenesis of SI-ALI, oxidative stress plays a major role, because high-temperature smoke contains a high concentration of powerful oxidants. Oxidants including ROS and RNS can lead to the damage of cells and deoxyribonucleic acid, increased capillary permeability and lung injury, and induction of a resultant inflammatory response.

The present study found that vitexin pretreatment attenuated SI-ALI in rats. Although pretreatment in SI-ALI patients is not common in clinical practice, these results are valuable in searching for an effective therapy to treat SI-ALI. Smoke inhalation injury is normally predictive. Therefore, it is possible to take the preventive drug before SI-ALI occurs, particularly by members of special groups, including soldiers and firemen, to decrease further lung injury. Otherwise, these results indeed showed that vitexin had anti-inflammatory and anti-oxidative effects in SI-ALI. These protective effects might bear little relationship with the timing of vitexin administration. It is speculated that vitexin treatment after smoke inhalation may have the same therapeutic effect as reported in this present study, and the effect of vitexin administration after smoke inhalation injury will be evaluated in further experiments.

In the present study, the rats received an intraperitoneal injection of vitexin of 3 mg/kg for 5 days before smoke inhalation. The pharmacokinetics of vitexin have shown that its oral absorption is incomplete, the first pass effect in the intestine is > 90 %, and the bioavailability is only approximately 5 % [10]. Therefore, the rats were pretreated with vitexin via

intraperitoneal injection. The results showed that vitexin at a dose of 3 mg/kg could attenuate the levels of inflammation and oxidative stress in SI-ALI rats. However, it is unclear whether vitexin at 3 mg/kg is the optimal dosage. Therefore, multiple groups with different vitexin doses will be established to determine the optimal vitexin dosage to attenuate SI-ALI in future experiments.

It has been reported that H_2S and gamma-tocopherol could attenuate SI-ALI through their antioxidant effects [3]. However, no study has compared the effectiveness of vitexin against gamma-tocopherol or H_2S in the treatment of SI-ALI. This issue is indeed of interest to design a separate but more extensive experiment to identify the equivalence or non-inferiority between vitexin and gamma-tocopherol or H_2S in treating SI-ALI. In the future, an extensive and detailed experiment will be carried out to investigate this issue further.

The adaptor protein p66Shc can regulate oxidative stress and is affected by PKC β [4]. Activation of p66Shc could affect mitochondrial respiratory chain function, oxidize cytochrome c, and produce excessive H_2O_2. Excessive H_2O_2 increases the levels of oxidative stress. Previous studies showed that p66Shc expression and activation increased significantly in the lung tissue of rats with ischemia-reperfusion injury [17]. The results of this study showed that p66Shc, p-p66Shc, and PKC β expression were increased in SI-ALI. Furthermore, vitexin can exert similar effects as the PKC β inhibitor LY333531, thereby inhibiting PKC β expression and p66Shc activation. These results confirmed the hypothesis that vitexin can inhibit the PKC β/p66Shc signaling pathway.

However, it was observed that the combined application of vitexin and LY333531 did not appear to increase inhibition compared to when treated with vitexin or LY333531 alone. Similarly, combined treatment with smoke and PMA also did not significantly increase PKC β activation than treatment with smoke or PMA alone. The reasons may be because PKC β was completely inhibited by treatment with vitexin or LY333531 alone, thus the combined application of vitexin and LY333531 could not further increase the inhibition. The same was true for the agonist effects. Another possible reason could be because the agonistic effect of PMA is bidirectional. When the PMA activation time is too long, it will induce PKC degradation [18].

LY333531, a PKC β-selective inhibitor, has been widely used in the improvement of oxidative damage and apoptosis. Vitexin, a flavonoid with anti-inflammatory and antioxidant effects, is commonly used in the treatment of diseases caused by inflammation or excessive oxidation. At present, there is no associated literature relating to the counteraction between LY333531 and vitexin. Under non-stimulation conditions, both vitexin and LY333531 have no deleterious effect on lung injury [10]. Therefore, a vitexin + LY333531 group was not established in the present study.

The results of the present study confirmed that vitexin can attenuate the levels of oxidative stress by inhibiting the PKC β/p66Shc signaling pathway. In earlier literature, it was reported that vitexin could enhance Nrf2 expression and inhibit NF-κB activation in inflammation, oxidative stress, and hypoxic-ischemic injury [6]. Therefore, vitexin can also regulate Nrf2 and NF-κB expression in the SI-ALI rat model, and, thus the modulation of Nrf2 and NF-κB expression also plays a role in the protective effect of vitexin on SI-ALI. However, the measurement of Nrf2 and NF-κB expression is insufficient to explain the relationship between the PKC β/p66Shc pathway and Nrf2/NF-κB pathways. A siRNA or inhibitors of Nrf2 and NF-κB might be used to explore this issue further. Moreover, the mechanisms relevant to the protective effect of vitexin and the vitexin-activated signaling pathway are very complex in addition to the PKC β/p66Shc, Nrf2, and NF-κB signaling pathways. Consequently, the measurement of Nrf2 and NF-κB expression is of interest with respect to the protective mechanisms of vitexin. Therefore, a separate but more extensive experiment should be performed on this subject in the future.

CONCLUSION

Vitexin decreases the mortality rate, attenuates lung tissue exudation, pulmonary edema, lung tissue inflammation levels and oxidative stress levels after smoke inhalation in rats. It also attenuates SI-ALI in rats by inhibiting oxidative stress via PKC β/p66Shc signaling pathway. Thus, the findings of this study provide a scientific basis for the development of vitexin as a therapy that could improve the prognosis of SI-ALI.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. CX and HZ conceived the present study. WF and CH performed the experiments; LH and LM collected and analyzed the data; CH, CX, and MJ interpreted the data and wrote the manuscript.

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