

Original Research Article

Regulatory effect of resveratrol on the expressions of factors and surface markers in alveolar macrophages of *Aspergillus fumigatus*-infected COPD rat model, and the associated mechanism

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Abstract

Purpose: To study the influence of resveratrol on chronic obstruction in *Aspergillus fumigatus* infection-induced chronic lung disease (COPD) in rats, and the process involved.

Methods: Seventy-five Sprague Dawley (SD) rats were assigned to blank control, COPD model, *Aspergillus fumigatus*-infected COPD model, low (100 mg/kg) and high-dose (200 mg/kg) resveratrol groups, with 15 rats in each group. Enzyme-linked immunosorbent assay (ELISA) was used to assay IL-6, IL-8 and TNF- α in bronchoalveolar lavage fluid (BALF), while lung protein concentrations of AMPK and PGC-1 α were assayed by immunoblotting.

Results: In low- and high-dose resveratrol groups, inflammatory factor levels were significantly lower than the corresponding levels in COPD model and *Aspergillus fumigatus*-infected COPD model ($p < 0.05$), while lung tissue proteins of AMPK, PGC-1 α , and AQP5 of the rats given high-dose resveratrol were significantly raised, relative to corresponding levels in rats given low-dose resveratrol ($p < 0.05$).

Conclusion: Resveratrol modulates expression levels of secretory factors and surface markers in alveolar macrophages in COPD model rats infected with *Aspergillus fumigatus*. Resveratrol exerts this effect through the regulation of AMPK/PGC-1 α signaling pathway.

Keywords: Chronic obstructive pulmonary disease (COPD), Resveratrol, Adenylate-activated protein kinase, Peroxisome proliferator-activated gamma receptor coactivator 1- α , Alveolar macrophages

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic illness often seen in respiratory medicine. It occurs mostly in middle-aged and elderly people, with clinical characteristics such as recurrent attacks, disease persistence, and

long duration [1]. Patients with COPD often have type II respiratory failure, a complication which results in significantly decreased ventilation manifested in respiratory muscle fatigue, shortness of breath, tachypnea, chest tightness and other clinical symptoms. In severe cases, hypercapnia, hypoxemia and other acute

complications may occur, resulting in high mortality [2]. Bacteria and viruses are the principal culprits responsible for the deterioration of COPD patients. Indeed, *Aspergillus fumigatus* is the most common pathogen that causes pulmonary fungal infection.

Studies have found that inhalation of spores of *Aspergillus fumigatus* aggravated airway inflammation in COPD patients and affected prognosis of the disease [3]. Therefore, it is clinically important to identify effective interventions for COPD patients with *Aspergillus fumigatus* infection so as to reduce airway inflammation and clinical symptoms in patients.

Alveolar macrophages are inflammatory cells widely present on the bronchial surface and alveoli. Studies have found that excessive release of inflammatory cytokines from alveolar macrophages is responsible for COPD lung tissue damage [4]. Resveratrol is a polyphenolic molecule with good antioxidant, anti-inflammatory, antibacterial and cytokine-inhibitory effects [5].

The present research was carried out to study the regulatory influence of resveratrol on secretory factors and surface markers in pulmonary alveolar macrophages of COPD rats infected with *Aspergillus fumigatus*, and the involvement of the AMPK/PGC-1 α signal pathway in the process.

EXPERIMENTAL

Animals and reagents

Seventy-five male Sprague Dawley (SD) rats, aged 6 to 8 weeks, weighing 180 to 220 g, were purchased from the animal management center of the TianJin Medical University General Hospital, Tianjin, China. The reagents used, and their suppliers were: Resveratrol (Shanghai Aladdin Reagent Co. Ltd), hematoxylin and eosin (H&E) staining kit (Shanghai Biyuntian Co. Ltd), TRIzol RNA extraction kit and reverse transcription kits for inflammatory factors, and ELISA kit (Shanghai Biyuntian), and antibodies for AMPK and PGC-1 α (Abcam Biotechnology Co. Ltd).

Establishment of rat model of COPD

The 75 SD rats were equally divided into 5 groups: blank control, COPD model, *Aspergillus fumigatus*-infected COPD model, low-dose resveratrol (100 mg/kg), and high-dose resveratrol (200 mg/kg) groups. Establishment of COPD model involved exposing the animals to

cigarette smoke using a home-made animal smoking box. In this process, 4 cigarettes were lit at each time in the box for 5 min, after which another set of 4 cigarettes was lit. This cycle was continued until 12 cigarettes were lit. This procedure was repeated twice daily for 28 days. The *A. fumigatus*-infected COPD model was established using *A. fumigatus* spores derived from sputum samples from *A. fumigatus* infection after organ transplantation. A spore suspension containing 1×10^7 cfu/mL was prepared.

One week after COPD model establishment, 50 μ L spore suspension was dropped into each side, once a week on Mondays and Fridays, for 5 weeks. After establishment of the above model, each group was given the corresponding dose of resveratrol or saline via continuous gavage for 30 days. This research was approved by the Animal Ethical Committee of TianJin Medical University General Hospital (approval no. 2022012), and conducted in line with the guidelines of "Principles of Laboratory Animal Care" [6].

Sample preparation

Under chloral hydrate anesthesia, the chest cavity of each rat was opened. Sterile PBS solution was injected into the bronchoalveolar region through endotracheal intubation, and bronchoalveolar lavage fluid (BALF) was collected for examination. The chest cavity was cut open and the lung tissue on the right side was excised and transferred to a refrigerator at -80 °C prior to investigation. The left lung was fixed overnight with 4 % paraformaldehyde solution and processed routinely using H&E staining.

Morphological observation of lung tissue

After cutting, dehydrating, embedding, sectioning, H&E staining and sealing, morphologic changes in bronchial texture in lung tissues of rats were examined under a light microscope.

Assessment of biochemical indices

The BALF collected was centrifuged at 3000 rpm for 20 min, and the supernatant was used for examination. Some of the cells were suspended in 1 mL of Hanks solution, and 100 μ L was used for counting the numbers of white blood cells and neutrophils using a blood smear counting plate. The other portion of the supernatant was used for assay of levels of IL-6, IL-8 and TNF- α with ELISA kits strictly in accordance with the kit instructions.

Expressions of surface markers in alveolar macrophages

Following centrifugation of the BALF at 3000 rpm for 20 min, the lower liquid portion was reserved for analysis. Sputum cells at the bottom of the tube were collected after centrifugation. Macrophages in sputum were separated using Rosette Sep cell separation method, and miRNA was extracted after culturing the macrophages. The miRNA was reverse-transcribed into cDNA using One Step PrimeScript miRNA cDNA synthesis kit. The levels of toll-like receptor 4 (TLR4), mucin 5AC (MUC5AC) and aquaporin 5 (AQP5) were determined using miRNA fluorescence quantitative PCR kit, and the cycles were completed according to the kit instructions. At the end of the reactions, the expression levels of these markers were calculated as indicated in the software.

Image analysis of airway remodeling

Image analysis software (Image-Pro Plus 5.1) was used to analyze airway remodeling in lung tissue sections. Three complete small and medium bronchi were randomly selected from each section and enlarged 200 times under the microscope. The bronchial airway smooth muscle area (Wam), inner duct wall area (Wai), total duct wall area (Wat) and basement membrane circumference diameter (Pbm) were measured. The measured Wam, Wai and Wat were normalized with Pbm and represented as Wat/Pbm, Wai/Pbm and Wam/Pbm, respectively.

Determination of protein expressions

Total protein was extracted from lung tissue by homogenizing with RIPA buffer at 4 °C to form a 10 % homogenate which was centrifuged to obtain a supernatant (lysate). Lysate protein content was quantified with BCA assay procedure. Thereafter, equal amounts of protein were resolved with SDS-polyacrylamide gel electrophoresis, followed by transfer to PVDF membranes. The membranes were sealed by incubation with non-fat milk solution, after which it was incubated overnight at 4 °C with primary antibodies. This was followed by incubation with

HRP-linked secondary antibodies at room temperature for 2 h. The protein expression levels were analyzed with Bio-RAD image laboratory software.

Statistical analysis

This was done with SPSS 20.0 software. Data are presented as mean \pm SD, and ANOVA was used for comparison amongst groups, while least significant differences (LSD) test or Tamhane test was used for inter-group comparison. Statistical significance was set at $p < 0.05$.

RESULTS

Morphological features

Results from H&E protocol revealed intact lung structure of rats in blank control. Cilia were orderly arranged, with normal alveolar volume and number, normal alveolar structure, near-normal spacer, and intact airway epithelial structure. In the COPD model group, the alveolar cavity was expanded, with thinner alveolar walls, and parts of alveoli were ruptured and fused to form bullae. The airway smooth muscle was thickened, with massive presence of inflammation cells around bronchioles and submucosa. Other features comprised goblet cell proliferation, mucous membrane degenerative necrosis, cilia adhesion, and lodging.

Image analysis of airway remodeling

There were significantly higher levels of Wat, Wam and Wai in COPD model than in control. Moreover, Wat, Wam and Wai concentrations were significantly raised in *Aspergillus fumigatus*-infection COPD model rats, relative to COPD model rats. In contrast, although Wat, Wam and Wai were significantly up-regulated in resveratrol-treated rats, relative to blank control group, they were significantly lower than those in COPD model and *Aspergillus fumigatus*-infected COPD model groups. However, Wat, Wam and Wai concentrations were significantly lower in high-level than in low-level resveratrol group ($p < 0.05$). These data are presented in Table 1.

Table 1: Image analysis of airway remodeling in each group ($\mu\text{m}^2/\mu\text{m}$, $n = 15$)

Group	Wat/Pbm	Wai/Pbm	Wam/Pbm
Blank control	34.24 \pm 3.52	16.43 \pm 2.58	2.63 \pm 0.43
COPD model	54.38 \pm 5.04 ^a	33.92 \pm 2.75 ^a	6.75 \pm 1.12 ^a
<i>Aspergillus fumigatus</i> -infected COPD model	65.00 \pm 5.34 ^{ab}	40.75 \pm 3.26 ^{ab}	8.16 \pm 1.42 ^{ab}
Low-dose resveratrol	47.47 \pm 4.54 ^{abc}	27.82 \pm 2.90 ^{abc}	4.19 \pm 0.95 ^{abc}
High-dose resveratrol	40.55 \pm 3.70 ^{abcd}	21.92 \pm 2.61 ^{abcd}	3.25 \pm 0.69 ^{abcd}

^{a,b,c} $P < 0.05$: ^avs control; ^bvs COPD model; ^cCOPD model group vs *Aspergillus fumigatus*-infected COPD model group; ^d $p < 0.05$; compared with resveratrol low-dose group

White blood cell count and neutrophil proportion

White blood cell count and neutrophil proportion in COPD model group were significantly higher than those in blank control group. Moreover, there were significantly higher white blood cell count and neutrophil proportion in *Aspergillus fumigatus*-infected COPD model rats than in COPD model rats. However, white blood cell count and neutrophil proportion were significantly lower in low-dose and high-dose resveratrol groups than in COPD model and *Aspergillus fumigatus*-infected COPD model groups ($p < 0.05$). There were significantly lower white blood cell count and neutrophil proportion in high-level resveratrol than in low-level resveratrol group. These data are presented in Table 2.

Relative protein concentrations in rat lung tissues

Pulmonary protein expressions of AMPK and PGC-1 α were significantly lower in untreated COPD model than in control. Moreover, AMPK and PGC-1 α concentrations were significantly lower in lung tissues of *Aspergillus fumigatus*-infected COPD model rats than in COPD model

rats. However, although the amounts of these proteins were significantly lower in pulmonary tissues in the 2 resveratrol doses than in blank control group, they were significantly higher than those in COPD model and *Aspergillus fumigatus*-infected COPD model group. Significantly up-regulated expressions of AMPK and PGC-1 α in lung tissues of rats were observed in the high-level than in low-level resveratrol treatment (Table 3).

Blood concentrations of inflammatory factors in alveolar macrophages

The serum levels of IL-8, IL-6 and TNF- α were significantly raised in COPD model group, relative to those in control group. Moreover, serum concentrations of these factors in *Aspergillus fumigatus*-infected COPD model group were significantly raised, when compared to those in COPD model rats ($p < 0.05$). However, the serum amounts of these factors were significantly decreased in low-dose and high-dose resveratrol groups, relative to those in COPD and *Aspergillus fumigatus*-infected model rats. There were significantly lower serum levels of IL-8, IL-6 and TNF- α in the higher resveratrol dose than in lower resveratrol dose (Table 4).

Table 2: Leucocyte count and neutrophil proportion in each group (n = 15)

Group	White blood cell count (10 ⁵ /L)	Proportion of neutrophils (%)
Blank control	8.24±2.52	22.43±3.58
COPD model	15.00±3.34 ^a	33.75±5.26 ^a
<i>Aspergillus fumigatus</i> -infected COPD model	18.38±5.04 ^{ab}	38.92±6.75 ^{ab}
Low-dose resveratrol	13.47±2.54 ^{abc}	28.82±4.90 ^{abc}
High-dose resveratrol	10.55±2.70 ^{bcd}	23.92±3.61 ^{bcd}

^{a,b,c,d} $P < 0.05$: ^avs control; ^bvs COPD model; ^cCOPD model vs *Aspergillus fumigatus*-infected COPD model group; ^dvs low-level resveratrol

Table 3: Relative protein concentrations of AMPK and PGC-1 α in lung tissues of each group (n = 15)

Group	AMPK	PGC-1 α
Blank control	0.86±0.28	0.76±0.29
COPD model	0.37±0.09 ^a	0.45±0.14 ^a
<i>Aspergillus fumigatus</i> -infected COPD model	0.28±0.04 ^{ab}	0.34±0.11 ^{ab}
Low-dose resveratrol	0.47±0.17 ^{abc}	0.52±0.23 ^{abc}
High-dose resveratrol	0.63±0.24 ^{abcd}	0.63±0.21 ^{abcd}

^{a,b,c,d} $P < 0.05$: ^avs control; ^bvs COPD model; ^cCOPD model vs *Aspergillus fumigatus*-infected COPD model; ^dvs low-dose resveratrol

Table 4: Comparison of inflammatory factors secreted by alveolar macrophages amongst the groups (μ g/L or ng/L; mean \pm SD)

Group	IL 8	IL 6	TNF- α
Blank control	2.76±0.29	97.86±11.28	50.71±8.22
COPD model	3.45±0.74 ^{ab}	150.37±16.09 ^{ab}	121.41±13.14 ^{ab}
<i>Aspergillus fumigatus</i> -infected COPD model	4.34±1.11 ^a	180.28±18.04 ^a	170.33±17.11 ^a
Low-dose resveratrol	3.12±0.53 ^{abc}	131.47±14.17 ^{abc}	97.55±9.13 ^{abc}
High-dose resveratrol	2.93±0.41 ^{bcd}	113.63±12.24 ^{abcd}	71.64±7.24 ^{abcd}

^{a,b,c,d} $P < 0.05$: ^avs control; ^bvs COPD model; ^cCOPD model vs *Aspergillus fumigatus*-infected COPD model group; ^dvs low-dose resveratrol group

Table 5: Surface marker levels in alveolar macrophages of rats in each group (mean \pm SD, n = 15)

Group	AQP5	MUC5AC	TLR4
Blank control	6.76 \pm 2.29	1.86 \pm 0.68	0.81 \pm 0.32
COPD model	3.34 \pm 1.11 ^a	4.28 \pm 1.04 ^a	2.84 \pm 1.26 ^{ab}
<i>Aspergillus fumigatus</i> -infected COPD model	1.45 \pm 0.64 ^{ab}	6.37 \pm 2.09 ^{ab}	3.69 \pm 1.47 ^a
Low-dose resveratrol	4.52 \pm 1.23 ^{abc}	3.47 \pm 0.87 ^{abc}	2.24 \pm 0.81 ^{abc}
High-dose resveratrol	5.63 \pm 2.21 ^{abcd}	2.63 \pm 0.74 ^{abcd}	1.69 \pm 0.55 ^{abcd}

^{a,b,c} $P < 0.05$: ^avs control; ^bvs COPD model; ^cCOPD model vs *Aspergillus fumigatus*-infected COPD model group; ^d $p < 0.05$; compared with resveratrol low-dose group

Expressions of surface markers in alveolar macrophages

The levels of MUC5AC and TLR4 were significantly higher in COPD model group than in blank control group, and they were significantly elevated in *Aspergillus fumigatus*-infected COPD model, relative to COPD model rats. However, the expression levels of MUC5AC and TLR4 were smaller in low-dose and high-dose resveratrol groups than in COPD rats and *Aspergillus fumigatus*-infected rats. The expression levels of MUC5AC and TLR4 in high-dose resveratrol group were significantly lower than that in low-dose resveratrol group ($p < 0.05$). The expression of AQP5 was down-regulated in COPD model and *Aspergillus fumigatus*-infected COPD groups, relative to control group. However, resveratrol treatment resulted in significant up-regulation of AQP5 expression, with higher expression in higher resveratrol dose, as shown in Table 5.

DISCUSSION

Chronic obstructive pulmonary disease (COPD) is caused by many factors. It is a chronic recurrent disease characterized by mild symptoms such as cough and sputum. Thus, the early clinical symptoms of COPD in patients are not very definitive. However, as the disease develops, patients may manifest symptoms of respiratory failure, with varying degrees of damage to lung function, resulting in poor ventilation. In severe cases, this may lead to pulmonary heart disease which brings serious economic burden to patients and their families [7]. *Aspergillus fumigatus* is widely distributed in indoor and external environments, and is a common parasitic fungus of carrion. Spores with highly hydrophobic surface structure are produced, and these are easily inhaled into the lower respiratory tract and into the terminal airway [8]. Alveolar macrophages in lung tissue engulf and remove the spores. Indeed, the mucin-ciliary system removes most of the inhaled spores. Thus, the inhalation of *Aspergillus fumigatus* spores in normal people does not cause infection or allergic inflammation. However, clinical studies have shown that due to

low immunity, COPD patients are susceptible to infection with *Aspergillus fumigatus* spores. Once the number of *Aspergillus fumigatus* spores inhaled by COPD patients exceeds the excretory capacity of the body, it easily leads to invasive fungal infection [9]. Studies have demonstrated that inhalation of *Aspergillus fumigatus* spores exacerbated airway inflammation in COPD patients, leading to airway remodeling which affected lung function in the patients [10].

Resveratrol is a polyphenolic molecule with good antioxidant, anti-inflammatory, antibacterial and cytokine-inhibitory effects [11]. This study has shown that levels of Wat, Wam and Wai were significantly higher in COPD model group than those in blank control group. Moreover, the levels of Wat, Wam and Wai were significantly higher in *Aspergillus fumigatus*-infected model group than those in COPD model rats. However, resveratrol treatment led to significant reductions in levels of Wat, Wam and Wai, when compared to the COPD and *Aspergillus fumigatus*-infected model groups. There were significantly higher levels of Wat, Wam and Wai in low-dose and high-dose resveratrol groups than in blank control group, but their levels were smaller in the higher resveratrol dose than in the lower dose. These results suggest that inhalation of *Aspergillus fumigatus* spores aggravated airway inflammation and enhanced airway remodeling in COPD patients. However, the severity of these effects was mitigated by resveratrol.

The pathogenesis of COPD has not been fully elucidated. However, recent studies suggest that imbalance between activities of proteases and antiproteases, as well as oxidative stress, tissue fibrosis and inflammation may be crucial in the pathogenesis of COPD, with abnormal inflammatory response playing a key role in the process [12]. In addition, inflammation is an important factor in immunity against *Aspergillus fumigatus* infection. Macrophages are the most abundant immune cells; they are widely distributed on the alveolar and airway surfaces of COPD patients, and they induce local inflammatory reactions by secreting cytokines and phagocytizing *Aspergillus fumigatus* spores [13].

Alveolar macrophages not only participate in lung inflammation by activating and releasing inflammatory cytokines, but also synergize with neutrophils in the removal of *Aspergillus fumigatus* spores. It is known that TGF- β 1, IL-6, and TNF- α constitute major regulatory and inflammation-inducing cytokines. In particular, TNF- α is an endogenous cytokine which enhances the release of pro-inflammatory factors by activating lymphocytes, macrophages, neutrophils and other immune inflammatory cells. Acute inflammatory response is induced by IL-6 by promoting lymphocyte activation in the blood. *Aspergillus fumigatus* spores generate IL-8 by activating airway epithelial cells. The main neutrophil chemokine, IL-8, induces morphological changes in neutrophils and chemotaxis, leading to aggravation of airway inflammation [14,15]. In addition, macrophages recognize *Aspergillus fumigatus* spores through pattern recognition receptors on their cell surfaces, and they mediate phagocytosis. Changes in the expression levels of AQP5, MUC5AC, TLR4 and other surface markers in alveolar macrophages are closely related to the occurrence and development of COPD.

Bacterial proteins activate macrophages through TLRs, and up-regulate the expressions of TGF- β 1, IL-6, TNF- α and other inflammatory cytokines, thereby mediating the aggregation of inflammatory cells, enhancing the function of macrophages, and eliminating pathogens. The mucin protein, MUC5AC, produced by respiratory epithelial cells is most sensitive to pathophysiological changes in the lungs, and it is highly inducible. Increased levels of MUC5AC cause excessive secretion of mucin which provides a good culture medium for bacterial reproduction, thereby aggravating airway inflammation in COPD patients. In addition, AQP5 is an important water transport channel in the airway and lung tissues, and reductions in levels of AQP5 decrease mucus secretion, thereby increasing the concentration of mucin and providing a good culture medium for bacterial reproduction [16,17]. The findings of this study suggest that *Aspergillus fumigatus* spore infection aggravated COPD airway inflammation and led to airway remodeling. Resveratrol up-regulated the expression levels of secretory factors and surface markers in COPD rats infected with *A. fumigatus*, thereby alleviating airway inflammation.

The AMPK/PGC-1 α signal route is widely used in humans, and it is crucial in regulation of cell multiplication, differentiation, migration and programmed deaths [18]. The serine/threonine protein kinase AMPK regulates cellular energy

and maintains energy balance [19]. Studies have found that when there is imbalance between metabolism and energy level, activation of AMPK regulates the expression of downstream malonyl-CoA and genes involved in lipogenesis through phosphorylation, thereby inhibiting inflammatory and oxidative stress responses, and restoring energy balance [20].

It is known that PGC1- α is transcription co-activator of mitochondrion-related genes. As a nuclear transcription co-activator, PGC1- α regulates important physiological processes such as fatty acid oxidation, oxidative phosphorylation and mitochondrial biogenesis by combining with other co-activators to act on different target genes, thereby increasing transcription efficiency [21]. It is mainly expressed in tissues rich in mitochondria and tissues with high energy requirements such as lung, kidney, liver, skeletal muscle and heart, and in renal tissues where it is expressed in the cytoplasm of renal tubular epithelial cells. It (PGC1- α) activates the Recombinant Transcription Factor A, Mitochondrial (TFAM) of mitochondrial DNA. Then, the activated TFAM crosses the mitochondrial membrane into the mitochondrial matrix and binds to mitochondrial DNA to form a transcription initiation complex with mitochondrial transcription factor B (TFBM) and mitochondrial RNA polymerase. This complex plays a crucial role in regulating mitochondrial biogenesis and function [22].

The results obtained in this study showed lower pulmonary concentrations of AMPK and PGC-1 α in COPD model rats than in control rats, while protein concentrations of AMPK and PGC-1 α in lung tissues of *Aspergillus fumigatus*-infected COPD model group were significantly lower than those in COPD model group, but they were significantly lower in the two resveratrol doses than in control rats. However, the amounts of AMPK and PGC-1 α in lung tissues of rats in high-dose resveratrol group were significantly higher with the higher resveratrol dose than with the lower drug dose.

CONCLUSION

Aspergillus fumigatus spore infection aggravates COPD airway inflammation and lead to airway remodeling. However, resveratrol improves the expression levels of secretory factors and surface markers in COPD rats infected with *A. fumigatus*, thereby alleviating airway inflammation through regulation of AMPK/PGC-1 α signaling pathway.

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 performed 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. Limei Du and Xinglan Huo designed the study, supervised the data collection, and analyzed the data. Limei Du interpreted the data and prepared the manuscript for publication. Xinglan Huo and Ying Liu supervised the data collection, analyzed the data and revised the draft of the manuscript. Limei Du and Xinglan Huo contributed equally to this work and should be considered as co-first authors.

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