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Article

Based on SLC7A11/GPX4 signaling pathway, the mechanism of inhibiting cell iron death in the treatment of asthma was investigated

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Abstract: Objective: To investigate the effects of Pingchuanning prescription (PCN) and Ferrostatin 1 inhibitors on airway inflammation in asthmatic rats from the perspective of cell iron death. **Methods:** Seventy SD rats were randomly divided into 7 groups: normal group, model group, Pinbuening group, Ferrostatin 1 inhibitor group, Pinbuening + Ferrostatin 1 inhibitor group, dexamethasone group, and Guilong Kechuanning group. 10% chicken egg albumin (OVA) was sensitized by peritoneal and limb subcutaneous injection. The asthmatic rat model was stimulated by 2% OVA atomization combined with cold (2–4 ℃) air stimulation. Pingchuanning (6.43 g/kg), Ferrostatin-1 (10 mg/kg), Pingchuanning (6.43 g/kg) + Ferrostatin-1 (2.5 μmol/kg), dexamethasone (0.5 g/kg), Guilong Kecchuanning (10g/kg) by gavage and atomization, Continuous intervention for 3 weeks. After the last stimulation, the lung tissues of rats were stained with hematoxylin-eosin (H&E) to observe airway inflammation and cell proliferation. The contents of IL-10, IL-22, IL-33 and ALOX15 in serum and LF of asthma were detected by enzyme-linked immunosorbent assay (ELISA). Real-time fluorescence quantitative polymerase chain reaction (RT-PCR) was used to detect the mRAN expression levels of SLC7A11 and GPX4, and Western blot was used to detect the protein expression levels of SLC7A11 and GPX4. **Results:** Compared with blank group, the diet, body weight, emotional irritability, respiratory shortness, airway inflammatory cell infiltration, goblet cell hyperplasia, serum and serum LF IL-10, IL-22, IL-33, ALOX15 inflammatory factors increased significantly in model group. The mRNA and protein expression levels of SLC7A11 and GPX4 were decreased. Compared with the model group, the diet of the rats in the Pinbuening, Ferrostatin 1 inhibitor and Pinbuening +Ferrostatin 1 inhibitor groups was gradually improved, wheezing was relieved, and airway inflammatory cell infiltration was significantly reduced. IL-10, IL-22, IL-33 and ALOX15 inflammatory factors in serum and LF of asthma were decreased ($P < 0.001$), while the mRNA and protein expressions of SLC7A11 and GPX4 were promoted ($P < 0.005$). **Conclusions:** Pinbuterin and its Ferrostatin 1 inhibitors can significantly improve airway inflammation induced by OVA combined with cold stimulation in asthmatic rats, and are related to SLC7A11/GPX4 signaling pathway and cell iron death. The efficacy of Pinbuterin combined with Ferrostatin 1 inhibitors is more obvious. It is suggested that the effect of combined treatment is better than that of single compound or western medicine.

Keywords: Pinchuanning; Ferrostatin 1 inhibitor; asthma; SLC7A11/GPX4

1. Foreword

Asthma is a common respiratory disease, a pulmonary inflammatory disease characterized by airway hyperresponsiveness, airway remodeling, airway stenosis, and airway mucus hypersecretion. Recurrent wheezing, shortness of breath, cough, chest tightness and other symptoms are commonly seen in clinic [1]. The disease affects a wide range of people. According to the 2024 report of the Global Asthma

Initiative (GINA), there are about 300 million asthma patients in the world, and children account for a large proportion, with a high mortality rate and high treatment costs [2].

Airway inflammation, as a core pathological feature of asthma, has a close interaction with airway remodeling. Long-term chronic inflammation leads to the release of Th2 cytokines such as IL-4, IL-5 and IL-13 from the airway epithelium, which promotes subepithelial fibrosis, smooth muscle cell proliferation and eosinophilic activation and infiltration, while activating fibroblasts and smooth muscle cells, and exacerbating their proliferation and activation. In addition, chemokines such as C-C chemokine ligand 5 (CCL5), CCL7 and CCL11 guide inflammatory cells to adhere, chemokine and infiltrate, release active mediators, exacerbate inflammatory reactions such as mucosal edema and increased secretions, and promote airway remodeling. Environmental stimuli such as allergens, cytokines, microbial proteins, etc. promote airway epithelium to release thymus stromal lymphopoietin (TSLP), IL-33, IL-25 and transforming growth factor (TGF-β), recruit and activate immune cells, form type 2 inflammatory response, and jointly drive the occurrence and development of airway remodeling [3].

In traditional Chinese medicine, asthma belongs to “wheezing disease”, it is believed that the fundamental reason of wheezing disease is that the body feels exogenous evil, the evil gas induces “croup phlegm”, the spittoon and gas strike each other, which affects the promulgation and descent of lung qi and causes cough and wheezing. At present, the purpose of drug treatment of asthma is mainly to control, need long-term use and can not be radical treatment of asthma, such as inhaled corticosteroids, leukotriene receptor antagonists, β2 receptor agonists, anticholinergic drugs, theophylline drugs and so on. Although the above drugs can effectively control the occurrence of asthma in the short term, long-term use is prone to adverse reactions, including endocrine, cardiovascular, respiratory, digestive, skeletal and other system damage, and reduce the quality of life of patients. The treatment of asthma in China varies from person to person, and each patient is treated after syndrome differentiation. The treatment methods include traditional Chinese medicine, moxibustion, acupuncture and massage. Among them, Chinese medicine treatment is the most widely used, according to the principle of “cure the disease”, in addition to the treatment in the asthma attack period, the most important thing is to regulate the patient in the remission period, enhance the patient’s own immunity, reduce the number of asthma attacks and the severity of the attack [4].

2. Theory overview

2.1. Pyranol

Prescription Middle ephedra combined with asarum, has the effect of relieving surface cold, warming lung, relieving cough and relieving asthma, and is the royal medicine. Bitter almond and Perilla seed have the effect of reducing qi, relieving cough and asthmatic asthma, and can reduce lung qi, and the combination of two medicines and ephedra, one rise and one fall, complement each other, and regulate lung qi significantly; Pinellia pinellia and Thunberg fritillaria have significant phlegm reducing effect, pinellia can dry dampness and phlegm reducing reverse, Thunberg

Fritillaria can clear heat and phlegm relieving cough; In addition, Dilong has the effect of expelling wind and relieving spasmodic asthma; The above five drugs are minister medicine. Tangerine peel has the effect of regulating qi and strengthening spleen, drying dampness and eliminating phlegm, and pinellia plays the effect of Qi shun phlegm qing, phlegm qing Qi drops from itself, and phlegm qing under Qi; Astragalus beneficial qi tonifying the effect of deficiency, anti-wind has the effect of dispelling wind and relieving spasmosis, the combination of astragalus and anti-wind is taken from jade Pingfeng powder, a powder and a supplement, the power of supplementing Qi and strengthening the surface is significant, and the Zi Zi ginseng can tonify the spleen soil, the three drugs used together have the effect of aiding the body's positive Qi, in addition, it can also help enhance the efficacy of Junyao Chen medicine; The above four drugs are adjuvants. Because of its good walking and channeling meridians, it can draw drugs to reach lung meridian, enhance the play of the efficacy of drugs, and also make the use of drugs [5].

2.2. Iron death

Iron death is a specific mode of cell death mediated by lipid peroxidation and associated with free iron-mediated Fenton reactions, the modulators of which may be cells and secretions of the immune system. Relevant studies have shown that iron death is closely related to the occurrence and development of asthma, and iron death can participate in airway inflammation by affecting the mucosal barrier, which promotes the occurrence and development of asthma. However, the current research on the relationship between iron death and asthma is not deep enough. Further research on the interaction between asthma inflammation and iron death signaling pathway will help to determine the regulatory factors of iron death pathway, and thus provide a basis for the treatment of asthma. glutathione peroxidase 4 (GPX4) and Solute Carrier Family 7 Member 11 (SLC7A11) are key regulatory proteins in the pathway associated with iron death [6]. GPX4 is a negative regulator of the iron death pathway, which protects cells from oxidative damage and reduces the occurrence of iron death [7]. Glutathione is mainly regulated by the cystine/glutamate transporter xc-system, and an important component of the XC-system is SLC7A11, which is mainly involved in the uptake of cystine, increases the amount of reducing substances in the body, plays an antioxidant role, and protects cells from death [8]. At the same time, previous studies have found that in the process of inflammatory diseases, in addition to the joint involvement of immune system and immune cells, iron death is also involved. However, the relationship between iron death and inflammatory diseases remains unclear and needs further discussion [9,10].

Under physiological conditions, cells can express and secrete a variety of proteins, such as SOD, nitric oxide oxidoreductase (NADPH), ubiquinone-1 (NQO1), catalase (CAT), GSH-Px and HO-1, to clear a large number of ROS and maintain the body in the oxidation equilibrium state [11,12]. When the body is stimulated by ALI/ARDS risk factors, a large number of neutrophils infiltrate and protease secretion increases, resulting in significant accumulation of oxidative products [13]. Excessive ROS exceeds the scavenging capacity of antioxidants in the body, resulting in the accumulation of a large amount of unsaturated fatty acids (PUFA) in the cell

membrane, reducing the fluidity of the cell membrane, increasing its permeability, and causing pulmonary edema and lung dilation [14]. A large number of lipids are oxidized to produce cytotoxic lipid peroxides under the action of free radicals or lipid peroxidase, which increases the formation and accumulation of lipid ROS, promotes the generation of MDA and 4HNE oxidation products, promotes cell iron death, and increases damage [15].

GSH is an important antioxidant, which can effectively remove intracellular free radicals. GPX4, also known as phospholipid peroxide glutathione peroxidase, is an enzyme that inhibits lipid hydroperoxides synthesized with GSH as substrate. When GSH is depleted or the activity of GPX4 is reduced, lipid hydroperoxides cannot be effectively reduced, resulting in their accumulation in the cell, and the accumulated lipid hydroperoxides will react with Fe^{2+} on the fenton. Produce a large number of ROS, cause cell membrane oxidative damage, and lead to iron death [16,17]. The synthesis of GSH and GPX4 depends on the cysteine/glutamate reverse transport system (System xc-), which consists of two amino acid chains of solute carrier family 3 member 2 (SLC3A2) and SLC7A11, which are transmembrane amino acid transporters on the cell surface. SLC7A11, as the light chain subunit of xc-system, is an important regulator of iron death. It trantranses cystine from extracellular to intracellular, reduces it to cysteine, collaborates with glutamic acid and glycine to synthesize GSH, participates in intracellular anti-oxidation, and maintains normal physiological state [18].

Interleukin-33 (IL-33) can activate a variety of immune cells to produce further inflammatory response, which is closely related to the persistent inflammatory state of asthma [19]. IL33 is an alarmin released on cellular damage from, for example, epithelial cells. Extracellular IL33 induces signaling via the heterodimeric receptor complex IL1RL1/IL1RAP. Airway IL33 levels have been associated with type 2 cytokines levels, and a positive correlation with eosinophil numbers in patients with asthma was recently reported. High IL33 levels have been found in induced sputum and bronchial biopsies of patients with asthma compared with in those of nonasthmatic controls. Moreover, IL33 may have a paracrine effect on the airway epithelium, as this epithelium has been shown to be responsive to IL33. These data suggest a connection among epithelium-derived IL33, eosinophilic inflammation, and asthma [20].

IL-22 belongs to the IL-10 cytokine family and also plays an important role in airway inflammation of asthma, promoting airway inflammation by stimulating the release of a variety of inflammatory cytokines. In asthma, Th2 inflammation is usually characterized by eosinophilic infiltration. Bullone et al. [21] reported that the increase of bronchial neutrophils in patients with asthma was related to the number of cells expressing IL-22. Although the mechanism of IL-22 in airway neutrophil inflammation is currently unknown, IL-22 is an important factor in neutrophil recruitment. In a mouse model of asthma, IL-22 not only induces neutrophils to recruit chemokines, but also promotes the secretion of CXCL1 and CXCL5 by airway epithelial cells. IL-22 significantly enhances neutrophil recruitment related chemokines, including CXCL1, CXCL2, CXCL8, and IL-1β. In addition, IL-22 can induce the expression of chemokines in airway epithelial cells and airway smooth muscle cells, and produce synergistic proinflammatory effects on airway neutrophils. In conclusion, IL-22 can promote airway inflammation in asthma by stimulating the

release of various inflammatory cytokines [22].

DAMPs is an important pathway to exert the biological effect of iron death, which can directly or indirectly mediate the pathophysiological process of allergic asthma. IL-33 plays an important role in the occurrence and development of allergic asthma. When bronchial epithelial cells are injured, a DAMPs, IL-33, is released. The inflammatory response caused by the release of IL-33 can be inhibited by Fer-1, and IL-33 is involved in iron death.

ALOX15 has been identified as being closely related to the development of asthma. Studies have shown that ALOX15 is present in airway epithelial and may be associated with chronic airway inflammation in asthma by catalyzing the conversion of arachidonic acid to 15-hydroxyeicosatetraenoic acid (15-HETE), while 15-HETE is associated with multiple airway inflammatory diseases [23]. Previous studies have found that the expression of ALOX15 in bronchial epithelium increases with the severity of asthma, and the high level of ALOX15 activity may contribute to the production of mucin MUC5AC in asthma [24]. Airway inflammation was reduced in mice with ALox15-deficient asthma compared to mice with wild-type asthma models. ALOX15 was also found to be highly associated with posterior ptosis. There are three main metabolic pathways associated with iron death, including iron metabolism, lipid peroxidation, and glutathione metabolism. Studies have shown that ALOX15 selectively catalyzes the oxidation of PE-AA to lipid hydrogen peroxide, which ultimately leads to iron death [25].

Traditional Chinese medicine treatment of asthma syndrome type Middle lung heat or lung dryness asthma may be related to oxidative stress, mainly due to the invasion of heat and dryness, the conflict between good and evil, the body produces a large number of ROS, destroys the cell membrane, and then induces iron death. GSH and GPX4 as important antioxidant factors, their upregulation can inhibit iron death. Lung stasis in traditional Chinese medicine is mainly Qi and blood stasis. Modern medicine believes that it has a great factor in causing red blood cell overflow outside the pulse due to inflammatory factor storm, which can reduce oxidative stress and inhibit iron death by regulating inflammatory response. Exogenous wind evil and internal disturbance of phlegm heat are related to the imbalance of cell metabolism, and metabolic disorder is an important factor inducing iron death. SLC7A11 plays an important role in cystine metabolism, and its normal function can maintain the balance of cell metabolism and reduce iron death.

3. Materials and methods

3.1. Animals

A total of 70 SPF-grade healthy male SD rats, aged 3–4 weeks and weighing 80–100 g, were purchased from Henan SCXK (Henan) 2020-0005 Biotechnology Co., LTD. (License No.: SCXK (Henan) 2020-0005. The rats were in good health condition. The animals were reared in the Geriatric Experimental Center, School of Nursing, Anhui University of Chinese Medicine, with indoor temperature of 20~25 ℃ and relative humidity of 45%~75%. The animals were reared for 7 days before the experiment. This study was reviewed by the Experimental Animal Ethics Committee of Anhui University of Chinese Medicine, and the relevant regulations and principles

were strictly observed during the whole process of the experiment, and it was confirmed that it could be carried out (Animal ethics number: AHUCM-rats-2024044).

3.2. Drugs

It is a decoction piece of traditional Chinese medicine (all purchased from the Chinese Medicine Hall of Anhui University of Traditional Chinese Medicine), and the traditional Chinese medicine compound decoction is converted according to "Formulology" [26]. Calculation formula: Db = Da × Rab, 9.8 raw drug quantity/kg rat body mass, the herbs were soaked, decocked for 3 times, mixed and concentrated to 0.98 g/mL; Ferrostatin-1 (Fer-1), Ferrostatin-1 (article No. S81461), Shanghai Yuanye Biotechnology Co., LTD.; Dexamethasone, 0.75 mg per tablet (Zhejiang Xianzhu Pharmaceutical Co., LTD.), 150 mL normal saline with a configured concentration of 0.005 g/L; Guilong Kechuanning, 0.5 g per tablet, (Guilong Pharmaceutical Co., LTD.), 2 tablets with 100 mL, the configured concentration is 0.032 g/mL.

3.3. Reagent

Egg albumin (OVA, US Sigma, lot number: A5235); Xylene (Tianjin Kaitong Chemical Company, lot number: 20240216); Anhydrous ethanol (Sinopsin Group Chemical Reagent Co., LTD., Lot No. 20240525); Trizol (Life technogies, lot number: 99088701); Novostart SYBR qPCR SuperMix Plus (novoprotein, lot number: 05285501); RIPA cell lysate (Beyotime, lot number: 09271919023); PBS buffer powder (Zs-BIO, lot number: 22121401); PVDF membrane (Millipore, lot number: 0000185693); Predyed protein Marker (Thermo, lot number: 91299553); Swestern Rapid Film transfer Solution, ECL supersensitive luminescence kit (Beyotime, lot No.: 052223230928, 23158476); Goat Anti-Mouse IgG and Goat Anti-Rabbit IgG (Zsbio, lot No. 140193, 202700514); Rat Interleukin-10 (IL-10), rat interleukin-22 (IL-22), Rat Interleukin-33 (IL-33), Rat arachidonic acid 15 lipoxygenase (ALOX15) (Wuhan Genmei Technology Co., LTD., Model number: JYM0651Ra & 20240506, JYM1204Ra & 20240525, JYM0832Ra & 20240510); Enzyme marker (Radu Corporation); Centrifuge (Anhui Jiawen Company); Swirl mixer (Qi Limber Instrument Manufacturing Co., LTD.); Pipette (eppendorf Corporation); And electric thermostats (Shanghai Sanfa Company). Glutathione (GSH) (A006-2-1), Serum iron (Fe) (A039-1-1), Malondialdehyde (MDA) (A003-1), Reactive oxygen species (ROS) (E004-1-1) kits, purchased from Nanjing Jiengcheng Institute of Biological Engineering.

3.4. Reagent for animal grouping and asthmatic model establishment

The asthma model was established according to the previous experimental method of the research group: 70 rats were divided into 7 groups according to random classification, which were: Blank group (A), model group (B), Pingchuanning group (C), dexamethasone group (D), Guilong Kechuanning group (E), Ferrostatin 1 inhibitor group (F), Pingchuanning + Ferrostatin 1 inhibitor group (G), with 10 rats in each group. Except the blank group, the rats in the other 6 groups were sensitized and stimulated with OVA, and 10% OVA mL was injected subcutaneously into the

abdominal cavity and both sides of the groin for sensitization on the 1st and 8th days, respectively. Then on the 15th and 28th days, the rats were placed in a cold tank at 0~2 ℃ with ultrasonic atomization of 2% OVA solution, once a day for 30 min each time. In the blank group, OVA was replaced by normal saline for intraperitoneal injection and atomization according to the above methods. On days 29 to 49, all the rats continued cold stimulation and nebulization, and were given gavage (10 mL/kg) 0.5 h after nebulization for 21 days, once a day. Blank group A and model group B were given normal saline according to the body mass of the rats; Group C was given intragastric administration at 6.43 g/kg; Ferrostatin-1 inhibitor group D was given intragastric administration at 10 mg/kg; Group E plus Ferrostatin 1 inhibitor group was given the dose of 6.43 g/kg of Pinterol first, and then the Ferrostatin 1 inhibitor group was given the dose of 10 mg/kg. Dexamethasone group F was given 0.5 g/kg intragastric administration; Group G of Guilong Kechuanning was given 10 g/kg gavage. On the 52nd day, group G was killed and local sampling was performed.

3.5. Sampling and preparation of specimens

After fasting for 24 h, samples were taken from rats, OVA stimulation was performed for the last time, 2 mL of abdominal aorta blood was taken after anesthesia, serum was taken by centrifuge and stored at −20 ℃ for ELISA assay. After devertebrae were killed, 6 rats in each group were exposed to the thorax and organs, and the alveoli were repeatedly lavage with normal saline (0.5 mL × 3 times), and 1.0–1.5 mL of alveolar lavage fluid was extracted, and the alveolar supernatant was retained after centrifugation. The left lung of the remaining rats was extracted, rinsed, fixed in 4% paraformaldehyde solution for H&E and PAS staining, and the right lung was placed in liquid nitrogen, stored and transported to a refrigerator at −80 ℃ for RT-PCR and protein determination.

3.6. Index detection

3.6.1. H&E staining was used to observe the lung histopathological changes

The left lung lobe tissue was removed and fixed with 10% formaldehyde for 24 h. After dehydration and embedding, sections of 3~4 μm thick were prepared, dewaxed with xylene and ethanol and hydrated with ethanol, H&E staining was performed according to routine procedures, and phenol-xylene, xylene I and xylene II were treated to transparency. The inflammatory cell infiltration around the airway was observed by light microscope after sealing.

3.6.2. PAS staining was used to observe the lung histopathological changes

The sections of lung tissue were dewaxed, oxidized and dyed, washed and dried with running water, dropped with Schiff dye, cultured at 37 ℃, stained with hematoxylin for 2–5 min, differentiated for 10 s, washed with water until reverted to blue for 1 min, and then dehydrated and transparent according to the above steps. The hyperplasia of goblet cells and mucus in lung tissue were observed by light microscope.

3.6.3. Western blot assay was used to detect the protein expressions of SLC7A11 and GPX4 in lung tissue

The total protein of rat lung tissue was 0.1 g, and RIPA cell lysate (0.6 mmol·L-

1 PMSF) was jinxing for cleavage. The supernatant was centrifuged at 12,000 × g for 15 min, and the total tissue protein was collected. 5X SDS-PAGE protein loading buffer was added into the collected protein samples at the rate of 1:4. Heat the boiling water bath for 15 min to fully denature the protein. After the sample is cooled to room temperature, the protein sample can be directly fed into the SDS-PAGE gluing hole. Add 5–10 uL per well. Electrophoresis at constant pressure 80 V for 1 h, the protein was transferred to PVDF membrane, added to Western sealing liquid shaking for 2 h, and then washed with PBST. After that, SLC7A11 (1:1000), GPX4 (1:1000) and GAPDH (1:2000) were added and incubated in a shaker at 4 ℃ overnight. The next day, after PBST washing, diluted dianti-horseradish peroxidase (1:20,000) was added and incubated in the greenhouse for 1.2 h. After PBST washing, the protein was detected by ECL luminescence kit, and stripe analysis was performed by Image J software.

3.6.4. The concentrations of IL-10, IL-22, IL-33 and ALOX15 inflammatory factors in serum and alveolar lavage fluid were detected by ELISA

According to the instructions, IL-10, IL-22, IL-33, ALOX15 reagents, serum and alveolar lavage solution were placed at room temperature, and different loading orders were marked. Standard well and sample well were added with standard solution and sample solution, 50μL enzyme labeled reagent was added, sealed and incubated at 37 ℃ for 30 min, left for 30 s to shake well, incubated at 37 ℃ for 10 min in the dark, stop solution was added, OD value was read with enzyme labeled instrument (450 nm), and standard curve was drawn.

3.6.5. The expression levels of SLC7A11 and GPX4 in rat lung tissue were detected by RT-qPCR

Total RNA was extracted from lung tissue by weighing 50 mg, adding Trizol (1mL) for cleavage, and the RNA was transcribed into cDNA using TaKaRa reverse transcription kit at 37 ℃ for 15 min. The cDNA was stored at −80 ° C for 85 ° C for 5 s. The cDNA was taken 1 μL and added to the upstream and downstream primers 1 μL each for fluorescence quantitative PCR reaction. The reaction system was 10 μL, and the reaction conditions were predenatured at 95 ℃ for 1 min, denatured at 95 ℃ for 20 s, and extended by annealing at 60 ℃ for 60 s, for 40 consecutive cycles. After the reaction, amplification and dissolution curves were obtained. Using β-actin as the internal reference, the detection was repeated for 6 times in each sample. The relative expressions of SLC7A11 and GPX4 were calculated by 2-ΔΔCt method. The primers were provided by Shenggong Bioengineering (Shanghai) Co., LTD., and the primers were sequenced in **Table 1**.

Table 1. Primer sequence of RT-PCR.

Gene	Amplicon Size (bp)	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Beta actin	150	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC
SLC7A11	161	AGGGCATACTCCAGAACACG	GGGACCAAAGACCTCCAGAA
GPX4	172	AATCCTGGCCTTCCCTTGCA	GCCCTTGGGCTGGACTTTCA

3.6.6. Detection of iron death-related markers in lung tissue

The expression levels of ROS, MDA, GSH and Fe^{2+} in lung tissues of 15–20 mg mice were detected according to the kit instructions.

3.7. Statistical analysis

SPSS 26.00 was used for statistical analysis of the experimental results, the measurement data were described as mean ± standard deviation (sx), one-way ANOVA was used for comparison among multiple groups, LSD test was used for homogeneous variance, Tamhane's T2 test was used for uneven variance. $P < 0.05$ was considered to be statistically significant.

4. Results

4.1. Effect on the general condition of rats

Before modeling, there was no difference between the groups of rats, and their spirit, eating and activity were normal. During the sensitization period, except for the blank group, the other rats had reduced food and water intake, weight loss, accompanied by wheezing, shortness of breath, irritability and dark and dirty hair color. After drug intervention, the symptoms of each treatment group were improved and relieved compared with the model group.

4.2. Lung histopathological changes in asthmatic rats

H&E staining group: Compared with blank group (A), asthmatic model group (B) had narrow lumen, thickened tube wall and septum, increased inflammatory cells and changed infiltration; In Pingchuanning group (C), Ferrostatin 1 inhibitor group (D), Pingchuanning + Ferrostatin 1 inhibitor group (E), dexamethasone group (F) and Guilong Kecchuanning group (G), the thickening state of tube wall and septum was reduced, the stenosis of the lumen was changed, the number of inflammatory cells was reduced, and the status of mucosal edema was improved. These results indicate that Pingchuanning, Ferrostatin 1 inhibitor, Pingchuanning + Ferrostatin 1 inhibitor, dexamethasone and Guilong Kechuanning can effectively improve the pathological status of asthmatic rats, and Pingchuanning group is better than Ferrostatin 1 inhibitor group, dexamethasone group and Guilong Kechuanning group. And Pingchuanning +Ferrostatin 1 inhibitor group is the best, as shown in **Figure 1**.

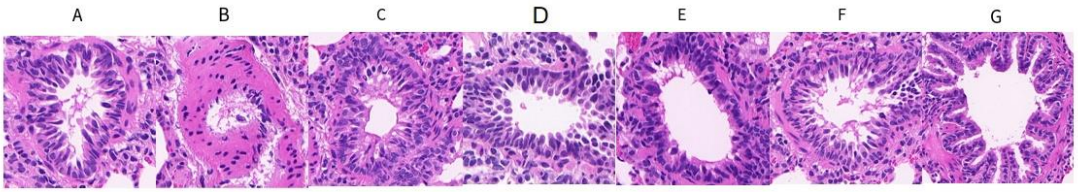

Figure 1. Effects of Pingchuanning, Ferrostatin 1 inhibitor and positive control drugs on lung histopathology of asthma rats induced by OVA combined with cold stimulation（HE, ×400）. **(A)** blank group; **(B)** model group; **(C)** Pingchuanning group; **(D)** dexamethasone group; **(E)** Guilong Kechuanning group; **(F)** Ferrostatin 1 inhibitor group; **(G)** Pinbuterol + Ferrostatin 1 inhibitor group.

PAS staining group: Compared with blank group (A), asthmatic model group (B) showed obvious goblet cells and mucus; Bronchial and airway mucus decreased in Pinchuening group (C), Ferrostatin 1 inhibitor group (D), Pinchuening group +Ferrostatin 1 inhibitor group (E), dexamethasone group (F), Guilong Kecchuening group (G) and control group, and goblet cell hyperplasia changes were significantly delayed. This indicates that Pingchuanning, Ferrostatin 1 inhibitor, Pingchuanning +Ferrostatin 1 inhibitor, dexamethasone and Guilong Keczhanning can effectively reduce bronchial and airway mucus and improve the trophy cell hyperplasia in asthmatic rats, and the Pingchuanning +Ferrostatin 1 inhibitor group has the best effect, as shown in **Figure 2**.

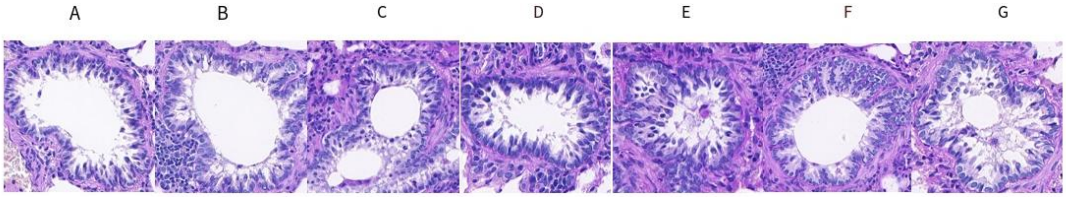

Figure 2. Effects of Pingchuanning, Ferrostatin 1 inhibitor and positive control drugs on lung histopathology of asthma rats induced by OVA combined with cold stimulation（PAS, ×400）**. (A)** blank group; **(B)** model group; **(C)** Pingchuanning group; **(D)** dexamethasone group; **(E)** Guilong Kechuanning group; **(F)** Ferrostatin 1 inhibitor group; **(G)** Pinbuterol + Ferrostatin 1 inhibitor group.

4.3. Effects of serum levels of IL-10, IL-22, IL-33 and ALOX15 in rats

Table 2. Effects of Pingchuanning and its administration groups on the contents of IL-10, IL-22, IL-33 and ALOX15 in serum of asthmatic rats (n = 6, $\bar{x} \pm s$).

group	Dosage(g/kg)	il-33	il-22	ALOX15	il-10
A blank group	-	60.1600 ± 8.45967^{cfehdg}	59.1700 ± 2.65451^{cfehdg}	28.1150 ± 3.15110^{cfehdg}	45.0617 ± 5.01369^{cfehdg}
B model group	-	353.8150 ± 9.26682^{afbhg}	320.4917 ± 7.77300^{afbhg}	122.4217 ± 2.83458^{afbhg}	178.1117 ± 3.65881^{afbhg}
C Pingchuanning group	6.43	205.9350 ± 11.26480^{acbed}	204.6683 ± 5.42003^{acbed}	79.2117 ± 4.67814^{acbed}	111.9733 ± 3.66437^{acbed}
D dexamethasone group	5×10^{-3}	210.4017 ± 14.32121^{acbed}	213.3083 ± 15.01205^{acbed}	81.3233 ± 2.02120^{acbed}	107.7900 ± 4.57479^{acbed}
E Guilong Kechuanning group	10	208.5667 ± 11.05496^{acfbed}	127.9633 ± 37.99420^{acfbehdg}	42.6567 ± 4.20517^{acfbehdg}	70.5683 ± 1.35640^{acfbehdg}
F Ferrostatin 1 inhibitor group	10	196.4767 ± 9.45924^{aced}	213.3450 ± 9.29539^{acbed}	84.0883 ± 4.11490^{acbed}	115.1467 ± 6.48861^{acbed}
G Pinbuterol + Ferrostatin 1 inhibitor group	6.43+10	107.3750 ± 10.94357^{acfbehdg}	207.6700 ± 14.54577^{acbed}	70.9033 ± 4.15772^{acfbehdg}	107.6150 ± 7.70270^{acbed}
F		439.624	134.215	409.930	408.882

Compared with blank group, IL-10, IL-22, IL-33 and ALOX15 in model group were significantly increased ($P < 0.001$); Compared with model group, IL-10, IL-22, IL-33 and ALOX15 in all groups were significantly decreased ($P < 0.001$); Compared with Pingchuanning group, IL-10, IL-22, IL-33 and ALOX15 in Ferrostatin 1 inhibitor group were slightly decreased ($P < 0.005$); The levels of IL-10, IL-22, IL-33 and ALOX15 in the Pinchuening + Ferrostatin 1 inhibitor group were significantly

decreased ($P < 0.005$); Compared with dexamethasone, the IL-22, IL-33 and ALOX15 were significantly decreased ($P < 0.05$), while the IL-10 was slightly increased ($P > 0.005$); Compared with Guilongkechuanning, the levels of IL-10, IL-22, IL-33 and ALOX15 were significantly decreased ($P < 0.001$), as shown in **Table 2**.

4.4. Effect on Gpx4 and SLC7A11 protein expression in rats with large lung tissue

Compared with blank group, the expression levels of SLC7A11 and GPX4 in lung tissue of model group were significantly decreased ($P < 0.001$). Compared with model group, the mRNA expressions of SLC7A11 and GPX4 in lung tissue of rats in administration group were significantly increased ($P < 0.001$ in Pingchuanning group and dexamethasone group, $P < 0.005$ in Guilong Kechuanning group). Compared with Pingchuanning, the expressions of Guilong Kechuanning group and Ferrostatin 1 ($P < 0.005$) inhibitor group were increased, and the expressions of dexamethasone group and Pingchuanning + Ferrostatin 1 inhibitor group were significantly decreased ($P < 0.001$), as shown in **Table 3** and **Figure 3**.

Table 3. Effects of Pingchuanning and its administration groups on the expression of SLC7A11 and GPX4 mRNA in lung tissue of asthmatic rats ($n = 6$, $\bar{x} \pm s$). The dose unit of Ferrostatin-1(Fer-1) iron death inhibitor group in 2 columns and 7 rows was: μmol/kg.

group	Dosage(g/kg)	SLC7A11	GPX4	group	Dosage(g/kg)	SLC7A11	GPX4	group
A blank group	-	$1.0017 \pm 0.04708^{cfedg}$	$1.000 \pm 0.01789^{cfehdg}$	A blank group	-	$1.0017 \pm 0.04708^{cfedg}$	$1.000 \pm 0.01789^{cfehdg}$	A blank group
B model group	-	$0.5683 \pm 0.02787^{afbg}$	$0.5400 \pm 0.03899^{afbhg}$	B model group	-	$0.5683 \pm 0.02787^{afbg}$	$0.5400 \pm 0.03899^{afbhg}$	B model group
C Pingchuanning group	6.43	$0.7433 \pm 0.04676^{acbed}$	$0.8267 \pm 0.04131^{acbed}$	C Pingchuanning group	6.43	$0.7433 \pm 0.04676^{acbed}$	$0.8267 \pm 0.04131^{acbed}$	C Pingchuanning group

Note: Compared[a] with the normal group, $P < 0.05$, [b] $P<0.001$; Compared[c] with model group $P < 0.05$, [d] $P < 0.01$, [e] $P <0.001$; Compared[f] with Pingchuanning group, $P < 0.05$, [g] $P < 0.01$, [h] $P < 0.001$.

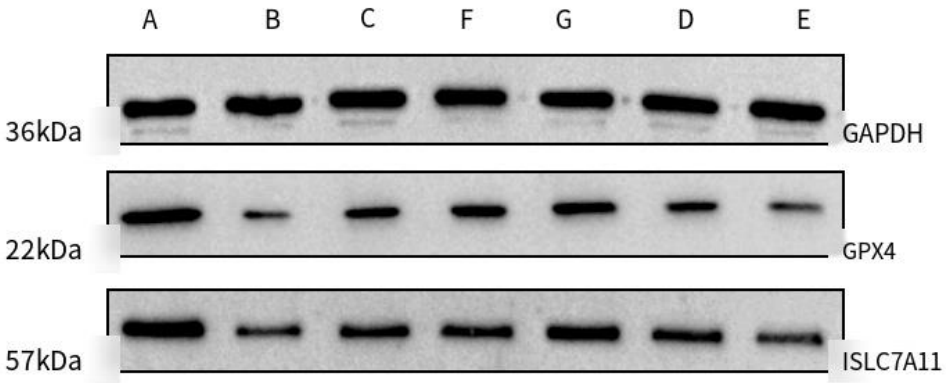

Figure 3. Effects of Pingchuanning on the expression of SLC7A11 and GPX4 mRNA in asthmatic rats. ($n = 6$, $\bar{x} \pm s$).

4.5. ROS, MDA, GSH, Fe^{2+} levels in lung tissue

As shown in **Figure 4**, the detection results of iron death markers ROS, MDA, GSH and Fe^{2+} showed that compared with the normal group, the contents of ROS, MDA, GSH and Fe^{2+} in the model group were significantly increased. Compared with

the model group, MDA, GSH and Fe^{2+} contents in the administration group were decreased, while ROS contents in the Guilong Kechuanning group were still increased; Compared with Ping Chuanning group, ROS, MDA, GSH and Fe^{2+} in dexamethasone group and Guilong Kechuanning group were increased, MDA, GSH and Fe^{2+} in Ferrostatin 1 inhibitor group were decreased, and ROS, MDA and Fe^{2+} contents in Ping Chuanning + Ferrostatin 1 inhibitor group were decreased.

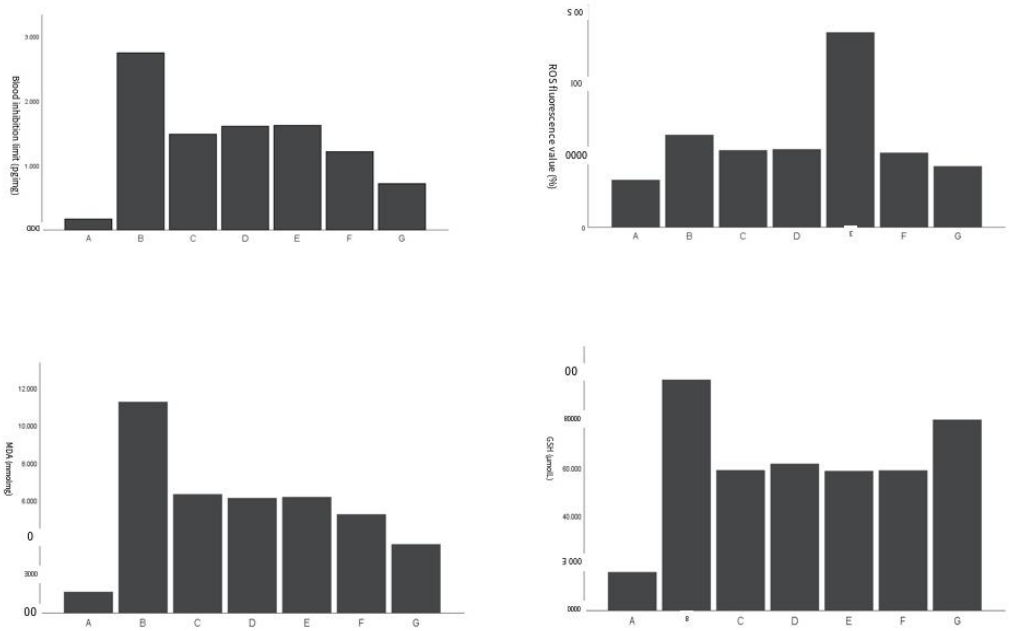

Figure 4. Detection results of iron death in mouse lung tissue. **(A)** normal group; **(B)** model group; **(C)** Pingchuanning group; **(D)** dexamethasone group; **(E)** Guilong Kechuanning group; **(F)** Ferrostatin 1 inhibitor group; **(G)** Pinbuterol + Ferrostatin 1 inhibitor group.

4.6. Observation results of behavior in mice

Before modeling, there were no differences among all groups of rats, and their spirit, eating and activity were normal. During the sensitization period, except the blank group, the other rats had reduced food and water intake, weight loss, wheezing, shortness of breath, irritability, dark and dirty hair color and so on. After drug intervention, the symptoms of each treatment group were improved and relieved compared with the model group.

5. Discuss

Asthma is prolonged and difficult to cure, caused by various pathogenesis stimulation of lung health is not solid, in case of allergen or cold air stimulation, wheezing, phlegm is obstructed, but the earth is the mother of gold, lung gold disease for a long time to the spleen, resulting in spleen soil deficiency; Gold is the son of the earth, spleen soil deficiency, lung gold no water valley of the fine and run it, then lung wei deficiency, in the case of cold asthma is easy to do. Pingchuanning consists of Sanzi Yangqin Decoction, a famous prescription for eliminating phlegm in Korean Yitong, Shegan Mahuang Decoction, a classic prescription for treating asthma in synopsis of golden chamber, and Yupingfeng Powder, a famous prescription for

benefiting health and strengthening exterior from Danxi Heart Method. The medicines are ephedra, almond, perilla, asarum, dried tangerine peel, Pinellia tuber, Saposhnikovia divaricata, earthworm, Fritillaria thunbergii, Radix Pseudostellariae and Astragalus. Together with the effect of expelling wind and stopping spasm, relieving asthma of lung, reducing qi and eliminating phlegm, Yiwei and stopping spasm to treat asthma. In this study, OVA induction combined with cold stimulation was used to construct an asthmatic rat model. The inflammatory cells of the lung tissues of the rats showed obvious infiltration, excessive secretion of mucus, constriction of bronchial lumen, shortness of breath accompanied by wheezing, dirty and dull hair, irritability and other manifestations, suggesting that the model of asthma was successfully constructed. After the intervention of Pingchuanning and Ferrostatin 1 inhibitor, the inflammatory cell infiltration, bronchial tube wall thickness and mucus secretion in the lung tissue of asthmatic rats were improved to varying degrees. Among them, the effect of Pingchuanning plus Ferrostatin 1 inhibitor group was the best, suggesting that the effect of combined treatment was stronger than that of single compound or western medicine.

Dexamethasone, a glucocorticoid drug, is one of the main drugs in the treatment of asthma at present, but there are many side effects, which have more negative effects on the treatment of patients. Guilong Kechuanning is a Chinese patent medicine, which has the effect of relieving cough and phlegm, reducing qi and relieving asthma. It can be used to treat cough, asthma and excessive phlegm. Its clinical effect is obvious in the treatment of asthma, and it can also be used as a positive control group of Pingchuan Ning prescription for comparison.

Iron death is a new type of programmed non-apoptotic cell death caused by iron-dependent lipid peroxidation after the inactivation of SLC7A11. It is associated with a variety of diseases. It is a lytic and pro-inflammatory cell death mode. In iron death, the antioxidant capacity of cells decreased, resulting in lipid peroxidation and metabolic dysfunction, and increased lipid reactive oxygen species (ROS). As a key marker of iron death, GPX4 plays a crucial regulatory role in the process of iron death. Its function is to convert lipid hydroperoxides into non-toxic lipid alcohols, thereby inhibiting lipid peroxidation. Therefore, GPX4 is also regarded as an important protein to resist iron death [27]. Ferrostatin 1 is a synthetic compound isolated by high-throughput screening of small molecule libraries that resists the occurrence of iron death by inhibiting lipid peroxidation. Animal studies have demonstrated iron death in allergic asthma, OVA inhalation can induce lung iron death and lipid peroxidation in mice, and iron death inhibitor Fer-1 can alleviate allergic asthma by inhibiting lung iron death and lipid peroxidation. In vitro studies have shown that inducing ROS dependent lipid peroxidation and disrupting iron homeostasis can promote iron death in bronchial epithelial cells, and treatment with Fer-1 can reverse lipid peroxidation and iron death in bronchial epithelial cells [28].

In the of this study, in the OVA-induced rat asthma model, compared with the model group, the lung tissue protein expression and mRNA expression of rats in the Ferrostatin 1 inhibitor group and the asthma + Ferrostatin 1 inhibitor group were significantly reduced. The concentrations of inflammatory factors such as IL-10, IL-22 and IL-33 in serum and alveolar lavage fluid were reduced to varying degrees. Pathological specimens showed that inflammatory cell infiltration around bronchus in

lung tissue and airway mucus secretion were also reduced to varying degrees, among which Pinbuterin + Ferrostatin 1 inhibitor group was better.

In summary, Pingchuanning can effectively inhibit iron death of cells through SLC7A11/GPX4 signaling pathway, alleviate pathological injury of lung tissue in asthmatic rats, alleviate inflammatory response, significantly reduce protein and mRNA expression, reduce the release of inflammatory factors, and improve airway inflammation in asthmatic rats. Iron death is a new direction in the treatment of asthma. This study will help to determine the relationship between asthma airway inflammation and iron death, and provide guidance for further understanding and effective treatment of asthma. The specific molecular mechanism of iron death needs further research.

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Ethical approval: The study was conducted in accordance with the Declaration of Helsinki, and approved by Animal Ethics Committee of Anhui University of Traditional Chinese Medicine (AHUCM-rats-2024044).

Conflict of interest: The authors declare no conflict of interest.

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