Original Article

Quercetin inhibits ferroptosis through the SIRT1/Nrf2/HO-1 signaling pathway and alleviates asthma disease

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Background: Quercetin (QCT) is a bioflavonoid derived from vegetables and fruits that has anti-inflammatory and anti-ferroptosis effects against various diseases. Previous studies have shown that QCT modulates the production of cellular inflammatory factors in asthma models and delays the development of chronic airway inflammation. However, the regulatory mechanism of QCT, a traditional Chinese medicine, in the treatment of asthma has not been elucidated. The aim of the present study is to investigate whether QCT can inhibit ferroptosis via the SIRT1/Nrf2 pathway and play a therapeutic role in asthma.

Methods: An ovalbumin-induced mouse asthma model was established, and its function was verified by hematoxylin eosin staining, enzyme linked immunosorbent assay, ferric ion assay, malondialdehyde and superoxide dismutase assays, dihydroethidium staining, immunohistochemical staining, western blotting, and quantitative real-time polymerase chain reaction.

Results: Our results indicated that an ovalbumin-induced asthma mouse model had been successfully established and that QCT inhibited inflammation, reduced serum levels of inflammatory factors IL-4, IL-5 and IL-13, increased superoxide dismutase levels in lung tissue homogenates, and reduced malondialdehyde and ferric ion production in asthmatic mice. In addition, we found that QCT was able to reverse the expression of SIRT1, Nrf2 and HO-1 in an *in vivo* asthma mouse model.

Conclusions: The data from this study indicate that QCT can alleviate asthma, and its mechanism is related to the regulation of ferroptosis, oxidative stress, and the expression of SIRT1 protein.

Keywords: Quercetin (QCT); asthma; ferroptosis; signaling pathway

Submitted May 20, 2024. Accepted for publication Oct 09, 2024. Published online Oct 28, 2024.
doi: 10.21037/tp-24-193
View this article at: https://dx.doi.org/10.21037/tp-24-193

Introduction

Allergic diseases are a series of diseases mediated by innate and adaptive immune responses, including asthma, allergic rhinitis, conjunctivitis, etc. In recent years, the prevalence of allergic diseases has been on the rise, bringing considerable medical and social-economic burden (1,2). Asthma, as a global health problem, affects more than 300 million people of all ages worldwide, including about 10–15% of school-age children (3).

Asthma is a heterogeneous disease with endotypes that exhibit different immune system characteristics, severity and response to current therapies (4). Two main

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subtypes of immune responses driving asthma have been defined, namely type 2 (T2)-high and T2-low (5,6). T2-high asthma is characterized by eosinophilia, eosinophilia counts in peripheral blood have been shown to correlate with asthma severity in children with T2-high asthma, and are also a predictor of a patient's good response to inhaled corticosteroids (4,7). When allergens enter the airway, the epithelial barrier is impaired, prompting airway epithelial cells to release alarmins such as thymic stromal lymphopoietin (TSLP), interleukin-25 (IL-25) and interleukin-33 (IL-33) (8). TSLP activates dendritic cells and induces the differentiation of naïve T cells into T helper 2 (Th2) cells, which release interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-13 (IL-13) (9). IL-4 regulates the proliferation and survival of Th2 cells and immunoglobulin E (IgE) synthesis, and is critical in initiating anaphylaxis (10). IL-5 triggers a series of intracellular signal transduction events that promote production and maturation of eosinophils (11). IL-13 directly stimulates bronchial smooth muscle contraction and induces epithelial cells to secrete mucin and fractional exhaled nitric oxide (FeNO). Asthma endotype helps to realize precision medicine (12).

Ferroptosis is an iron-dependent regulatory cell death characterized by excessive oxidized phospholipids, often accompanied by a large amount of lipid peroxidation (13,14). More and more studies have shown that ferroptosis plays a key role in the pathogenesis of asthma and is involved in regulating airway inflammation of asthma (15). Ferrostatin-1 (Fer-1) can reduce the levels of IL-4, IL-5 and IL-13 in house dust mite asthma model (16). The nuclear factor erythroid 2-associated factor 2 (Nrf2) is a very important nuclear transcription factor. When cells are exposed to oxidative stress, Nrf2 moves from the cytoplasm to the nucleus and increases the expression of heme oxygenase-1 (HO-1) to maintain the intracellular REDOX balance. Nrf2 is an important transcriptional regulator of anti-ferroptosis genes (17). Silent information regulator 1 (SIRT1) is a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase involved in the regulation of inflammation and oxidative stress. SIRT1 can deacetylate Nrf2 and regulate the activity of Nrf2 to inhibit oxidative stress. The role of SIRT1 in regulating intracellular ferroptosis through Nrf2 has been validated in other diseases.

Traditional Chinese medicine has been used to treat allergic diseases in Asia for hundreds of years. Quercetin (QCT) is a natural flavonoid that contains no carbohydrates and is the most abundant flavonoid in vegetables and fruits (18). QCT has been shown to have a variety of pharmacological activities, including neuroprotective (19), antioxidant (20), anti-inflammatory (21), anticancer (22), as well as anti-allergic and anti-apoptotic effects (23), and QCT is a pro-apoptotic compound with few harmful effects on normal cells (24). It plays an immunomodulatory role in the two stages of sensitization and response of allergic diseases (25). In atopic dermatitis models of human keratinocytes and mouse models, QCT has been shown to inhibit the expression and secretion of TSLP and interleukin-33 (IL-33) (26). QCT's regulatory effect on epithelial cytokines was observed in experimental models of asthma, significantly reducing levels of interleukin-25 (IL-25), IL-33 and TSLP (27), and reducing the occurrence of allergic airway inflammation. The aim of this study was to investigate the effect of QCT treatment on asthma and its potential regulatory mechanisms. We present this article in accordance with the ARRIVE reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-24-193/rc).

Highlight box

Key findings

- Quercetin (QCT) is able to alleviate asthma through the SIRT1/Nrf2/HO-1 signalling pathway by a mechanism that may be related to ferroptosis.

What is known and what is new?

- QCT has been shown to possess a variety of pharmacological activities, and in experimental models of asthma, QCT was able to reduce the production of cellular inflammatory factors and lower the level of oxidative stress. However, it appears that the specific mechanism by which QCT alleviates asthma remains to be investigated.
- This study provides a comprehensive overview of the potential mechanisms of QCT, a traditional Chinese medicine ingredient, in the treatment of asthma.

What is the implication, and what should change now?

- QCT is a promising drug for the treatment of asthma and provides new ideas for clinical asthma drug development.

Methods

Animal assays

Female BALB/c mice (16–20 g, 6–8 weeks old) were purchased from Beijing HFK Bio-Technology. Co., LTD (China) and housed in a temperature- and humidity-controlled room under a 12/12 h light/dark cycle with free

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access to water and food. Experiments were performed under a project license (No. CMU20231099) granted by institutional ethics board of the Laboratory Animal Welfare and Ethics Committee of China Medical University, in compliance with the Laboratory Animal Welfare and Ethics Committee of China Medical University institutional guidelines for the care and use of animals.

Treatment protocols

The specific method of the *in vivo* experiment was as follows: Thirty-six mice were randomly divided into six groups: control group, model group, QCT treatment group (40 mg/kg, HY-18085, MedChemExpress, USA), QCT treatment group (80 mg/kg), dexamethasone (Dex) treatment group (1 mg/kg, HY-147220, MedChemExpress, United States) and Fer-1 treatment group (1 mg/kg, S7243, Selleck Chemicals, China). The grouping of animals involved in this experiment, the location of the animals, and the treatment of the animals were performed in a standardised order to effectively control (28) confounding factors. The sample size calculation was determined by the Power and sample size software. The labelling and randomisation of the mouse numbers was done by a function "Rand ()" in the Excel sheet. The animals were allocated, housed and handled by people who were not responsible for analysing the results of the experiments, and the researcher responsible for the experimental manipulation was only aware of the number of mice and not the grouping of the mice. A protocol was prepared before the study without registration. The drugs used in this study were determined from previous studies (28-31). Animal models of asthma were established following the ovalbumin (OVA) method (A5503, Sigma-Aldrich, USA). Briefly, on the first, second, third and fourteenth days, the mice were intraperitoneally injected with 200 µL OVA intraperitoneal sensitizer [50 µg OVA + 0.8 mg $AL(OH)_3$], then on the twenty-first to twenty-fifth day, the mice were placed in a homemade closed container, and ultrasound aerosol inhalation stimulation was performed with 1% concentration of ovalbumin for thirty minutes each time, during which the mice were intraperitoneally injected with drugs every day one hour before provocation. After twenty-four hours, the mouse arterial blood was collected using the eyeball blood collection method. Subsequently, the mice were euthanized. Finally, we fixed a portion of the lung tissue in 4% formaldehyde, and stored the rest at −80 ℃ for further experiments. It was worth stating that in the course of the experiment, it may lead to the mice's mobility, serious infection, severe pain and other adverse consequences, anaesthesia, analgesia and other care methods would be adopted, and observation would be made twice a day, if the symptoms were still not relieved after the care, the animals would be humanely euthanised, and euthanasia would be carried out by the way of inhalation of excessive carbon dioxide.

Hematoxylin eosin (H&E) staining

The 4% neutral buffered formalin-soaked lung tissues were subjected to gradient alcohol dehydration, paraffin embedded, and then cut into 4 µm thin sections. Next, the sections were incubated with hematoxylin staining solution (C0105, Beyotime, China) for fifteen minutes. Then, the tissues were washed with pure water and stained with eosin for one minute. Finally, pathological changes were observed and histologically analyzed by two unbiased observers using a 200× light microscope (Leica, Germany).

Enzyme linked immunosorbent assay (ELISA)

Mouse IL-4 ELISA kit (KE10010, Proteintech, China), mouse IL-5 ELISA kit (KE10018, Proteintech, China) and mouse IL-13 ELISA kit (KE10021, Proteintech, China) were used to detect the expression of these factors in mouse serum. Measurements were performed according to the manufacturer's instructions. In short, serum samples from each group were added to the wells, reacted with horseradish peroxidase (HRP)-labelled detection antibody, and the plates were incubated at 37 ℃ for one hundred and twenty minutes. The liquid was removed and the plates were washed. Substrates A and B were added, and the plates were incubated in the dark at 37 ℃ for fifteen minutes. A total of 100 µL of termination solution was added to each well and the optical density (OD) value was measured at 450 nm within five minutes.

Detection of ferric ion

Lung tissue was mixed and homogenized at a ratio of 1:9 using total iron detection buffer. Centrifuge at 12,000 revolution per minute (rpm) at 4 ℃ for fifteen minutes, and the supernatant was taken as the sample to be tested. Protein concentrations were measured with the Bicinchoninic Acid Assay (BCA) method (P0009, Beyotime, China). The sample dilutions to be tested and the serial

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dilutions were incubated at 37 ℃ for fifteen minutes in the corresponding system according to the instructions of the total iron detection kit (G4301, Servicebio, China). A special probe was precisely added to the measuring hole. This was followed by a constant incubation at 37 ℃ for forty minutes protected from light. Centrifugation was performed at 12,000 rpm for five minutes. The supernatant 200 μL was placed in a microplate and the absorbance at 593 nm was detected by a microplate reader. Finally, based on the absorbance measurement, the corresponding iron content was calculated.

Detection of superoxide dismutase (SOD)

An appropriate amount of tissue samples was taken and homogenized under ice bath conditions at the ratio of 100 μL of SOD sample preparation solution per 10 mg of tissue. Centrifuge at 4 ℃ at 12,000 rpm for five minutes, and the supernatant was taken as the sample to be tested. Protein concentrations were measured with the BCA method. Sample wells and various blank control wells were set up using 96-well plates. According to the instructions of the SOD test box (S0131, Beyotime, China), add the sample to be tested and other solutions in turn. Mix well and incubate at 37 ℃ for thirty minutes. The absorbance was determined with a microplate reader at 450 nm. Finally, the total SOD activity content was obtained according to the recommended formula.

Detection of malondialdehyde (MDA)

Lung tissue was mixed in a ratio of 1:9 using normal saline and homogenized on ice. The homogenates were then centrifuged at 12,000 rpm for ten minutes at 4 ℃. Protein concentrations were measured with the BCA method. The homogenate 100 μL was taken and reacted under the same conditions as the standard curve, zeroed in a blank tube, and heated in a water bath at 100 ℃ for fifteen minutes. After cooling to room temperature, centrifuge at 3,000 rpm for ten minutes. The supernatant 200 μL was added to a 96-well plate, and the absorbance was measured at 532 nm with a microplate reader, and the MDA content was obtained according to the formula recommended by the manufacturer's instructions (S0101, Beyotime, China).

Dihydroethidium (DHE) staining

The frozen sections (5 μm) were fixed with 4% paraformaldehyde and incubated with 0.5% Triton X-100 (P0096, Beyotime, China) to enhance cell membrane permeability. Then, incubate with DHE staining solution (S0063, Beyotime, China) for twenty minutes in the dark and observe the fluorescence under a confocal microscope (Olympus, Japan). Fluorescence expression was measured using ImageJ 1.52p software (USA).

Immunohistochemical (IHC) staining

We embedded lung tissues in paraffin, cut the tissues into 4 um thin slices and subsequently deparaffinised these tissues with xylene. Afterwards, these tissues were boiled in 0.01 M sodium citrate buffer (pH 6.0) for ten minutes for antigen repair. Subsequently, the sections were cooled and incubated with SIRT1; Nrf2; HO-1 antibody (1:200, rabbit) at 4 ℃ overnight. After staining with anti-mouse/rabbit immunohistochemistry detection kit (PK10006, Proteintech, China), the samples were restained with hematoxylin and images were captured with a fluorescence microscope (Olympus, Japan).

Western blotting (WB)

Total protein was extracted with RIPA buffer (P0013, Beyotime, China). Next, the concentration of these samples was determined by the BCA method. Then, these proteins were separated by 10% SDS-PAGE gel (P0917, Beyotime, China). After that, these proteins were transferred to polyvinylidene fluoride (PVDF) membrane (ISEQ00005, Millipore, USA). These membranes were closed with 5% skimmed milk for two hours at room temperature and incubated with primary antibodies at 4 ℃ overnight. Primary antibodies used in this study were SIRT1 (WL00599, Wanleibio, China), Nrf2 (WL02135, Wanleibio, China), HO-1 (WL02400, Wanleibio, China) and β-Tubulin (WL01931, Wanleibio, China). The next day, these membranes were washed with PBST and incubated with secondary antibody goat anti-rabbit IgG (ab150077, Abcam, United States) and secondary antibody goat anti-mouse IgG (ab150113, Abcam, USA) for two hours. Finally, bands were developed using enhanced chemiluminescence (ECL) substrate (WBULP, Millipore, USA).

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from lung tissues using TRIzol reagent (G3013, Servicebio, China) following the

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manufacturer's instructions. Then, the extracted RNA was reverse-transcribed into cDNA using the ABScript Neo RT Master Mix for qPCR with gDNA Remover (RK20433, ABclonal, China). RT-qPCR was performed using an ABclonal 2X Universal SYBR Green Fast qPCR Mix (RK21203, ABclonal, China) on qPCR detection equipment (CG-05, Heal Force, China). The PCR thermocycling parameters were 95 ℃ for thirty seconds, 95 ℃ for fifteen seconds, and 60 ℃ for thirty seconds to collect fluorescence signals for forty cycles, and the results were used to calculate the relative expression of mRNA by the $2^{-\Delta\Delta CT}$ method. Amplification quantification primers are shown in *Table 1*.

Table 1 The sequences of primers for qPCR are presented

Name		Primers (5'-3')
SIRT1	Forward	TAATGTGAGGAGTCAGCACC
	Reverse	GCCTGTTTGGACATTACCAC
Nrf2	Forward	CAGTCCCAGCAGAGTGAT
	Reverse	GAAACCTCCTTCCAAAAC
HO-1	Forward	CCCTGGAAGAGGAGATAG
	Reverse	GTGGAGACGCTTTACATAG
GAPDH	Forward	GGTTGTCTCCTGCGACTTCA
	Reverse	TGGTCCAGGGTTTCTTACTCC

qPCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

All data in the study were analyzed using GraphPad Prism 8.0 (GraphPad Software, USA). The experiment for this study was repeated three times. Data were expressed as mean ± standard deviation (SD). Comparisons between groups were made using Student's *t*-test or one-way analysis of variance (ANOVA) test. Statistical significance was defined as a 95% confidence level (P<0.05).

Results

Quercetin alleviates in vivo inflammatory responses in asthmatic mice

As shown in *Figure 1A-1E*, histological examination of

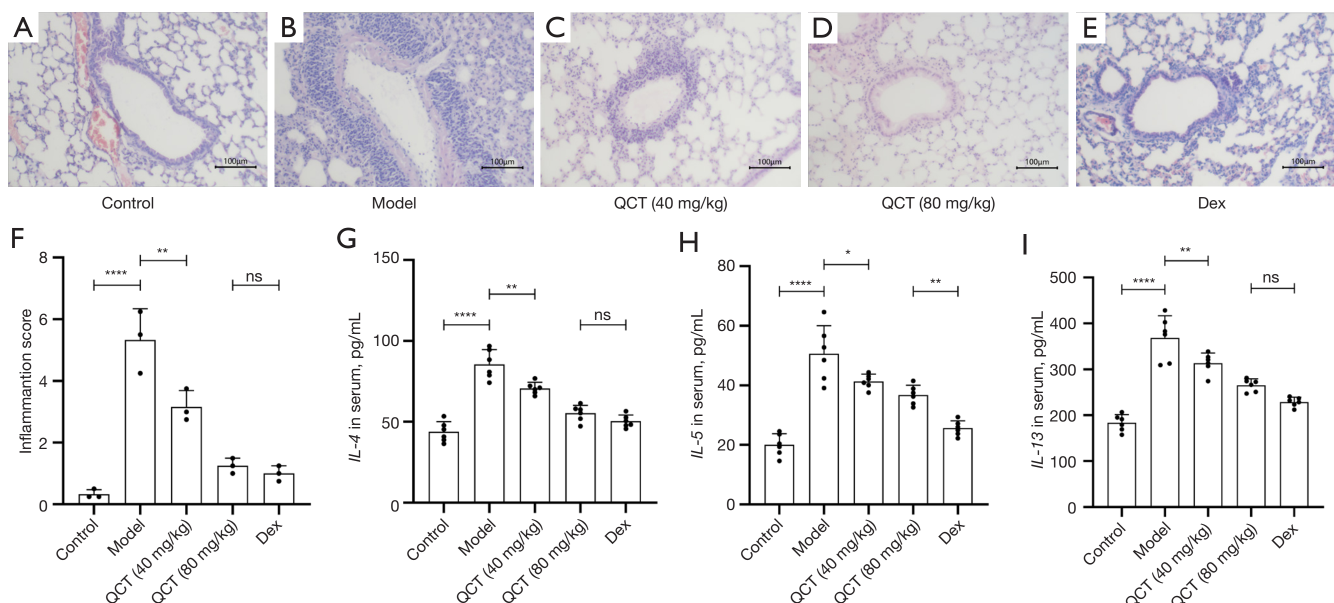

Figure 1 Effect of QCT on OVA-induced asthma inflammatory response. (A-E) Representative H&E-stained lung sections of mice, 200×. (F) Inflammation score of H&E-stained lung sections to estimate perivascular and peribronchiolar inflammation (n=3). (G-I) The IL-4, IL-5 and IL-13 levels in serum of each mice group measured by ELISA (n=6). *, P<0.05; **, P<0.01; ****, P<0.0001; ns, P>0.05. QCT, quercetin; Dex, dexamethasone; OVA, ovalbumin; H&E, hematoxylin eosin; IL-4, interleukin-4; IL-5, interleukin-5; IL-13, interleukin-13; ELISA, enzyme linked immunosorbent assay.

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the lungs showed that peribronchial and perivascular inflammation predominated in mice sensitised and attacked by OVA, whereas no such changes were detected in the control group. In order to observe the therapeutic effect of the drugs on asthmatic mice, we performed a semi-quantitative analysis of the pathological findings (*Figure 1F*) according to the H&E staining scoring criteria. The H&E staining scoring criteria were shown in Multimedia Appendix 1. Treatment of asthmatic mice with QCT at concentrations of 40 mg/kg and 80 mg/kg showed a trend of reduction of peribronchiolar and perivascular inflammation in the mice in a concentration-dependent manner. Pathological results of the high-dose QCT-treated group and the Dex-treated group showed no statistical difference between the two. In addition, we collected serum from mice in each group and performed ELISA, which showed that IL-4, IL-5, and IL-13 were significantly elevated in asthmatic mice compared with the control group, and QCT 80 mg/kg reduced the release of inflammatory factors, which was close to the effect of Dex (*Figure 1G-1I*).

Quercetin attenuates albumin-induced oxidative stress in vivo

In order to investigate the specific mechanism of asthma alleviation by QCT, we examined the MDA, SOD, and iron content in the lung tissues of asthmatic mice treated with QCT. As shown in *Figure 2A-2C*, there was significant oxidative stress in the OVA-induced asthma model compared with the control group, and the abnormally elevated iron content also drew our attention. However, after QCT treatment, the expression of oxidative stress-related indicators SOD and MDA was moving in a favourable direction, and the iron content was reduced. Our experiments showed that QCT at a concentration of 80 mg/kg seems to exert a better effect on the asthma model compared to 40 mg/kg. In order to further verify our idea, we performed frozen sections of lung tissues from control, model, low-dose QCT, and high-dose QCT mice, and detected the reactive oxygen species content in the lungs of the mice in each group by immunofluorescence, and the results were in line with our expectation that the high-dose QCT could alleviate the level of oxidative stress in the asthma mice in a certain extent (*Figure 2D,2E*).

Anti-ferroptosis effects of quercetin are associated with activation of the SIRT1/Nrf2/HO-1 signalling pathway

As shown in *Figure 3*, in order to investigate whether QCT exerts the mechanism of inhibiting ferroptosis by regulating the SIRT1/Nrf2/HO-1 signalling pathway, we set up four groups: control group, model group, QCT-treated group, and ferroptosis inhibitor-treated group. The expression of SIRT1, Nrf2, HO-1 was observed at three levels (i.e., pathological; protein; mRNA) using three techniques: IHC, WB, and qPCR. The results showed that the lung tissues of asthmatic mice showed low expression of the target genes compared to the control group, and it is noteworthy that the expression of the three target genes was relatively elevated in Fer-1 and high dose of QCT-treated asthmatic mice.

Discussion

Inhaled corticosteroid is the cornerstone of the treatment of asthma. With the further study of pathophysiology and endotype of asthma, biologics, such as mepolizumab and reslizumab targeting T2 asthma, have been developed for severe asthma that does not respond well to high doses of steroids. However, the efficacy evaluation of biological agents and the conversion of biological agents are still challenging in clinical application (32). Therefore, natural products with good antioxidant, anti-inflammatory and low toxicity have become an important resource for asthma (33). QCT has been extensively studied for its powerful anti-inflammatory and antioxidant properties (34). Study has shown that QCT can reduce eosinophilic recruitment, reduce the levels of IL-4 and IL-5, and inhibit nuclear transcription factor-κβ (NF-κβ) activation, thus playing an anti-inflammatory role (35). In addition, QCT can inhibit mast cell activation, inhibit histamine release from mast cells, relax airway smooth muscle, and alter TH1/TH2 differentiation, thereby reducing allergic airway inflammation and hyperreactivity. QCT is believed with the potential for being a possible application for allergic airway diseases such as asthma (34,36). Our study focuses on the role of QCT in ferroptosis and its possible therapeutic effect on asthma. QCT can reduce the inflammatory response of asthma by inhibiting ferroptosis, and this protective mechanism depends on the SIRT1/Nrf2 pathway (*Figure 4*).

SIRT1, belongs to the class III histone deacetylase

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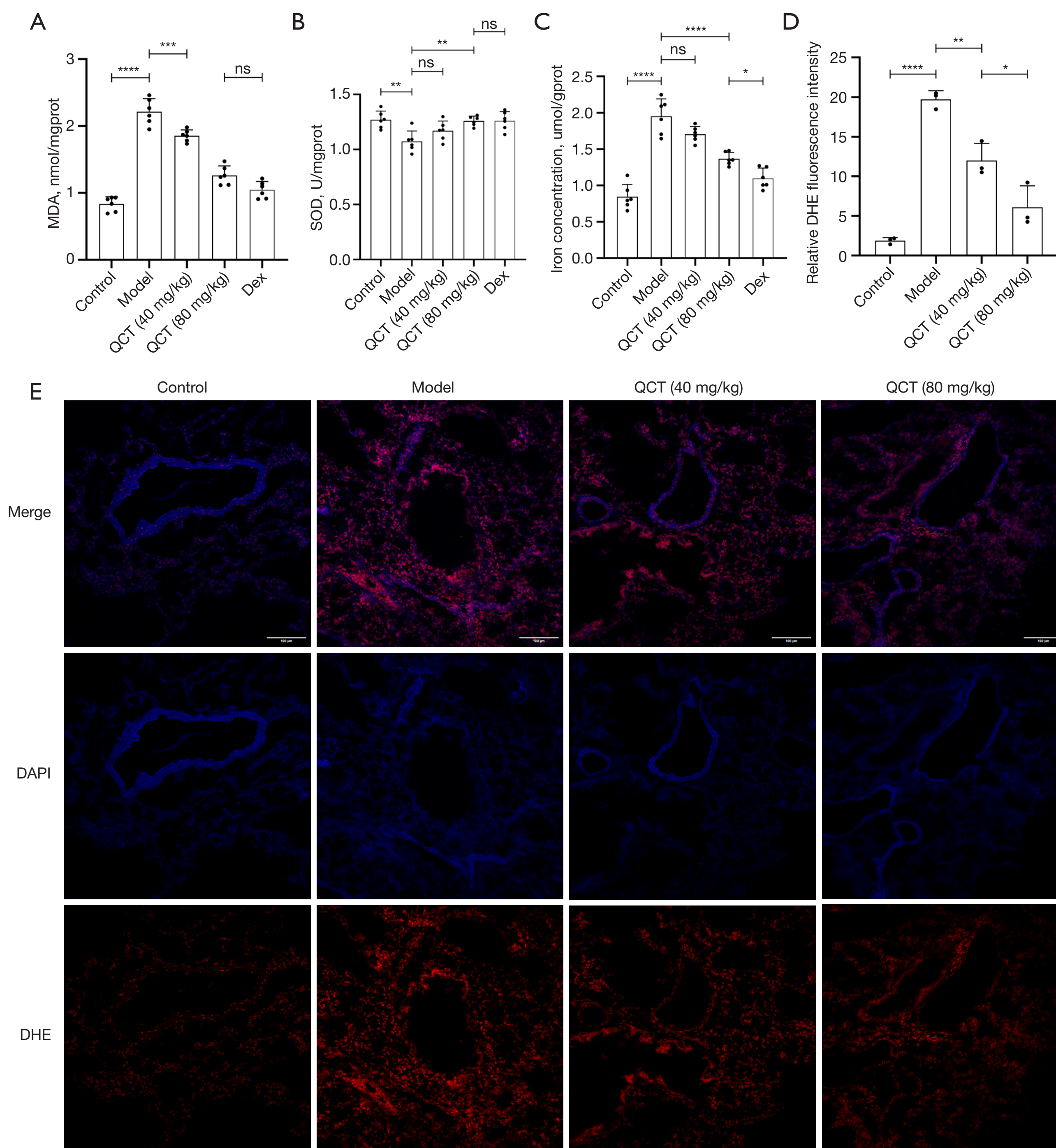

Figure 2 Effect of QCT on oxidative stress in asthma mice. (A-C) Quantitative analysis of iron ions, MDA and SOD concentrations in lung tissues of each group (n=6). (D) Quantification of the integrated intensity of DHE in control, model, QCT (40 mg/kg) and QCT (80 mg/kg) groups. (E) Lung tissue sections were stained with DHE (red; a ROS fluorescent probe) for immunofluorescence. Cell nuclei were stained with DAPI (blue; bar =100 μm). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, P>0.05. QCT, quercetin; Dex, dexamethasone; MDA, malondialdehyde; SOD, superoxide dismutase; DHE, dihydroethidium; ROS, reactive oxygen species; DAPI, 4’,6-diamidino-2-phenylindole.

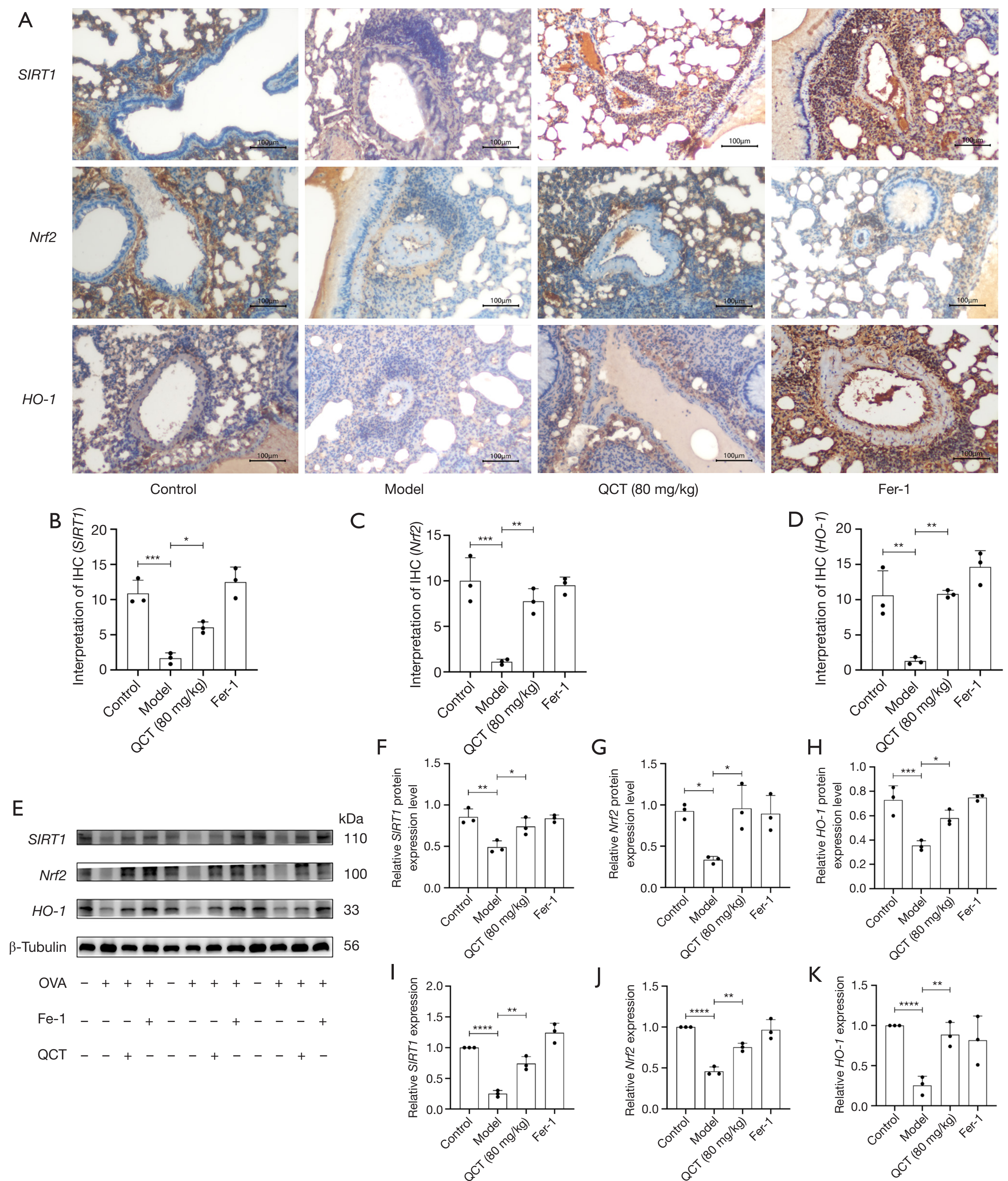

Figure 3 Effect of QCT on SIRT1/Nrf2/HO-1 signaling pathway in asthma mice. (A) IHC analysis for the expression and distribution of SIRT1, Nrf2 and HO-1 in the lung tissues of mice. (B-D) The quantitative results of the expression and distribution of SIRT1, Nrf2 and HO-1 in the mouse lung tissues (n=3). (E) Representative images of WB analysis of SIRT1, Nrf2 and HO-1 in mouse lung tissues. β-Tubulin

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was used as the loading control. (F-H) Densitometric quantification of proteins from the control, model, QCT (80 mg/kg) and Fer-1 groups (n=3). (I-K) qPCR examination for the mRNA expression of SIRT1, Nrf2 and HO-1 genes (n=3). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. QCT, quercetin; SIRT1, silent information regulator 1; Nrf2, nuclear factor erythroid 2-associated factor; HO-1, heme oxygenase-1; WB, Western blotting; Fer-1, ferrostatin-1; qPCR, quantitative real-time polymerase chain reaction.

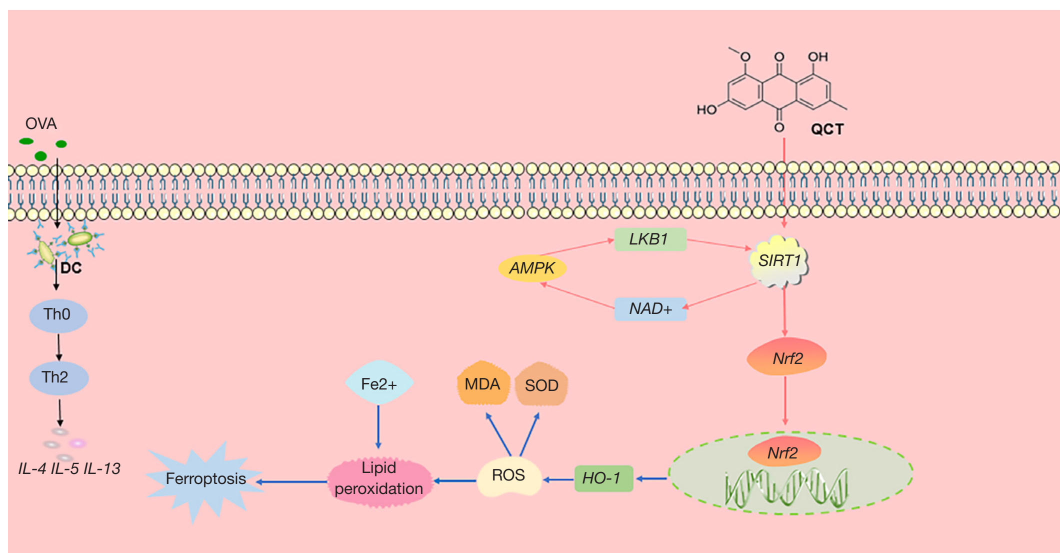

Figure 4 Potential mechanisms of asthma alleviation by QCT. QCT inhibits ferroptosis and attenuates OVA-induced asthma by activating the SIRT1/Nrf2/HO-1 pathway. OVA, ovalbumin; DC, dendritic cells; Th0, T helper 0 cell; Th2, T helper 2 cell; IL-4, interleukin-4; IL-5, interleukin-5; IL-13, interleukin-13; MDA, malondialdehyde; SOD, superoxide dismutase; ROS, reactive oxygen species; SIRT1, silent information regulator 1; Nrf2, nuclear factor erythroid 2-associated factor; HO-1, heme oxygenase-1; QCT, quercetin; AMPK, adenosine 5'-monophosphate (AMP)-activated protein kinase; LKB1, liver kinase B1; NAD+, nicotinamide adenine dinucleotide.

family (37). Only SIRT1 among members of the SIRT family (SIRT1-SIRT7) is reportedly associated with asthma. In OVA-sensitized mice, SIRT1 increased in serum and decreased in lung homogenates, and when SIRT1 expression decreased, the ability of the lungs to resist damage reduced. SIRT1 is a key regulator in the activation of genes related to oxidative stress or inflammation. The expression of SIRT1 is related to adenosine 5'-monophoric acid activated protein kinase (AMPK). Activation of AMPK can enhance SIRT1 activity in a NAD+ dependent manner, thereby inhibiting the downstream inflammatory pathway and ferroptosis pathway (38,39). SIRT1 positively regulates Nrf2 concentration, Wang, Le (40) found that toluene diisocyanate impaired Nrf2 nuclear translocation and activation, potentiating reactive oxygen species (ROS) accumulation, and allopurinol can induce target gene transcription and promote Nrf2 nuclear translocation in a SIRT1-dependent manner to clear ROS (40). AMPK directly phosphorylates Nrf2 at Ser550, promoting nuclear accumulation of Nrf2 (41). Nrf2 is an important transcriptional regulator of anti-ferroptosis genes, and targeting Nrf2 prevents lipid peroxidation and the accumulation of free iron (17). In traumatic brain injury, the SIRT1/Nrf2 pathway mitigates ferroptosis after brain injury by activating glutathione peroxidase 4 (GPX4), a key factor in ferroptosis (42).

The pharmacological effect of QCT shows that it has broad therapeutic potential (43). Many studies have confirmed that QCT can play an anti-oxidative stress and anti-inflammatory role by enhancing SIRT1 deacetylase mechanism. QCT can inhibit ferroptosis of alveolar epithelial cells through SIRT1/Nrf2/Gpx4 signaling pathway, and has therapeutic effect on acute lung injury induced by lipopolysaccharide (44). In osteoarthritis rat model, QCT inhibited endoplasmic reticulum stress through activating SIRT1/AMPK pathway, attenuated oxidative stress-induced apoptosis, and prevented the progression of osteoarthritis (45). In our study, OVA-induced asthma

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increased levels of Th2 cytokine, when treated with QCT or Fer-1 in asthmatic mice, QCT reversed iron overload and lipid peroxidation, and significantly improved peribronchial inflammatory cells and mucus secretion in the airway to relieve asthma, as did Fer-1. In addition, WB analysis showed that QCT treatment resulted in an increase in SIRT1, Nrf2 levels.

An increasing number of studies suggest that a variety of signaling pathways related to ferroptosis play an important role in asthma (14,46), such as iron metabolism (47), lipid metabolism, ROS production (48), amino acid metabolism and other potential signaling pathways. The effect of ferroptosis on asthma is mainly achieved by three types of cells. In asthma, pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) regulate the synthesis of ferritin and affect the storage of iron in cells (49). Iron catalyzes the formation of highly destructive hydroxyl radicals and ROS through Fenton reaction, leading to ferroptosis in airway epithelial cells, and ROS induces ferroptosis, promoting airway epithelial cells to release T2 cytokines (47). In addition, over-infiltrated eosinophils in asthma have been found to produce lipid mediators that contribute to airway inflammation and airway hyper reactivity (AHR) through lipid peroxidation pathways (50). Michaeloudes *et al.* (51) showed that the up-regulation of extracellular matrix protein periostin (POSTN) in smooth muscle increases the sensitivity of cells to ferroptosis. In our study, SIRT1/Nrf2/HO-1 affects iron metabolism, leading to the accumulation of a large number of iron ions and ROS, thereby increasing lipid peroxidation and leading to ferroptosis. In addition to the SIRT1/Nrf2/HO-1 pathway, QCT can also regulate ferroptosis through other signaling pathways to alleviate disease, for example, in gastric cancer, QCT activates the phospho (p)-calcium/calmodulin-dependent protein kinase 2 (Camk2)/p-dynamin-related protein 1 (DRP1) axis via solute carrier family 1 member 5 (SLC1A5) to promote ferroptosis (52). Dihydro-QCT, a derivative of QCT, alleviates acute liver failure by inhibiting ferroptosis through the SIRT1/p53/apoptotic protease activating factor-1 (Aaf-1) axis (53). Although QCT has several pharmacological benefits, its clinical use is restricted due to its poor water solubility, substantial first-pass metabolism, and consequent low bioavailability (54). In some clinical studies, such as metastatic prostate cancer, QCT can partially restore the development of resistance to the first-line chemotherapy drug docetaxel and prolong the overall survival of patients. And QCT has been utilized as a nutraceutical in the United States (34).

There have been many studies on QCT's use in asthma, but to our knowledge, this is the first study to verify that QCT can inhibit ferroptosis through the SIRT1/Nrf2/HO-1 signaling pathway. Our results suggest that QCT is a promising asthma treatment, which is consistent with the conclusions in published studies. However, there are some limitations in this study. First, our study was conducted only in vivo, not in vitro; Second, the small sample size we used for the in vivo experiments does not provide a good indication of the interaction between QCT and the SIRT1/Nrf2-mediated ferroptosis pathway.

Conclusions

In summary, the data from this study suggest that QCT is able to alleviate asthmatic airway inflammation *in vivo* and inhibit ferroptosis through a mechanism of action of the SIRT1/Nrf2/HO-1 signalling pathway. This may provide a new strategy for clinical development of medications to treat asthma.

Acknowledgments

Funding: This study was supported by the Liaoning Provincial Department of Education (No. JYTMS20230090).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-24-193/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-193/dss

Peer Review File: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-193/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-24-193/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were

performed under a project license (No. CMU20231099) granted by institutional ethics board of the Laboratory Animal Welfare and Ethics Committee of China Medical University, in compliance with the Laboratory Animal Welfare and Ethics Committee of China Medical University institutional guidelines for the care and use of animals.

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Cite this article as: Sun B, Cai F, Yu L, An R, Wei B, Li M. Quercetin inhibits ferroptosis through the SIRT1/Nrf2/HO-1 signaling pathway and alleviates asthma disease. Transl Pediatr 2024;13(10):1747-1759. doi: 10.21037/tp-24-193

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