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Original Research Article

Effect of tangeretin on ovalbumin-provoked allergic respiratory asthma in Swiss albino mice

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Abstract

Purpose: To evaluate the effect of tangeretin on ovalbumin (OVA)-induced allergic respiratory asthma in Swiss albino mice.

Methods: Mice were injected intraperitoneally with 50 µg OVA dissolved in water and 1 mg of aluminium hydroxide (alum) on the first day, and on day 12. On days 22, 26 and 30, the mice were exposed to 1 % OVA for 30 min. Mice in the asthma control group received saline via the same route. Tangeretin (50 or 100 mg/kg body weight) was given orally from day 1 to day 30. On the day of sensitization and challenge, tangeretin (50 or 100 mg/kg body weight) was injected 30 min prior to the treatment. Bronchial function, degree of inflammation, broncho-alveolar lavage fluid (BALF), levels of IL-4, IL-5, IL-13 and IFN-γ in BALF, serum total IgE, OVA-specific IgE, IgG1 and IgG2a, as well as histopathology of lungs were determined.

Results: Tangeretin administration led to significant reduction in airway sensitivity, and significant decreases in leukocyte, macrophage, and eosinophil counts ($p < 0.05$). It also brought about significant decreases in the levels of the pro-inflammatory cytokines (IL-4, IL-5 and IL-13) in both BALF and serum ($p < 0.05$). In addition, tangeretin reversed the OVA-induced histopathological anomalies in the lungs.

Conclusion: These results demonstrate that tangeretin exerts protective effects against OVA-induced allergic respiratory asthma in Swiss albino mice, and that the drug can potentially be developed for the management of allergic asthma.

Keywords: Allergic asthma, Inflammation, Cytokines, Ovalbumin, Tangeretin

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INTRODUCTION

Asthma is a severe, long-lasting inflammatory ailment characterised by reversible respiratory airway congestion. Presently, it affects about 300 million persons globally, and its incidence

increases at the rate of 50 % per decade [1]. Respiratory inflammation results in intermittent wheezing, shortness of breath and chest congestion, particularly during the evening period, and in the early hours of the day. These events are normally connected with respiratory

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blockage. Allergic respiratory inflammation is thought to be aggravated by changes in the levels of cytokines [2]. Presently, the precise pathway for the development of asthma is uncertain. Despite the use of extant drugs for asthma (mostly β_2 receptor antagonists), exacerbations of asthmatic symptoms still occur, probably because these agents do not cause alterations in the causative pathological pathways [2].

Several animal models for screening anti-asthmatic effects of new pharmacological interventions have been developed. In the present study, allergy-based animal model was used.

Tangeretin is one of the important polymethoxylated flavones abundantly available in citrus fruits. Studies have shown that tangeretin possesses anti-cancer, antioxidant and anti-inflammatory properties [3-5]. Apart from its antioxidant property, tangeretin has been shown to reduce the development of hepatic cancer *in vivo* as well as *in vitro* [6].

In the present study which was based on the extensive beneficial properties of tangeretin, the anti-inflammatory and immunomodulatory activities of tangeretin were investigated in a mouse model of allergic asthma. The chemical structure of tangeretin is shown in Figure 1.

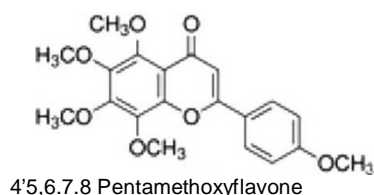


Figure 1: Structure of tangeretin

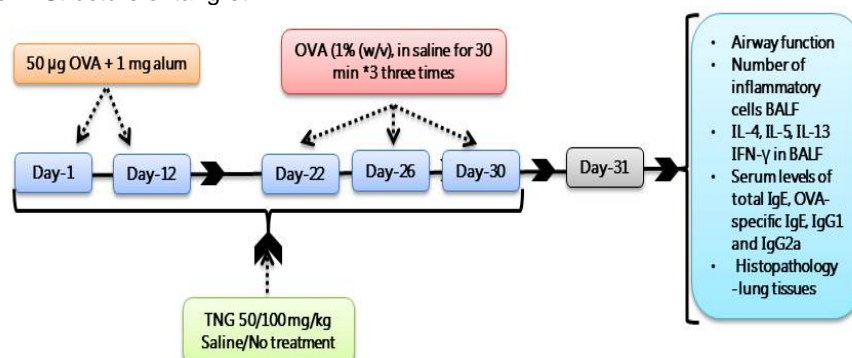


Figure 2: Schematic diagram of the study. Animals were actively sensitized by intraperitoneal injections of 50 µg OVA with 1 mg alum on days 1 and 12. On 22nd, 26th and 30th days, they were exposed to OVA (1 % (w/v) in sterile physiological saline) for 30 min, each day. The negative control group mice were exposed to saline in a similar manner. Twenty-four hours after the final antigen challenge (on 31st day), bronchial responsiveness to Ach was measured; BALF was collected, and lung sections were removed for histopathology. (Tangeretin was administered orally from day 1 to day 30. On the day of sensitization and challenge, tangeretin was given 30 min before the treatment)

EXPERIMENTAL

Animals

In this study, 6-8 week-old male BALB/c mice were used. The experimental animals were housed in cages in a well-ventilated room at with average temperature of 23 ± 2 °C, and 61 ± 4 % moisture. The mice were given normal laboratory feed and water *ad libitum*, and were assigned to four different groups. Group 1 mice were injected with saline (intra peritoneal (i.p.) route) and served as normal control group, and group 2 (OVA) group was treated with OVA (i.p.), and served as bronchial airway asthma control group. Mice in group 3 were treated with OVA (i.p.) and 50 mg/kg tangeretin, while mice in group 4 received OVA (i.p.) and 100 mg/kg tangeretin. The study was approved by the Institutional Ethical Committee (approval no. 2017/2033). It was carried out in accordance with Good Clinical Practice and the Declaration of Helsinki Guidelines and all applicable regulatory requirements [7]. A schematic diagram of the study is shown in Figure 2.

Chemicals and reagents

In this study, the following chemicals and reagents were commercially obtained: OVA and 100 % pure, endotoxin-free tangeretin (Simcere Doyea Pharmaceutical Co., China); bovine serum albumin (BSA, Atlanta Biologicals, Lawrenceville, GA, USA); pancuronium bromide (Xi'an Miracle Biotechnology Co., Ltd.); anti-dinitrophenol (anti-DNP) and IgE mab (Atlanta Biologicals, Lawrenceville, USA); peroxidase-labeled anti-mouse IgE and goat IgG polyclonal antibody (Atlanta Biologicals (Lawrenceville,

USA); rat anti-mouse IgE mab (Santa Cruz, USA); goat anti-mouse IgG1 polyclonal antibody (Santa Cruz, USA); rat anti-mouse IgG2a mab (Invitrogen, Carlsbad, CA, USA); and peroxidase-conjugated streptavidin (Santa Cruz, USA).

Sensitization and challenge

The experimentations in this study were performed according to the procedures described by Balaha *et al* [8]. The experimental animals were sensitized by administering 50 µg OVA (intraperitoneally) and 1 mg of alum on day 1 and day 12. Furthermore, on days 22, 24 and 30, the animals were injected with 1 % w/v solution of OVA in sterilized saline, 3 times each day. Mice in the normal control group were administered saline in the same manner. On day 31, after the last antigenic injection, the sensitivity of the respiratory mucous muscle to acetylcholine (Ach) was measured. Bronchial-alveolar lavage fluid (BALF) and lungs were collected for histopathological studies. Tangeretin was injected for 30 days (from day 1 to day 30). On the sensitization day, tangeretin was administered 30min prior to treatment (Figure 2).

Measurement of airway function

Bronchoconstriction was measured in accordance with the procedure of Balaha *et al* [8]. The experimental animals were anesthetized by intraperitoneal administration of sodium pentobarbitone (60 mg/kg), and Ach was administered through jugular vein cannulation. Furthermore, intravenous pancronium bromide (0.1 mg/kg) was injected to suppress inhalation, and the mice were allowed ventilation from oxygen-filled air at 120 beats/min, with beat size of 0.3 ml/mouse. Respiratory constriction was tested by employing respiratory spasm transducer coupled to tracheal cannula. To determine respiratory tract sensitivity towards Ach, anomalies in bronchial excess volume were determined by employing ascending doses of Ach. Excess respiratory airflow exhibited by Ach was demonstrated as percentage of the maximal excess flow volume (100 %) attained by fastening the respiratory tract cannula. The area under curve was measured from a dose-response curve of Ach (range: 30.25 to 2,000 µg/kg). Each dose was transformed logarithmically, and the area under curve was measured and depicted as arbitrary units.

Tests on BALF

The accumulation of inflammation mediators in BALF was tested to determine respiratory tract

inflammation. The experiments were performed according to the method of Balaha *et al*. [8]. Twenty-four hours after the terminal inhalational dose of the antigenic agent, the experimental animals were sacrificed under intraperitoneal sodium pentobarbitone (100 mg/kg) anaesthesia. The left bronchial tissue was knotted for histopathological studies, and the right airway tract was splashed four times with 0.5 ml of phosphate buffered saline (PBS). The BALF from each mouse was collected in a plastic tube, chilled in icepack, and centrifuged at 150 × g for 10 min at 4 °C. The resultant cell capsules were suspended in an equivalent volume of buffer (1 mL). One part of the cell suspension was then suspended in Türk solution, and the nucleated cells were measured in a Bürker cubicle. A differentiating count was done on a cover slip smear made using a cyto-centrifuge, and then marked with Diff-Quick solvent. According to standard diagnostic selection, 300 cells were measured at an amplification of × 500.

Determination of cytokine concentrations in BALF

The cytokine content of BALF suspension was analysed utilizing Biovision enzyme-linked immunosorbent assay (ELISA) kits for interleukin-4 (IL-4), interferon-γ, interleukin-5 (IL-5) and interleukin-13 (IL-13).

Assay of serum immunoglobulin (Ig) concentrations

After administering the terminal dose on day 30, sera samples were subjected to cryo-centrifugation at -30 °C, and the resultant supernatants were used for further analysis. Total IgE, ovalbumin-specific IgE, ovalbumin-specific IgG1 and ovalbumin-specific IgG2a in the serum were determined by ELISA in line with the method of Balaha *et al* [8]. Total serum IgE was calculated by coating flat-bottomed microtiter plates with rat anti-mouse IgE mab (5 µg/mL). After blocking with 1 % Block-Ace, the reference and serum dilutions were left to stand for one hour and then treated with peroxidase-labeled anti-mouse immunoglobulin E goat IgG polyclonal antibody. Diluted anti-DNP IgE mab was used as a reference.

Serum ovalbumin-specific IgE was determined by coating the microtiter plates with rat anti-mouse IgE antibody mab (5 µg/mL). Blocking was done with 1 % BSA, and serum dilutions were left to stand for about one-hour. Serum ovalbumin-specific IgG1 was assayed by coating the microtiter plates with goat anti-mouse IgG1 polyclonal antibody (2 µg/mL). On coating with 1

% BSA, serum dilutions were kept for 1 h followed by treatment with biotinylated-ovalbumin and peroxidase-conjugated streptavidin. Serum ovalbumin-specific immunoglobulin G2a was determined by layering the microtiter plates with ovalbumin solvent (20 µg/mL). Similarly, serum dilutions were kept for 1 h followed by treatment with peroxidase-conjugated monoclonal rat anti-mouse IgG2a antibody. Sequentially diluted monoclonal anti-OVA IgE, IgG1, and IgG2a were employed as reference standards. Absorbance was measured in a programmed ELISA detector at 492 nm. The limit of detection was 1 ng/mL for aggregate IgE, anti-ovalbumin IgE, and anti-ovalbumin IgG1. For anti-ovalbumin IgG2a, the limit of detection was 3 ng/mL.

Histopathology

The left-side lung tissue sections of the animals were separated and fixed in newly-prepared 10 % formalin for about 24 h. The fixed tissues were thereafter embedded in paraffin, and 5µm sections were cut and stained with Hematoxylin and Eosin (H & E) for histopathological examinations.

Statistical analysis

Data are expressed as mean ± SEM, and were analysed by SPSS Statistics. Correlations were determined by one-way analysis of variance (ANOVA), followed by Tukey's test where appropriate. $P \leq 0.05$ was considered as indicative of statistical significance.

RESULTS

Effect of tangeretin on antigen-induced airway hyper-responsiveness

Inhalation of OVA caused airway hyper-responsiveness to Ach exposure, when compared to normal saline-treated animals. Exposure to tangeretin during OVA inhalation substantially prevented the OVA-provoked airway hyper-responsiveness in a dose-dependent fashion (Figure 3 A and B).

Effect of tangeretin on antigen-provoked airway eosinophilic inflammation

The inhalation of OVA significantly increased total amount of inflammation mediatory cells, including macrophages and eosinophils in BALF. The accrual of inflammation mediatory cells in BALF was significantly prevented by 100 mg/kg tangeretin (Figure 3 C – F). Compared with inflammatory infiltrates, the concentrations of cytokines (Th2) such as IL-4, IL-5 and IL-13 in BALF were substantially increased, while BALF levels of Th1 cytokines and interferon-γ were significantly reduced (Figure 4). In line with its effect on respiratory eosinophilic inflammation, tangeretin administration was associated with reduction in proinflammatory cytokines and Th2 cytokines, and increased Th1 cytokine concentrations in BALF (Figure 3).

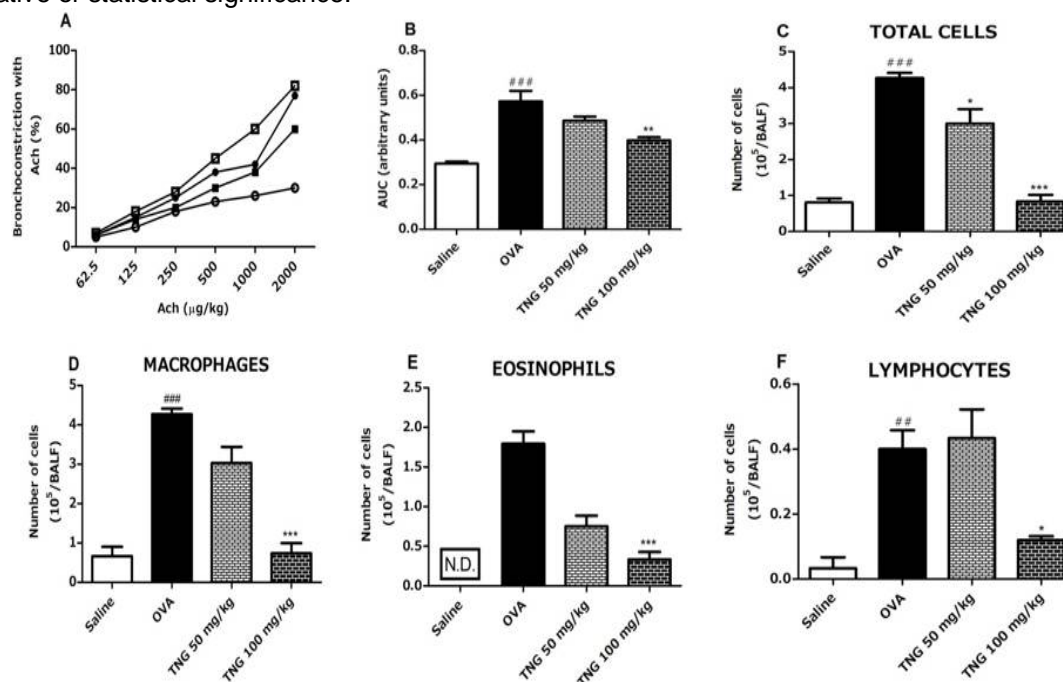


Figure 3: Effect of tangeretin on increased airway responsiveness to Ach and inflammatory infiltrates in BALF in OVA-induced bronchial asthma of BALB/c mice

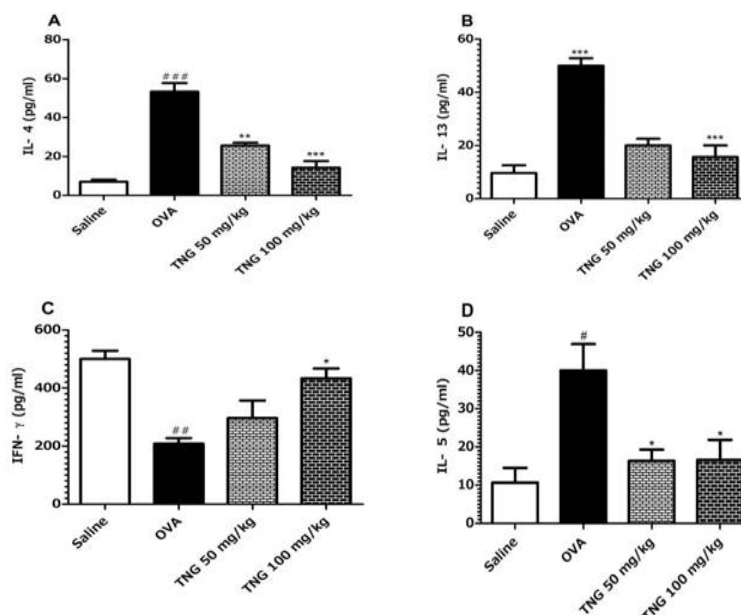


Figure 4: Effect of tangeritin on increased cytokine levels in BALF in OVA-induced bronchial asthma of BALB/c mice 24-h after the final antigen challenge. Values represent mean \pm SEM for six animals in each group. A= IL-4, B = IL-13, C = IFN- γ , D = IL-5. Saline = saline exposed group; OVA = non-treated bronchial asthma group; TNG 50 mg/kg = bronchial asthma group treated with 50mg/kg tangeritin. # $P < 0.05$, ## $p < 0.01$, ### $p < .001$ (vs. saline group); * $p < 0.05$, ** $p < 0.01$, *** $p < .001$ (vs. OVA group)

Effect of tangeretin on serum immunoglobulins

Parenteral and local ovalbumin insult markedly raised the levels of serum total IgE, ovalbumin-specific IgE, IgG1 and IgG2a. Pre-treatment with tangeretin significantly reversed the increases in concentrations of total IgE, ovalbumin-specific IgE and IgG1 in a dose-dependent fashion. On the other hand, tangeretin significantly increased the levels of ovalbumin-specific IgG2a at a dose of 100 mg/kg (Figure 5).

Effect of tangeretin on histopathological abnormalities

Figure 6 shows representative photomicrographs of lung tissue sections of the experimental animal groups (H&E). Ovalbumin (OVA) injection resulted in perivascular and peribronchial cellular infiltration, raised condensation of respiratory tracheal muscle, epithelial hyperplasia, and flaked epithelial lining. However, injection of tangeretin at a dose of 100 mg/kg substantially ameliorated the histopathological anomalies caused by OVA (Figure 6).

DISCUSSION

In this study, the immunomodulatory and anti-inflammatory activities of tangeretin were determined in a mouse model of bronchial asthma in relation to bronchial function, antigen-prompted, inflammation-mediated fluid secretion

in the bronchial airways, local cytokine (Th1/Th2) secretion, serum immunoglobulin contents, and lung histopathology. Several animal models have been developed for studying new pharmacological interventions for allergic asthma. In the present study, experimental mice model was chosen. This model can simulate all the characteristic traits of bronchial inflammation in humans [9].

In the present investigation, tangeretin administration in mice challenged with OVA substantially improved airway hyper-responsiveness, decreased total counts of eosinophils, macrophages and leukocytes, and reduced the concentrations of proinflammatory cytokines in BALF. Serum concentrations of OVA-specific IgE, total IgE and IgG1 were also significantly reduced by tangeretin.

Generally, Th-2 type 2 cytokines (IL-4, IL-5 and IL-13) act as crucial factors in the precipitation of bronchial asthma in humans [10]. Among these cytokines, IL-4 stimulates the separation and propagation of Th2-type T-cells, and promotes the secretion of IgG1 and IgE by B-cells. Stalling of IL-4 by monoclonal antibodies (MABs) reduces IgE concentration and bronchial eosinophilia in hypersensitive mouse [11]. Interleukin-5 can function as a vital contributor in the segregation, maturation and survival of eosinophils, which accounts for its rise in bronchial constriction. Previous studies have demonstrated that eosinophilia-induced inflammation would not

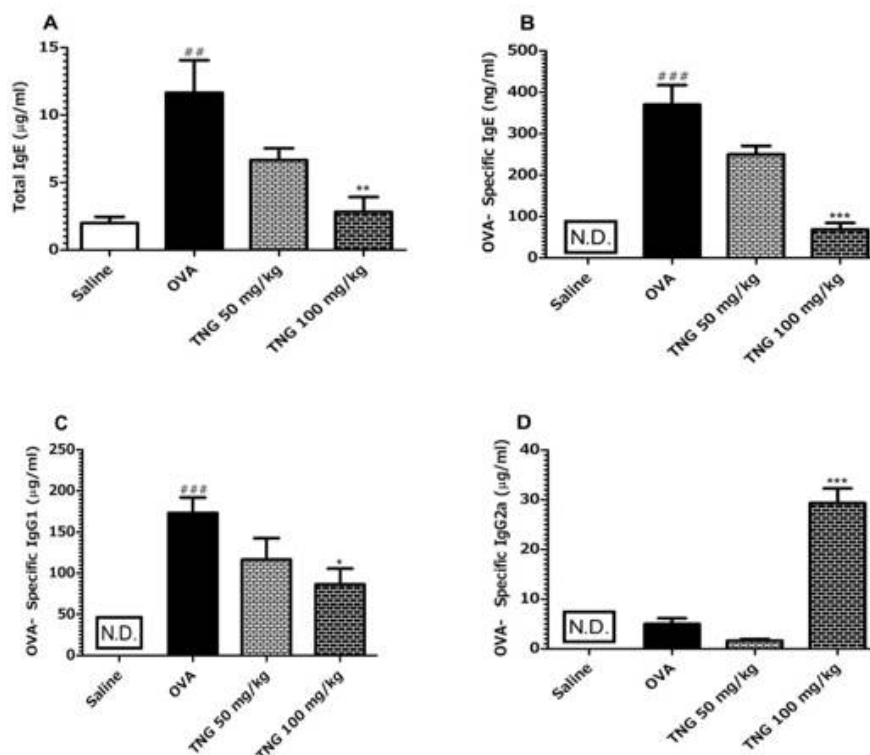


Figure 5: Effect of tangeritin on increased serum immunoglobulin levels in OVA-induced bronchial asthma of BALB/c mice after the final allergen challenge. Values represent mean \pm S.E.M. for six animals in each group. A = Total IgE, B = OVA-specific IgE, C = Ova-specific IgG1, D = OVA-specific IgG2a, Saline = saline-exposed group; OVA = non-treated bronchial asthma group; TNG 50mg/kg = bronchial asthma group treated with 50mg/kg tangeritin; N.D. = not detected. ^{##} $p < 0.01$, ^{###} $p < 0.001$ (vs. Saline group); $p < 0.05$, $p < 0.01$, ^{***} $p < 0.001$ (vs. OVA group)

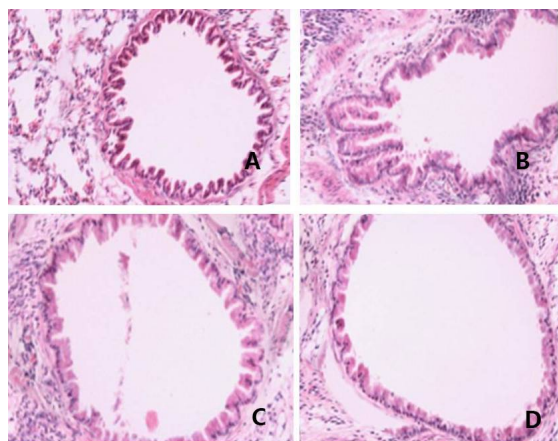


Figure 6: Effect of TNG on histopathological alterations of lung tissues in OVA-induced bronchial asthma in BALB/c mice. A: Saline exposed group, B: Non-treated bronchial asthma group, C: Bronchial asthma group treated with TNG 50 mg/kg, D: Bronchial asthma group treated with TNG 100 mg/kg (H&E staining, $\times 200$)

develop without the activity of interleukin-5 or without its function in the trachea of OVA-treated animals [12].

Interleukin-13 is implicated in airway hyper-responsiveness. It induces IgE isotype switch-over in B-cells and mucus secretion in the

bronchial airways. It has been shown that IL-13 knockout mouse failed to develop airway hyper-responsiveness from allergen exposure even after developing severe bronchial inflammatory reaction [13]. In contrast to the Th2 cytokines, interferon- γ secreted by Th1 cells can increase secretion of IgG2a, IgE and antigen-provoked eosinophils [14]. Consequently, replenishment of local cytokine homeostasis (balance in Th1/Th2 ratio) is an appropriate strategy for discovering new anti-asthma drugs.

In this study, tangeretin (4',5,6,7,8-pentamethoxyflavone) was tested as an intervention for asthma. Studies have revealed that tangeretin possesses potential anti-inflammatory, anti-proliferative and chemopreventive properties [15]. In addition, previous studies have also showed that tangeretin reduced inflammation-related cyclooxygenase (COX-2) activity in lung epithelial carcinoma cells [15], and provoked G1 cell cycle arrest in breast and colon carcinomas [16]. In view of these beneficial effects of tangeretin, its anti-asthma activity was investigated in the present study. The results showed that exposure of OVA-treated mice to tangeretin inhibited Th2 cytokine secretions (IL-4, IL-5 and IL-13) in a dose-dependant fashion, and

increased the secretion of Th1 cytokine (interferon- γ) in BALF. Increased production of local cytokines (Th1/Th2) is consistent with airway hyper-responsiveness, lowered bronchial inflammation and cellular infiltration, and decreased total IgE, ovalbumin-specific IgE and raised IgG2a [17]. The effect exhibited by tangeretin could be ascribed to its antioxidant and anti-inflammatory properties which have been reported in previous studies [15].

Except for IFN- γ production, all the cytokines were affected by tangeretin. This is in agreement with previous reports [8]. In this study, the animal group sensitized with OVA had lower levels of IFN- γ when compared to the saline group and drug-treated group. In contrast, a previous study reported that the concentration of IFN- γ in the blood of sensitized and challenged guinea pigs was comparatively higher than that of normal controls [18]. This difference in IFN- γ secretion could be due to difference in the experimental animal models used. There exist a diversity in immunization procedure, antigen used, and frequency and level of stimulating antigen in the various animal models of asthma. These differences could influence asthma-like symptoms [18]. Indeed, these conflicting observations were also witnessed in the current study. Nevertheless, since asthma produces diverse manifestations in different models, one should take note of the salient, characteristic features in a particular animal model such that each critical parameter is represented in that experimental model.

The animal model employed in this study is known to exhibit all the characteristic traits of allergic bronchial swelling in human beings. However, the model limits one to examination of bronchial remodelling, particularly sub-epithelial and respiratory airway hypertrophy and hyperplasia. Moreover, the OVA used for inducing asthma might not be an appropriate antigenic agent in humans. Hence, further studies are required on the possible ameliorating activity of tangeretin using a chronic experimental animal model [17].

CONCLUSION

The findings of this study demonstrate the anti-asthmatic effect of tangeretin against OVA-provoked respiratory airway allergic asthma in mice. The ameliorating effect of tangeretin may be mainly due to its effect on cytokine secretion. These results suggest that tangeretin may be a potential agent for treating bronchial airway allergic asthma.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this work

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Liang-Mei Li conceived and designed the study. Wei Liu collected and analysed the data. Liang-Mei Li and Wei Liu wrote the manuscript. The manuscript was read and approved for publication by all the authors.

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