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Research Article

Citrus Jabara Juice Diminishes Allergic Airway Inflammation in an Ovalbumin (OVA)-Induced Murine Asthma Model

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Keywords

- Citrus jabara juice
- Fruit
- Anti-allergy effects
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Abstract

Background: Citrus jabara is a type of citrus fruit originally grown in Kitayama-Mura in Wakayama, Japan, and is reputed to have anti-allergic properties. In this study, we tested whether citrus jabara juice inhibits inflammatory mediators in a mouse allergic asthma model.

Methods: Mice were sensitized and challenged with ovalbumin to induce chronic airway inflammation. Two groups were orally administered citrus jabara juice at a daily dose of 10 mg/ml or 100 mg/ml in sterile water from day -3 to day 28.

Results: Administration of citrus jabara juice significantly reduced the numbers of infiltrating inflammatory cells in bronchoalveolar lavage fluid, as compared to control mice. Histopathological studies of the lung using hematoxylin & eosin and periodic acid-Schiff staining showed that citrus Jabara juice inhibited inflammatory cell infiltration and mucus hypersecretion in ovalbumin-challenged mice. In addition, the juice reduced levels of IL-4 while increasing levels of INF-γ.

Conclusions: These findings provide new insight into the immunopharmacological effects of citrus jabara and suggest its potential in supportive therapies for asthma.

INTRODUCTION

Flavonoids are polyphenolic plant secondary metabolites ubiquitously present in vegetables, fruits and beverages, and possess anti-allergic activities as well as immunomodulatory traits [1-9]. For example, flavonoids inhibit activation of mast cells and basophils, thereby suppressing the release of chemical mediators and the synthesis of Th2 type cytokines such as interleukin (IL)-4 and IL-13 [10,11].

Bronchial asthma is a complex disease of the lung characterized by reversible airway obstruction, allergic airway inflammation, excessive mucus production and airway hyper-responsiveness (AHR) [12]. Citrus jabara is a type of citrus fruit originally produced in Kitayama-Mura in Wakayama, Japan (Figure 1). Anecdotal evidence suggests citrus jabara possesses anti-allergic properties, but there is no scientific evidence at the moment. To address that issue, in the present study, we examined the degree to which citrus jabara juice protects against the development of ovalbumin (OVA)-induced bronchial asthma in Balb/c mice.

METHODS

Animals and experimental protocol

Balb/c mice (6-8 weeks old) were purchased from Japan SLC (Sizuoka, Japan) and housed in separate cages according to the treatment protocol. Food and water were provided ad libitum. All experiments were carried out following the guidelines for the care and use of experimental animals formulated by the Japanese Association for Laboratory Animal Science in 1987.

Figure 2 illustrates the experimental protocol. Mice were sensitized to OVA by immunization on days 0 and 7 through intraperitoneal injection of 100 μg of OVA (Grade V, Sigma Chemical Co. St. Louis, MO, USA) plus 1.6 mg of aluminum hydroxide in 200 μl of 0.9% sterile saline (Otsuka, Tokyo, Japan). As a control, mice received only the aluminum hydroxide in saline. The OVA-sensitized mice were randomized into three groups (n=8 in each). Two groups were orally administered citrus jabara juice (Wakayama, Japan) at a daily dose of 10 mg/ml (Jabara L) or 100 mg/ml (Jabara H) in sterile water (0.3 ml/mouse) from day -3 to day 28. The third group received only the sterile water. The non-sensitized control mice (Sham; n=8) were also treated with

sterile water only. The OVA-sensitized mice were then exposed to an aerosol of OVA (10 mg/ml) in 0.9% saline for 30 min each day on days 25, 26 and 27, whereas the non-sensitized mice were exposed only to saline. All mice were sacrificed 24 h after the last aerosol challenge.

Cell counts in BALF

Bronchoalveolar lavage was performed by instilling 0.5 ml of saline into the lungs through a tracheal cannula and then gently aspirating the fluid. This was repeated three times. After staining with Wright's stain, total cell and eosinophil counts in the BALF were determined.

Peripheral blood cell counts

Peripheral blood cell counts were made using blood samples collected from the orbital artery after the mice were sacrificed on day 28.

Histological evaluation and immunohistochemical staining

After sacrificing the mice under anesthesia by intravenous injection with 30 mg/kg sodium pentobarbital on day 24, the lung tissues were fixed in 10% buffered formalin, embedded in paraffin, cut into 4-mm-thick sections and used for histological evaluation (H&E, PAS) or immunohistochemical staining. For immunohistochemical staining of IL-4, INF-γ and nitrotyrosine, phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 2% normal goat serum was used as the antibody diluent after blocking endogenous peroxidase by addition of 0.3% H_2O_2 in methanol. The sections were incubated overnight at 4°C with human monoclonal antibodies against IL-4, INF-γ or nitrotyrosine (500:1 dilution; Oncogene Science, Cambridge, MA), washed with PBS to remove excess primary antibody, and then incubated with biotinylated horse anti-mouse IgG (200:1 dilution; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Bound antibody was then visualized using the standard protocol for the avidin-biotin-peroxidase complex protocol.

The degree of inflammation evident in the H&E-stained sections was scored as follows: 0, no sign of inflammation; 1, light or dispersed infiltrate in only a few areas of the section; 2, moderate infiltrate surrounding <50% of the vessels and airways; 3, heavy and focused infiltrate surrounding the majority of vessels and airways [13]. Scoring for mucus production in PAS-stained sections was as follows: 0, no sign of mucus or increased numbers of PAS-positive cells in the airways; 1, no mucus in the airways, but a slight increase in the numbers of PAS-positive cells in a few airways; 2, some mucus is detectable in the airways, and ~50% of airway epithelial cells in multiple airways are PAS-positive; 3, several airways are plugged with mucus, and the majority of airway epithelial cells in multiple airways are PAS-positive. Scoring was carried out by two individuals blinded to the experimental protocol.

Statistical analysis

The data are presented as means ± SD. Multiple comparisons among groups were made using one-way ANOVA with post hoc Tukey's tests. Values of $p<0.05$ were considered significant.

RESULTS

Cell counts in BALF

We found that eosinophil counts were significantly higher in bronchoalveolar lavage fluid (BALF) from OVA-challenged mice than from saline-challenged control mice (Figure 3, A vs. B group). Moreover, administration of citrus jabara at a dose of 10 or 100 mg/ml (0.3 ml/day) significantly reduced eosinophil counts in BALF (Figure 3) from the OVA-challenged mice.

Lung histology

To assess the histological effect of citrus jabara juice on allergen-induced airway inflammation, we analyzed lung tissues on day 24. In untreated, OVA-sensitized/challenged mice, numerous mucus-producing cells were observed within the airways, which were not seen in mice exposed only to aerosol saline. There was a substantial reduction in airway infiltration by PAS-positive cells in mice administered in citrus jabara juice at 100 mg/ml (0.3 ml/mouse), but not in those receiving citrus jabara at 10 mg/ml (0.3 ml/mouse) (Figure 4).

Immunohistochemical staining

Numbers of periodic acid-Schiff (PAS)-positive mucus-producing cells within the airways were markedly higher in OVA-challenged mice than in control mice. Semiquantitative histological scores indicative of the degree of inflammation within lung tissue sections from OVA-challenged mice were significantly higher than the scores for corresponding control sections. OVA-induced infiltration of the lung tissue by inflammatory cells was significantly attenuated by 10 mg/ml or 100 mg/ml citras jabara juice, and there was a concomitant reduction in the inflammation scores in PAS-stained sections (Figure 5). Histological analysis

Figure 1 Jabara is a type of orange produced in Kitayama-Mura in Wakayama Prefecture, Japan.

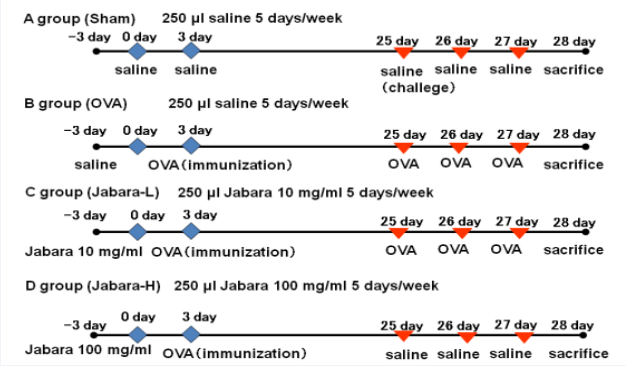

Figure 2 Experimental protocol.

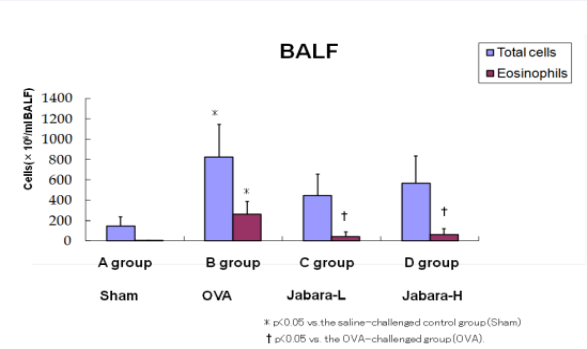

Figure 3 Total cell and eosinophil counts in bronchoalveolar lavage fluid (BALF).

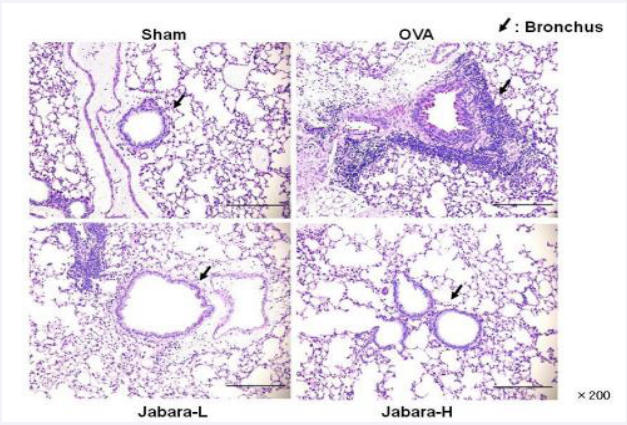

Figure 4 Histological analysis of lung tissue sections stained with PAS.

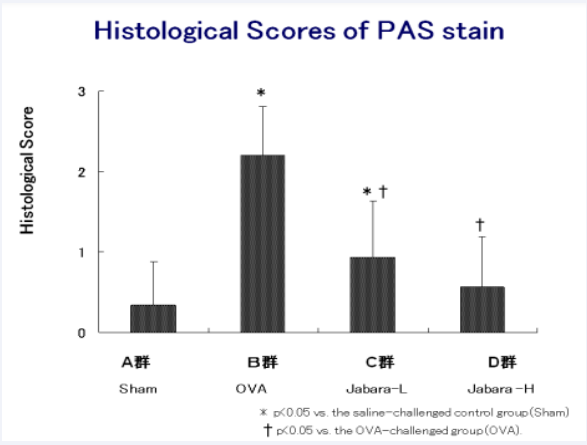

Figure 5 Histological scores for lung tissues stained with PAS.

showed that airway infiltration by IL-4- and nitrotyrosine-positive cells was markedly greater in OVA-challenged than control mice, and that citrus jabara dose-dependently reduced staining for both IL-4 and nitrotyrosine in the lungs (Figure 6). In addition, numbers of INF-γ-positive cells within the airways were markedly lower in the OVA-challenged mice than in the control mice, but citrus jabara juice reversed the effect of OVA on INF-γ expression within the lungs (Figure 7).

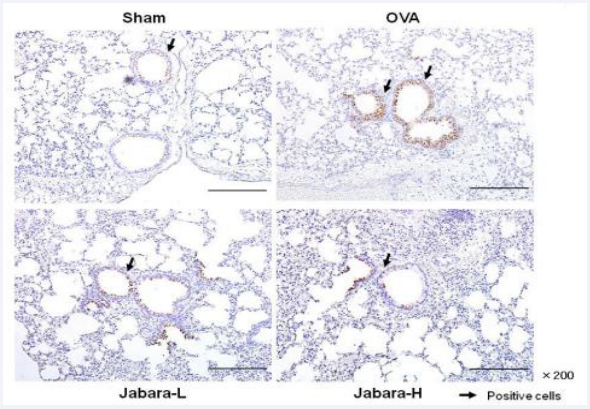

Figure 6 Histological analysis of lung tissues stained for IL-4.

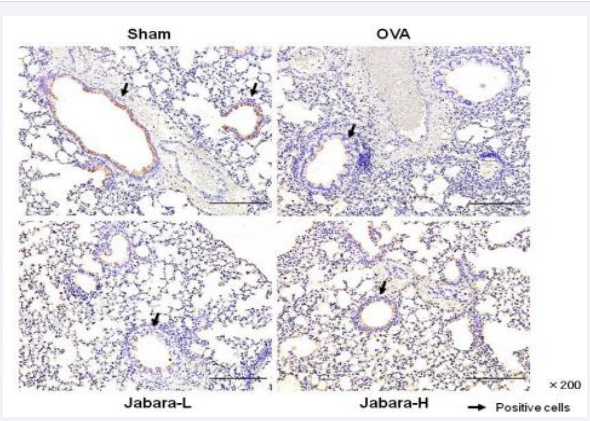

Figure 7 Histological analysis of lung tissues stained for IFN-γ.

DISCUSSION

Bronchial asthma is characterized by acute and chronic airway inflammation and by AHR, the severity of which correlates with the degree of inflammation. In OVA-sensitized/challenged mice, repeated inhalation of antigen leads to increases in the levels of Th2 cytokines, including IL-4 and IL-5, and in the numbers of inflammatory cells (mainly eosinophils) in the BALF [14]. The cytokines expressed are involved in stimulating B cells to produce IgE and in promoting the infiltration of target tissues by mast cells and eosinophils [15]. IgE receptor-mediated activation of mast cells within the airways leads to oxygenation of arachidonic acid by 5-lipoxygenase and generation of leukotrienes (LTs), which promote airway edema, bronchial smooth muscle contraction and proliferation, enhanced mucus secretion and further eosinophil recruitment [16]. Several studies have shown that administration of TH1 cytokines, such as IFN-γ, at the time of sensitization can inhibit the induction of AHR and TH2-driven inflammation [17-19].

In humans, as in our mouse model, induction of IL-4 results in AHR, and blockade of IL-4 can relieve some of the symptoms of asthma [20]. IL-4 is central to the allergic response, promoting isotype switching of B cells to IgE synthesis, directing T cells along the Th2 differentiation pathway, upregulating the expression of vascular cell adhesion molecule-1 (VCAM-1), and controlling

the expression levels of IgE Fcε and the various cytokines and chemokine receptors involved in the allergic cascade. IL-4 drives IgE synthesis by B cells, and IgE levels in serum are dependent upon IL-4 [21]. In addition, IL-5 reportedly regulates the differentiation, recruitment and activation of eosinophils, which contribute to airway inflammation [22].

We previously reported that Narirutin is a flavonoid present in citrus jabara that exerts strong anti-allergic effects in a mouse model of asthma, and that the symptoms of allergic rhinitis are relieved by ingestion of the fruit juice ingredient of citrus jabara juice [23]. In the present study, we observed that citrus jabara significantly reduced allergen-induced airway inflammation in a mouse model of asthma, and that this effect was associated with significantly reduced IL-4 levels and increased IFN-γ levels within the lung tissues. We also found that citrus jabara juice reduced eosinophil counts in BALF, which could account for the reduced IL-4 levels. These studies show that administration of citrus jabara juice significantly diminishes such asthmatic reactions as leukocyte recruitment to the lungs, AHR and the production of Th2 cytokines, and that it attenuates inflammatory cell infiltration of lung tissue and goblet cell hyperplasia within airways. Taken together, these findings suggest citrus jabara may relieve airway inflammation, AHR and goblet cell hyperplasia through actions affecting production of Th1 and Th2 cytokines.

In summary, citrus jabara inhibited allergen-induced airway inflammation in a mouse model of asthma. The mechanism of its anti-asthma effect can be attributed, at least in part, to its ability to reduce IL-4 levels and increase IFN-γ levels. Citrus jabara thus has the potential for use in supportive therapies for bronchial asthma.

AUTHORS' CONTRIBUTIONS

YuO, SM, JK, TSS, ST and KK performed research and analyzed data. YoO designed the study, analyzed data and wrote the manuscript. SS and YT supervised YuO's and SM's work and contributed to human sample collection.

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