Amelioration of asthmatic inflammation by an aqueous extract of Spinacia oleracea Linn

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Received October 6, 2009; Accepted December 18, 2009

DOI: 10.3892/ijmm_00000359

Abstract. Inflammation of the respiratory tract is a crucial process in immune diseases, including asthma, and atopic rhinitis. To establish whether an aqueous extract of Spinacia oleracea Linn (SoL) has a beneficial influence in terms of anti-asthmatic activity, we examined its effects on an ovalbumin-induced asthmatic model. Mice sensitized to ovalbumin were orally administered the SoL extract, and their lungs examined by hematoxylin and eosin staining to determine IL-4/13 cytokine expression. The SoL extract exerted strong anti-asthmatic effects by inducing a decrease in the CD4+ cell number, IL-4/13, and other molecular markers in the lung. Our results collectively indicate that the aqueous SoL extract ameliorates asthmatic symptoms effectively in a mouse ovalbumin-challenge model.

Introduction

Reactive oxygen species (ROS) are pivotal in triggering particular degenerative diseases in cells (1). To expedite the reduction of radicals, antioxidants are required for specific degenerative diseases, such as asthma and chronic obstructive pulmonary disorders (2). ROS can attack random cells. Chronic asthma-related disorders arise, in cases where the radicals attack bronchoalveolar cells (1,2).

Asthma is a classical disorder of the airway hyper-responses, originating from a T-cell imbalance leading to molecular inflammation (3). In bronchial asthma, various mediators induce the infiltration of mast cells, eosinophils and Th2 lymphocytes into lesions with downstream mediators, resulting in classical asthmatic phenotypes, such as mucous over-production, airway hyper-responsiveness, and submucosal thickness (4).

The spinach, Spinacia oleracea Linn, is well-known for its vitamin content. The alkaline vegetable contains carotene (the precursor of vitamin A), calcium, iodine and ferrous ions, and is considered suitable for children and pregnant women (5). Additionally, the vegetable has beneficial effects on anemia and constipation, due to its saponin, dietary fiber, ferric salt, and folic acid content. Folic acid and ferric salts in spinach have a potent anti-proliferation to combat stomach, colon, lung cancer (5,6). A recent study shows folic acid in spinach exerts anti-hyperglycemic effects by removing homocysteine in the blood. Moreover, the vegetable not only inhibits DNA breakage due to oxidative stress, but also reduces damage to ischemic brains (7).

Herein, we demonstrate the potential of Spinacia oleracea Linn in alleviating asthmatic symptoms in vivo. The major finding of this study is that spinach exhibits antioxidant and anti-asthmatic activities in vivo and in vitro, as confirmed with data from antioxidant assays and evaluation IL-4, IL-13, IgE, and CD4+ levels using several molecular biology experiments, including immunohistochemistry.

Materials and methods

Cell culture. For the MTT assay and antioxidant assay, murine melanoma B16 cells and SH-SY5Y neuroblastoma cells were cultured at 37°C under a humidified atmosphere of 5% CO2 and 95% air in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, and 100 μg/ml of penicilllin and streptomycin.

Animals and care. Mice weighing 25±2.5 g (male, 7-week, Samtaco, Osan, Korea) were utilized. Each group was singly housed in cages. Animal care was performed as described previously, with slight modifications (8). All procedures were performed in compliance with the Guiding Principles in...
Animals received tap water and food *ad libitum*, and were maintained in a room under standard laboratory conditions (23°C±1°C and 50±5% humidity) with a 12 h dark/light cycle. Rules for animal experiments, including ethical care, were strictly observed under guidance of the Committee.

Figure 1. Antioxidant and protective effects of an aqueous extract of *Spinacia oleracea* Linn. (A) Reduction of the ferric-tripyridyltriazine (Fe³⁺-TPTZ) complex into the ferrous (Fe²⁺) form was accompanied by an intense blue color. Absorption was recorded at 593 nm. (B) An aqueous extract of SoL enhanced cell proliferation activity under conditions of oxidative stress induced by hydrogen peroxide. Cells were subjected to oxidative stress with or without SoL extracts as indicated. After incubation, cell morphology was assessed using phase contrast microscopy or the MTT assay. No treatment (a and b), 500 μM H₂O₂ (c, d, e and f), and 25 antioxidant/ml of SoL extract (e and f). Scale bar, 100 μm; arrowheads, a crystal form of living cells; SoL, 25 μl/ml of *Spinacia oleracea* Linn extract. (C) Relative cell viability of SoL-treated SH-SY5Y neuroblastoma cells. *Significant differences from control, p<0.05.*
**Determination of FRAP value.** An FRAP assay was adopted to measure the ferric-reducing ability of plasma at a low pH (14). Reduction of the ferric-triprydyltriazine (Fe$^{3+}$-TPTZ) complex into the ferrous (Fe$^{2+}$) form was accompanied by an intense blue color. The FRAP reagent contained 10 mM TPTZ solution in 40 mM HCl and equal volume of 20 mM FeCl$_3$ and 10 times of 0.3 M acetate buffer. FRAP solution was freshly prepared. Samples (10 μl) were added to 200 μl of FRAP solution, and then measured each 100 sec at 0-1000 sec. The absorbance of the reaction mixture was at 595 nm. The FRAP value of samples were calibrated with a ferrous sulfate (Fe$_2$SO$_4$·7H$_2$O) curve.

**Preparation and fractionation of samples.** *Spinacia oleracea* Linn was obtained from a Farm in Chilgok, Korea. The leaf was incubated in an oven at 60°C for 12 h, and the dried leaf extracted with water (1:1; v/v) for 18 h, and centrifuged at 2,000 x g for 10 min to collect supernatant fractions before storage at -70°C. The supernatant fractions obtained were used for various in vitro and in vivo assays (data not shown). The plant was collected between September and November, 2005, and identified by a senior member of staff at the Department of Biology, Kyungpook National University, Daegu, Korea. Voucher specimens of the plant have been deposited in the Enzyme Biotechnology Lab, KNU.

**Cell protection by the SoL extract.** Cell protective effects of the SoL extract were analyzed using the CCK-2 kit (Dojindo, ML) as follows: cells (5x10$^5$ cells/ml) were plated on 96-well plates, and incubated for 24 h in 100 μl of RPMI medium. Various concentrations of extracts were added to cells and incubated for an additional 48 h. Next, 10 μl of MTT solution (5 mg/ml MTT in PBS) was added to each well and incubated for a further 4 h at 37°C. To terminate the reaction, 100 μl of 0.04 M HCl in isopropanol was added with vigorous mixing. Absorbance was determined on a VICTOR3 multilabel counter (Wallac, Turku, Finland) at 564 nm (9).

**IL-4, IL-13, and IgE levels.** The IL-4 and IL-13 concentrations were determined using sandwich ELISA with slight modifications (10,11). IgE was measured with a commercially available kit (AdB Serotec, Oxford, UK).

**Ovalbumin-induced animal model.** Mice underwent ovalbumin (OVA) sensitization and challenge using an earlier protocol with slight modifications (10-13). OVA (100 μg/ml in saline,
sterile filtered) was mixed with an equal volume of 10% (w/v) aluminum potassium phosphate (alum, Sigma), and the pH adjusted to 6.5 with 10 N NaOH.

**Histopathology and immunohistochemistry.** Tissues were embedded in paraffin, cut into 4-6-mm sections, and set overnight on a slide warmer at 37°C. Paraffin was removed by dipping in xylene 3 times for 10 min. Dehydration was additionally performed by dipping serially in 70-100% ethanol for 60 min. After washing, peroxidase bleaching was conducted via 1% BSA/ PBS for 30 min at 4°C. Hematoxylin and eosin (H&E) staining was performed as described previously (11,14). For each mouse, eight randomly-selected airways of the left lung were analyzed. For immunohistochemical analysis, lung tissues were fixed for 24 h in a 10% neutral-buffered formalin solution, and routinely processed. Paraffin sections were placed on Probe-On slides and incubated with methanol containing 3% hydrogen peroxide to inhibit endogenous peroxidase activity. Tissue sections were treated with 10% normal goat serum for 1 h at room temperature to block non-specific binding. Slides were subsequently incubated overnight at 4°C with rabbit anti-IL-4 or anti-IL-13 antibody (SantaCruz Biotechnology; 1:200). Periodic Acid Schiff (PAS) staining was carried out, as described previously (10).

**Eosinophil counts.** Eosinophil has a unique morphology in shape when the asthmatic event cause the number, therefore, we counted the numbers by comparing control and SoL-treated sample, as shown in detail with slight modifications (15).

**Statistical analysis.** Data are expressed as means ± standard deviation. Statistical significance was determined with the Student-Newman-Keuls method for independent means, using the Microsoft Excel program (16). The critical level for significance was set at P<0.05.

**Results and Discussion**

Herein, we examine whether an aqueous extract of *Spinacia oleracea* Linn (SoL) has the potential to improve asthmatic symptoms induced by ovalbumin challenge. Recently, we showed that SoL protects SH-SY5Y neuroblastoma cells against damage induced by hydrogen peroxide (100 μM) or amyloid β-peptide (20 μM) (9). We are convinced that SoL against damage induced by hydrogen peroxide (100 μM) or shown that SoL protects SH-SY5Y neuroblastoma cells grown exponentially 24 h after splitting, and cell viability examined (Fig. 1B and C, and data not shown). As shown in Fig. 1B, hydrogen-peroxide (500 μM) induced cell death. This phenomenon was clearly confirmed with the MTT assay (Fig. 1Bd). Conversely, following the addition of 25 μg/ml of SoL extract, the viable cell number recovered dramatically up to 45% higher than that observed in the presence of 500 μM hydrogen-peroxide onset (Fig. 1Be and Bf).

Next, anti-asthmatic effects were examined in vivo using an ovalbumin-induced mouse model. H&E staining exposed clean tissue in control (Fig. 2a), but several immune positive cells around small parts of the lung in ovalbumin-treated tissue (Fig. 2c). PAS staining revealed similar patterns (Fig. 2d). The total CD4+ cell number was additionally assessed by immunohistochemistry using a CD4 monoclonal antibody. The total CD4+ cell number was significantly reduced to that estimated using H&E and PAS staining (Fig. 3B, and data not shown).

At the same time, the number of bronchoalveolar lavage (BAL) eosinophils was additionally confirmed by counting the cells under a phase contrast microscope (Fig. 3C). The data showed that the number of eosinophils was significantly lower than that of the SoL-treated group, indicating that SoL treatment decreased BAL's eosinophil expression (Fig. 3D). We also examined the expression patterns of matrix metalloproteinases (MMPs) to compare the basal levels of asthmatic molecular markers under anti-asthmatic conditions (data not shown).

To further evaluate whether the molecular markers related to asthma decrease during SoL treatment, IL-4, IL-13 and IgE levels in serum were measured using ELISA (19,20). The IL-4 content was 91.5±20.9 pg/ml in the control group and 285.1±38.9 pg/ml in the ovalbumin-challenged group. This increase in the IL-4 level (up to 2.5 times) may be attributed to the ovalbumin-induced responses of immune cells. Treatment with SoL extracts suppressed the IL-4 level to approximately 45% (Fig. 4A). We clearly observed a 52% decrease in IL-13 expression in the group treated with ovalbumin plus SoL (Fig. 4B). ELISA data, confirmed by IgE expression in cells (Fig. 4C), show that the levels of asthma-related molecular markers are decreased by SoL in an ovalbumin-challenged mouse model. We also found that IL-4, IL-13, moreover IL-17E expression in SoL-treated lung tissues are lower than that in OVA-challenged tissues (data not shown).

In summary, we confirmed that the aqueous SoL extract exerts anti-asthmatic activity in vitro and in vivo by inhibiting asthma-related cytokine activity, as confirmed by H&E staining, IL-4/IL-13 ELISA and IgE excretion activity. The
precise atopic dermatitis mechanisms may be elucidated by manipulating anti-asthmatic activity via amelioration of asthma-related molecular signal(s). Future investigations should focus on the collection of potential anti-asthmatic compound(s) from SoL extracts, and subsequent purification on a large scale.
Acknowledgements

This work was supported by the Technology Development Program for Agriculture and Forestry, Ministry of Food, Agriculture and Forestry, Republic of Korea (S.-H.L.), and in part by a grant from MAF/ARPC through the Grape Research Projects Group on in vitro assays (S.-H.L.).

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