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ORIGINAL PAPER

In vitro and in vivo anti-allergic effects of 'benifuuki' green tea containing *O*-methylated catechin and ginger extract enhancement

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Abstract 'Benifuuki', a tea (*Camellia Sinensis* L.) cultivar in Japan, is rich in anti-allergic epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3″Me). 'Benifuuki' green tea and simultaneous addition of ginger extract remarkably suppressed cytokine (TNF-α and MIP-1α) secretion from mouse bone marrow-derived mast cells after antigen stimulation and, as expected, suppressed delay-type allergy. After drinking 'benifuuki' green tea containing 43.5 mg of EGCG and 8.5 mg of EGCG3″Me, the AUC (area under the drug concentration time curve; min μg/ml) of EGCG was 6.72 ± 2.87 and EGCG3″Me was 8.48 ± 2.54 in healthy human volunteers. Though the dose of EGCG was 5.1 times the dose of EGCG3″Me, the AUC of EGCG3″Me was higher than that of EGCG. A double blind clinical study on subjects with Japanese cedar pollinosis was carried out. At the 11th week after starting the study, in the most severe cedar pollen scattering period, symptoms, i.e., blowing the nose and itching eyes, were significantly relieved in the 'benifuuki' intake group compared with the placebo group, and blowing the nose, itching eyes and nasal symptom score, and at the 11th and 13th weeks, stuffy nose, throat pain and the nasal symptom medication score were significantly relieved in the 'benifuuki' containing ginger extract group compared with the placebo group. These results suggested that over one consecutive month, drinking 'benifuuki' green tea was useful to reduce some of the symptoms from Japanese cedar pollinosis, and did not affect any normal immune response in subjects with seasonal rhinitis, and the ginger extract enhanced the effect of 'benifuuki' green tea.

Keywords Anti-allergic effect · Mast cell · Bioavailability · Seasonal allergic rhinitis · 'Benifuuki' green tea · *O*-methylated catechin

Abbreviations

EGCG	Epigallocatechin-3-*O*-gallate
EGCG3″Me	Epigallocatechin-3-*O*-(3-*O*-methyl) gallate
AUC	Area under the drug concentration time curve

Introduction

Tea (*Camellia sinensis* L.) is consumed all over the world, and in large quantities in Japan and China,

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where it has been used for medicinal purposes for thousands of years. Tea has been found to exhibit various bioregulatory activities, such as anti-carcinogenetic (Kuroda and Hara 1999; Lin et al. 1999; Suganuma et al. 1999; Cao and Cao 1999; Ahmad et al. 2000; Lambert and Yang 2003), anti-metastatic (Isemura et al. 1993; Sazuka et al. 1995, 1997; Maeda-Yamamoto et al. 1999, 2003), anti-oxidative (Okuda et al. 1983; Bors and Saran 1987; Kimura et al. 2002; Hashimoto et al. 2003), anti-hypertensive (Yokozawa et al. 1994), anti-hypercholesterolemic (Murase et al. 2002; Chisaka et al. 1988; Matsumoto et al. 1998), anti-dental caries (Hattori et al. 1990; Sakanaka et al. 1992), anti-bacterial (Fukai et al. 1991), and to contribute to intestinal flora amelioration activity (Okubo et al. 1992). Catechins, a group of polyphenolic compounds, have been shown to be largely responsible for these activities.

Allergy has been defined as a disease of excessive immune activity, and in Japan, the morbidity of allergy is estimated to be about 30%. Many Japanese have misgivings about the use of anti-allergic medicine because of side effects and mounting medical expenses, so there is a demand for the development of physiological-functional foods for allergy prevention. An anti-allergic effect is a functional property in which catechins apparently play a significant role. We have reported that *O*-methylated EGCGs (epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3″Me) and epigallocatechin-3-*O*-(4-*O*-methyl) gallate (EGCG4″Me)) (Sano et al. 1999; Suzuki et al. 2000; Fujimura et al. 2002; Maeda-Yamamoto et al. 2004) and strictinin (Tachibana et al. 2001) had anti-allergic action and that the Japanese tea cultivar 'benifuuki' was rich in EGCG3″Me, which disappeared in black tea (Maeda-Yamamoto et al. 1998, 2001). Oral administration of these methylated catechins significantly and dose-dependently (5–50 mg/kg) inhibited type I allergic (anaphylactic) reactions in mice sensitized with ovalbumin and Freund's incomplete adjuvant. These catechins also strongly inhibited mast cell activation through the prevention of tyrosine phosphorylation (Lyn, Syk and Btk) of cellular protein and histamine/leukotriene release, interleukin-2 secretion after Fcepsilon RI cross-linking (Maeda-Yamamoto et al. 2004). In this paper, to examine the influence on not only immediate allergy but also delay-type allergy, we investigated the in vitro and in vivo effects of 'benifuuki' green tea containing *O*-methylated catechin and a combination of food components working synergistically with 'benifuuki' on inflammatory cytokine production from mast cells after antigen stimulation, symptom relief and safety in subjects with seasonal allergic rhinitis (a double-blind clinical trial), and the blood levels of unconjugated EGCG or EGCG3″Me after the administration of 'benifuuki' green tea to humans.

Materials and methods

Cells, stimulation, cytokine secretion

Bone marrow cells from the femurs of NC/Nga mice were cultured in 4 ng/ml of murine recombinant IL-3 (Peprotec, NJ, USA)-containing RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Life Technologies, CA, USA), 2 mM glutamine and 50 μM 2-mercaptoethanol in humidified 95% air/5% CO_2 at 37 °C. More than 95% pure mast cells were obtained as bone marrow-derived mast cells (BMMC) after 4 weeks of culture. BMMC cells were passively sensitized at a density of 2×10^6 cells/ml with 0.5 μg/ml anti-dinitrophenyl (DNP) mouse monoclonal IgE antibody (Sigma-Aldrich (MO, USA)) at 37 °C overnight. After washing in Tyrode buffer (Ca^{2+}-free; 10 mM HEPES, pH 7.4; Wako Chemical, Osaka, Japan; containing 0.8% NaCl, 0.02% KCl, 0.056% NaH_2PO_4, 0.1% glucose, 0.05% gelatin, and 1 μM $MgCl_2/6H_2O$), the cells were resuspended in Tyrode buffer at a density of 1×10^7 cells/ml, incubated for 20 min with samples at 37 °C, and then stimulated by 300 ng/ml of DNP-HSA (LSL Cosmo Bio, Tokyo, Japan) with 300 μM $CaCl_2$ at 37 °C. About 18 kinds of cytokines secreted into the Tyrode solution during 2–4 h stimulation were measured by Bio-plex protein suspension array system (Bio-Rad, USA).

Overall, 2.5 g of 'benifuuki' green tea powder were extracted at 95 °C for 6 min with 25 ml of distilled water. After centrifugation, the polyphenol (tannin) content of the supernatant was measured by colorimetry using the ferrous-tartrate method (Iwasa and Torii 1962) and 50 μg tannin content added per 1×10^7 cells of BMMC. About 5 g of vegetables (broccoli sprout, radish sprout, red cabbage sprout, rucola sprout, ginger) were added to 5 ml of 50% ethanol and ground well in a mortar. After

centrifugation at 6,000*g* for 15 min, the supernatant as vegetable extract was added to 50 μl of BMMC per 1×10^7 cells.

Blood levels of unconjugated GCG and EGCG3″Me after administration of 'benifuuki' green tea

Six healthy male and female subjects <40 years of age were recruited as participants in this study. Study participants were informed of all procedures and requirements for the study. All participants were required to refrain from ingesting tea or tea products for 3 days before this study. All procedures were in strict compliance with the study protocol, which was approved by the Institutional Review Board of Asahi Soft Drinks Ltd. on Human Research. Written informed consent was obtained from all participants.

The day before the study, all participants were instructed to fast after 9 p.m. except for drinking water. On the study day, all subjects skipped breakfast, came to the clinic and drank 'benifuuki' green tea containing 43.5 mg of EGCG and 8.5 mg of EGCG3″Me within 3 min. Blood samples (5 ml each) were collected before administration and 1, 6, 12, 24 h after 'benifuuki' green tea administration. After each blood collection, Japanese noodles without meat and vegetables were provided to all subjects. Blood samples were centrifuged at 4 °C and 0.4 ml of plasma was added to 0.2 ml of 0.2 M phosphate buffer (pH 6.0) supplemented with 0.5 mM EDTA and 0.2 ml of DW. The mixture was added to 0.8 ml of methylene chloride and mixed well with a vortex. The centrifuged supernatant (aqueous phase) was extracted with 5-fold ethyl acetate. The ethyl acetate fraction was dried by vacuum centrifugation. The dried sample was redissolved in 0.1 M NaH_2PO_4 buffer (pH 2.5) supplemented with 0.1 mM EDTA-acetonitrile (87:13) solution.

About 20 μl of the filtrate after filtration through a membrane filter (DISMIC-13HP-PTFE, pore size 0.45 μm, ADVANTEC, Tokyo, Japan) was injected by an autosampler (SIL-10Avp, Shimadzu, Kyoto, Japan) into HPLC apparatus (Shimadzu class VP HPLC system). HPLC was performed with a Shimadzu LC-10A pump coupled with an electrochemical detector (Coulochem II, ESA, USA) using a reverse-phase Wakopak Navi C18-5 column (150 × 4.6 mm i.d., particle size; 5 μm, Wako Chemical, Tokyo, Japan) with Wakopak Navi C18-5 column (10 × 4.6 mm i.d., particle size; 5 μm, Wako Chemical) as a guard column eluted with the eluent described below at a flow rate of 1 ml/min at 40 °C. HPLC analysis was performed using a linear gradient system with mobile phase A (H_2O-acetonitrile-H_3PO_4, 400:10:1) and mobile phase B (methanol-mobile phase A, 1:2). Linear gradient elution was performed as follows: 100% mobile phase A for 2 min; 20% mobile phase A for 27 min; maintained 20% mobile phase A for 10 min; and return to 100% mobile phase A for 7 min. The eluent was monitored by the Coulochem electrode array system with potential settings at −200 (E1) and 400 mV (E2). Quantification was carried out using the external standard method. Quantification of EGCG and EGCG3″Me was performed after data acquisition using an LC workstation (Class VP system, Shimadzu).

Human clinical trial on seasonal allergy rhinitis

About 18 male and 9 female subjects (>22 years of age) with a stuffy nose, itching eyes, a sore throat or persistent sneezing during cedar pollen season, and with a positive Japanese cedar pollen-specific IgE value without treatment at a medical institution were recruited as participants in this study. A researcher who did not participate directly in the final examination divided participants into three groups, a 'benifuuki' test group (7 men and 2 women, aged 39.1 ± 9.9 years old), a 'benifuuki' + ginger extract group (5 men and 4 women, aged 37.6 ± 10.3 years old) and a 'yabukita' placebo group (5 men and 4 women, aged 41.8 ± 12.3 years old), based on each cedar pollen-specific IgE value of blood. Study participants were informed of all procedures and requirements for the study. All procedures were in strict compliance with study protocol, which was approved by an Institutional Review Board of National Institute of Vegetable and Tea Science on Human Research. Written informed consent was obtained from all participants.

The placebo, 'yabukita' green tea, did not contain EGCG3″Me whereas 'benifuuki' green tea contained 1.49%DB of EGCG3″Me, but the total catechin content of both was approximately 14%. During the test period, all subjects consumed 2 × 1.5 g tea

bags with water every day. The 'benifuuki' + ginger test sample was supplemented with 30 mg of ginger extract per 1.5 g of tea powder and the ginger flavor was hardly noticed. This test started on December 17, 2004, approximately two months before cedar pollen season. Tea drinking started on December 22, 2004 and continued for 86 days (to March 18, 2005), and all tests finished on April 8, 2005. All subjects visited the hospital every 4 weeks for consultation, and blood and urine samples were taken each time for hematological examination, general biochemical examination, histamine content, IgE score, cedar pollen-specific IgE score, total IgG antibody titer, and serum iron content. During the test period, all subjects were required to write an 'allergy diary' which included the frequency of sneezing and nose blowing, a stuffy nose, itching eyes, the extent of eye watering, sore throat pain, difficulties in daily life, and use conditions of the medicine every day in accordance with the method proposed by the Japanese Society of Allergology Allergic Rhinitis Committee. Symptoms were evaluated from 0 (no symptoms) to 4 (severe symptoms present all day). The diary was collected at the end of examination and we calculated the Nose Symptom Score and Medication Score (pattern of taking medicine), and the Symptom Medication Score, the sum of both scores, according to the practical guidelines for the management of allergic rhinitis of the Japan Allergy Foundation.

Statistical analysis

We compared the test groups using the subjective symptoms of allergic rhinitis in the Mann-Whitney *U*-test as the object of analysis of the score frequency every 2 weeks.

Results and discussion

Cytokine secretion

Mast cells play a critical role in the effector phase of IgE-dependent immediate hypersensitivity and allergic diseases (Galli et al. 1999). Cross-linking of high-affinity IgE receptors (FcεRI) with IgE and allergen initiates the activation process, leading to the release of preformed and de novo synthesized vasoactive amines, proteases, leukotrienes, cytokines, and chemokines (Beaven and Metzger 1993; Kinet 1999; Kawakami and Galli 2002). These chemicals and polypeptide agents elicit various allergy-associated pathophysiological changes locally and systemically, for instance, amines, such as histamine and serotonin, enhance vascular permeability, and cytokines, such as TNF-α recruit inflammatory cells to the site of allergen exposure. Production increase by antigen stimulation was observed in IL-5, IL-6, IL-17, GM-CSF, TNF-α, MIP-1α from BMMC after antigen stimulation in 2 h, as shown in Table 1. Inflammatory cytokine TNF-α (tumor necrosis factor), eosinophil-migrating chemokine MIP-1α (macrophage inflammatory protein-1), and IL-6 were produced in particular abundance by antigen stimulation. Therefore, the inhibitory effect of 'benifuuki' green tea extract and the simultaneous addition of various vegetable extracts to 'benifuuki' green tea on cytokine (TNF-α or MIP1-α) production were investigated. As Fig. 1 shows, 'benifuuki' green tea inhibited TNF-α production by 38.9%, but only ginger showed 70.6% inhibition with the vegetable extract alone. Moreover, the inhibitory effect of 'benifuuki' on TNF-α production was enhanced approximately 2 times by adding broccoli sprout or white radish sprout extract, with 93.6% inhibition by administrating ginger and BF together. As mentioned above, MIP-1α production was suppressed 28.7, 55,

Table 1 Cytokine secretion from BMMC after DNP antigen stimulation

Antigen stimulation	IL-5	IL-6	GM-CSF	IL-17	TNF-α	MIP-1α
−	0.6 ± 0.03	58.4 ± 3.8	0.3 ± 0	2.9 ± 0.03	13.0 ± 1.1	31.3 ± 3.8
+	2.3 ± 0.19	1287 ± 225.3	27.4 ± 2.3	7.3 ± 0.07	1400 ± 9.6	90 ± 0.3

Data represent the average of cytokine contents (pg/ml) and SD measured by Bio-plex in BMMC (murine bone marrow-derived mast cells) culture supernatant after DNP-antigen stimulation or no stimulation for 4 h

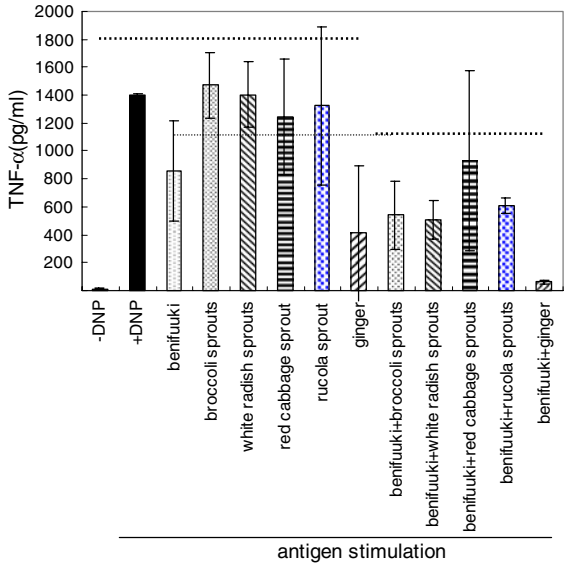

Fig. 1 The effect of 'benifuuki' green tea and vegetable extract on TNF-α production after DNP antigen stimulation of BMMC. 'Benifuuki' green tea extract and vegetable extract were administered at 50 μg (equivalent to tannin content) and 50 μl per 1×10^7 cells of BMMC, respectively

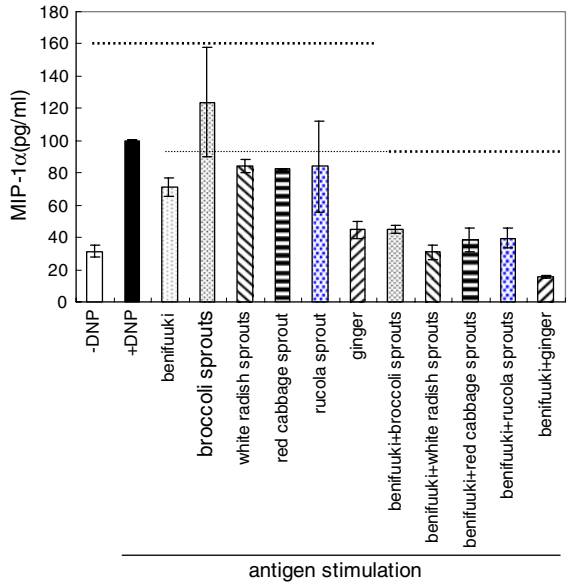

Fig. 2 The effect of 'benifuuki' green tea and vegetable extract on MIP-1α production after DNP antigen stimulation of BMMC. 'Benifuuki' green tea extract and vegetable extract was administered at 50 μg (equivalent to tannin content) and 50 μl per 1×10^7 cells of BMMC, respectively

and 84.2% by 'benifuuki', ginger and 'benifuuki' and ginger combination, respectively, as shown in Fig. 2.

'Benifuuki' green tea or the simultaneous administration of 'benifuuki' green tea and ginger extract strongly inhibited inflammatory cytokine production, such as TNF-α and MIP-1α, after antigen stimulation of BMMC. From these results, 'benifuuki' green tea or the combination of 'benifuuki' and ginger suppressed delay-type allergy by inhibiting inflammatory cytokine production.

Bio-availability

Figure 3 shows the average plasma unconjugated EGCG and EGCG3″Me concentration-time profile after 'benifuuki' green tea administration. After oral administration, plasma EGCG levels increased toward a peak and declined rapidly. On the other hand, the metabolism of EGCG3″Me was slow compared with EGCG. The AUC (area under the drug concentration time curve; min μg/ml) of EGCG was 6.72 ± 2.87 and EGCG3″Me was 8.48 ± 2.54. 'Benifuuki' green tea beverage contained 43.5 mg of EGCG and 8.5 mg of EGCG3″Me. Although the dose of EGCG was 5.1 times the dose of EGCG3″Me, the AUC of EGCG3″Me was higher than that of EGCG. Chow et al. (2001, 2003) reported that the peak of average plasma unconjugated EGCG was approximately 70, 75, 160 and 400 ng/ml at 4 h after oral administration of 200, 400, 600 and 800 mg of EGCG, respectively. From this result, it was suggested that free EGCG3″Me was more easily absorbed by blood in comparison with free EGCG; therefore, EGCG3″Me might be delivered in greater quantity to an inflammation location, with greater affect.

Clinical trial

A double blind clinical study on subjects with Japanese cedar pollinosis was carried out to evaluate the effect and safety of 'benifuuki' green tea, which contains EGCG3″Me and a combination of 'benifuuki' green tea and ginger extract, together with 'yabukita' green tea as a placebo. First, the effect of the simultaneous administration of 'benifuuki' green tea and various vegetable extracts on cytokine inhibition using mast cells was investigated, and the

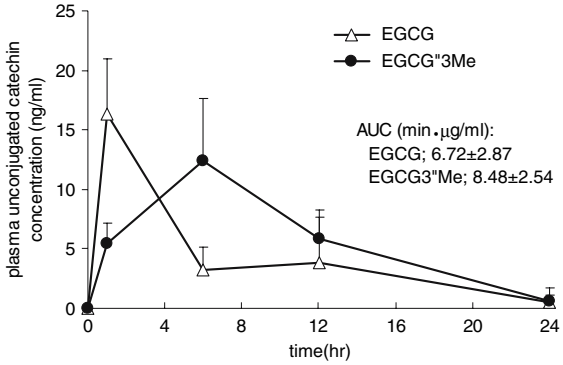

Fig. 3 Plasma unconjugated EGCG and EGCG3″Me concentration versus time profiles after oral administration of 'benifuuki' green tea beverage. Each point represents the average of six subjects, and the cross-vertical bars represent SD of the mean. All subjects drank 'benifuuki' green tea containing 43.5 mg of EGCG and 8.5 mg of EGCG3″Me within 3 min

simultaneous administration of 'benifuuki' green tea and ginger extract remarkably suppressed cytokine production, as described above. The subjects therefore drank 1.5 g of each tea powder, 'benifuuki' green tea, 'benifuuki' green tea containing 30 mg of ginger extract, and 'yabukita' green tea, with water twice a day for 13 weeks. 'Benifuuki' or 'yabukita' green tea contained 44.7 or 0 mg of EGCG3″Me, 176.1 or 202.8 mg of EGCG, 71.4 or 84.6 mg of caffeine and 432 or 425 mg of total catechin per 3 g, respectively. As cedar pollen increased, the symptoms of pollinosis worsened in the order: placebo group > 'benifuuki' group > 'benifuuki' supplemented with ginger group. About 11 weeks after starting the treatment, in the most severe cedar pollen period, the symptoms, i.e., nose blowing and itching eyes, were significantly relieved in the 'benifuuki' group compared with the placebo group ($p < 0.05$), as shown in Fig. 4a and b. In the 11th week after starting the treatment, nose blowing, itching eyes and nasal symptom score, and in the 11th and 13th weeks, a stuffy nose, sore throat and the nasal symptom medication score were significantly relieved in the 'benifuuki' supplemented with ginger extract group compared with the placebo group, as shown in Fig. 4c. Among test groups, there were no changes related to clinical problems in the hematological examination, general biochemical examination, total IgG antibody titer, serum iron content, and interview throughout the intake period.

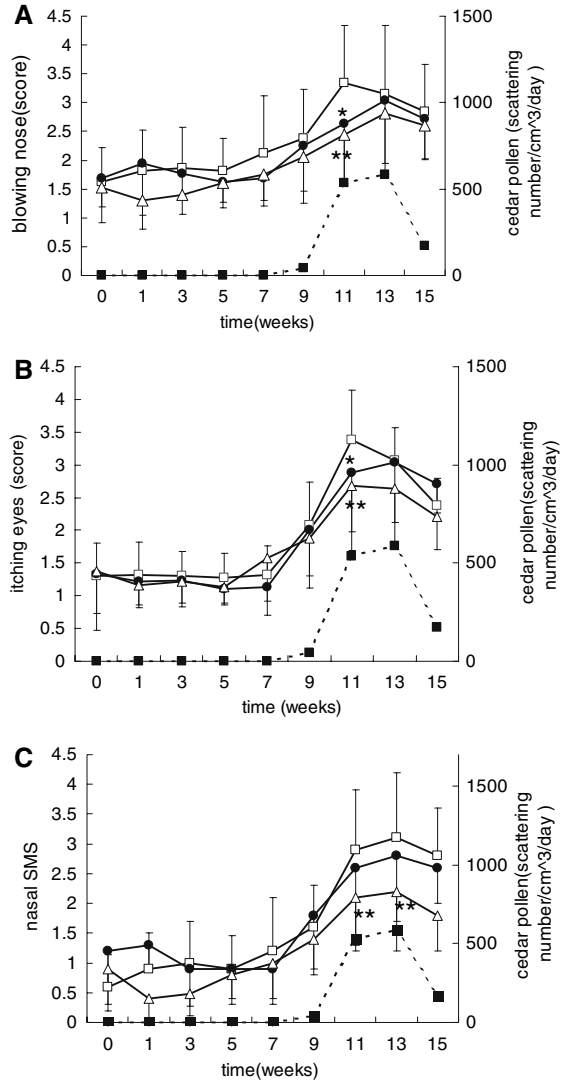

Fig. 4 The effects of 'benifuuki' green tea and additive ginger extract on the symptom score of seasonal allergic rhinitis. All subjects drank 1.5 g of each tea powder, 'benifuuki' green tea, 'benifuuki' green tea containing 30 mg of ginger extract, and 'yabukita' green tea, with water twice a day for 13 weeks. Each point represents the average of nine subjects every 2 weeks and the cross-vertical bars represent SD of the mean. (**a**) blowing nose (0 (0 time)–4 (more than 21 times)), (**b**) itching eyes (0 (no)–4 (severe)), (**c**) nasal symptom medication score. *,**Significantly different from the placebo group (*$p < 0.05$, **$p < 0.01$)

EGCG3″Me, an active factor in 'benifuuki', had an absorption ratio in the body about 6.4 times higher than EGCG, a high quantity of which is contained in general tea varieties such as 'yabukita'.

Ginger is used as a Chinese medicine, and has anti-inflammatory effects (Thomson et al. 2002; Grzanna et al. 2005), antipyretic action, increased salivation, antitussive effect, analgesic effect, antidigestive ulcer, transportation promotion in the intestinal tract, and cardiotonic action. [6]-Gingerol included in ginger is supposed to have anti-inflammatory action (inhibition of prostaglandin E2 production) (Young et al. 2005) so we surmised that the anti-inflammatory action of gingerol had a strong inhibitory effect on inflammatory cytokines and the relief of seasonal allergic rhinitis symptoms by 'benifuuki' supplemented with ginger extract.

These results suggested that more than one consecutive month intake of 'benifuuki' green tea was useful to reduce some of the symptoms of allergic rhinitis, but did not affect normal immune responses in subjects with Japanese cedar pollinosis, and that ginger extract enhanced the effect of 'benifuuki' green tea.

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