Research Note

In Vitro and *In Vivo* Anti-inflammatory Activities of Mixed Fruit and Vegetable Juice

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Abstract In this study, we attempted to develop fruit-based functional juice with anti-inflammatory activity for consumers living under heavy air pollution. At first, four different formulations for functional juice prepared by combining 10 kinds of fruits and other ingredients in various ratios were tested for NAD(P)H: quinoneoxidoreductase 1 (NQO1)-inducing activity in mouse hepatoma Hepa1c1c7 cells. The formulation showing the highest NQO1-inducing activity [named detoxification juice (DJ)] was investigated for its anti-inflammatory activity in a co-culture system of human lung carcinoma A549 cell line and a differentiated human monocytic cell line THP-1. DJ significantly reduced the LPS-induced production of proinflammatory cytokines, such as interleukin-6 (IL-6), IL-1 β , and tumor necrosis factor α (TNF- α). Furthermore, DJ administration suppressed the benzo[a]pyrene-induced elevation of plasma cytokine levels of C57BL/6 mice. However, the protective effect of DJ against inflammation in lung and trachea caused by air pollution needs to be assessed through clinical studies.

Keywords: detoxification juice, anti-inflammation, proinflammatory cytokines, lipopolysaccharide, benzo[a]pyrene

Introduction

Microdust, also known as fine particulate matter (PM), is an air pollutant commonly produced by combustion reaction. According to the particle size, PM can be classified into two types; PM₁₀ ($\leq 10 \mu\text{m}$ in diameter) and PM_{2.5} ($\leq 2.5 \mu\text{m}$ in diameter) (1,2). The air PM contains complex mixtures of polycyclic aromatic hydrocarbons (PAHs) that are considered to be one of the primary causes of health problems (3). Among PAHs, benzo[a]pyrene (B[a]P) is known to be a procarcinogen. B[a]P is metabolized into highly potent carcinogens such as benzo[a]pyrenediol-epoxide (BPDE) by cytochrome P450 (CYP) enzymes (4). These B[a]P metabolites can bind to DNA and form mutagenic DNA adducts, leading to tumor initiation. In addition, they can modulate the pulmonary inflammatory system (5,6). Particularly, BPDE promotes the production of inflammatory cytokines such as tumor necrosis factor α (TNF α), interleukin-6 (IL-6), and IL-1 β and stimulates MAPK/NF κ B-mediated pathway in human lung fibroblasts (6-8).

Intracellular detoxification system plays a critical role in metabolizing and detoxifying toxic compounds including carcinogens in polluted air. Phase I enzymes such as CYP1A1 and CYP1A2 metabolize toxins or procarcinogens to reactive electrophiles or ultimate carcinogens.

In contrast, nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-mediated phase II detoxifying enzymes including NAD(P)H: quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), and glutathione reductase metabolize the electrophiles and thereby protect cells from proinflammatory and detrimental oxidizing effects (9). Multiple studies have reported that functional food substances can modulate the Nrf2-antioxidant responsive element signaling pathway (10-13). The phytochemicals present in fruits and vegetables have been reported to exert beneficial effects under oxidative stress and on exposure to PAHs. The phenolic compounds in fruits and vegetables, including isomers of flavones, isoflavones, flavonols, catechins, tocopherols, and phenolic acids, influence important cellular signaling processes, such as NF- κ B activation, chromatin structure, glutathione biosynthesis, nuclear redox factor (Nrf2) activation, and reactive oxygen species (ROS) scavenging, and as a consequence, regulate inflammatory genes in macrophages and lung epithelial cells (14).

In the present study, we developed mixed fruit and vegetable juice [named as detoxification juice (DJ)] using ingredients with anti-inflammatory and/or detoxifying activity and highly acceptable sensory characteristics and examined whether the juice exhibits detoxifying capability by inducing phase II detoxification enzymes and reducing inflammatory cytokine production *in vitro* as well as *in vivo*.

Materials and Methods

Reagents and chemicals 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and lipopolysaccharides (LPS) from *Escherichia coli* 055:B5 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All cell culture reagents and fetal bovine serum (FBS) were obtained from HyClone Laboratories (Logan, UT, USA). Mouse hepatoma Hepa1c1c7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). A549 (human alveolar adenocarcinoma epithelial cell line) and THP-1 cells (human acute monocytic leukemia cell line) were from the Korean Cell Line Bank (Seoul, Korea).

Preparation of detox juice To formulate detox juice, various fruits and vegetables obtained from Korea, USA, or Vietnam (Table 1) were mixed considering their detoxifying capability reported in the literature as well as sensory preference (15). Korean black raspberry, MBA, and Campbell grapes, jujube fruit, mulberry, pear, balloon flower root, grape seed, and ginkgo nut originated from Korea; blueberry, cranberry, and cinnamon were from USA or Vietnam. The mixture was ground with balloon flower root extract (data not shown) at 60°C for 1 h, passed through a 60-mesh sieve, and sterilized at 70°C for 30 min.

Cell culture Hepa1c1c7 cells were maintained in minimum essential medium with alpha modification supplemented with 10% FBS and 1% penicillin-streptomycin (pen-strep). The A549 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS and 1% pen-strep. THP-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% pen-strep. For *in vitro* analysis, various cells were detached using 0.05% Trypsin-EDTA (Gibco BRL, Gaithersburg, MD, USA), harvested, plated onto prepared culture dishes, and maintained in appropriate culture media for a designated period in an incubator (37°C, 5% CO₂, humidified).

Co-culture system To determine the detoxifying and anti-inflammatory activities of DJ samples, A549 cells were plated in a six-well plate (Thermo Scientific Nunc, Rochester, NY, USA), and THP-1 cells were cultured on insert membranes (Corning Inc., Corning, NY, USA). Before combined in co-culture, THP-1 cells were activated with TPA for 48 h and A549 cells were treated with DJ samples for 24 h. The secretion of proinflammatory cytokines from the co-cultures was stimulated by LPS treatment for 4 h (16).

Animal experiment The animal study was conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of the Kyungpook National University (approval number: 2015-0126). C57BL/6J male mice (four weeks old) were purchased from Orient Bio Inc. (Seongnam, Korea). After adaptation for a week under the standard condition (temperature, 22±2°C; humidity, 50±5%; 12-h light-dark cycles) with free access to food and water *ad*

Table 1. Formulations of detox juice

Ingredient	Formulation			
	DJ-1 ¹⁾	DJ-2	DJ-3	DJ-4
Korean black raspberry	10			10
MBA grape juice	13			13
Campbell grape juice	6			6
Grape seed extract		2	2	2
Omija (<i>Schisandra chinensis</i>)	3			
Blueberry				4
Mulberry	5			5
Cranberry	5			5
Pear	30	39	40	30
Pineapple		37	18	
Kiwi			18	
Jujube fruit	5			5
Steamed garlic	2			
Garlic		1	1	
Cinnamon			1	
Na-alginate	1	1		
Balloon flower root extract ²⁾	19.75	19.75	19.75	19.75
Vitamin C	0.1	0.1	0.1	0.1
Enzymes ³⁾	0.15	0.15	0.15	0.15
Total (%)	100	100	100	100

¹⁾The constituents of each detox juice are given as percentage (% w/w).

²⁾The composition of balloon flower extract is as follows (% w/w): Korean bellflower root, 9.2; ginger, 4.6; grape seed, 0.2; milk vetch root, 0.7; licorice, 0.5; cinnamon, 4.6; cabbage, 0.2; ginkgo nut, 1.2; jujube fruit, 1.8; water, 77.0.

³⁾Enzymes are rapidase and cellulase.

libitum, five-week-old mice [17–19 g body weight (BW)] were used in this study.

Sixty mice were randomly assigned to six groups (10 mice per group): (i) no treatment (negative control; administered only with drinking water and corn oil); (ii) DJ administration (1.6 g DJ/kg BW); (iii) B[a]P treatment with no DJ pretreatment; (iv) B[a]P treatment after low dose of DJ (0.4 g DJ/kg BW); (v) B[a]P treatment after medium dose of DJ (0.8 g DJ/kg BW); (vi) B[a]P administration after high dose of DJ (1.6 g DJ/kg BW).

Sample treatment DJ liquid samples were freeze-dried (Freeze Dryer, ILSHIN Biobase Co. Ltd., Dongducheon, Korea) and dissolved in drinking water at the desired concentrations. Three different doses of DJ preparations were orally administered using a gastric sonde attached to a syringe every other day for 15 days. B[a]P dissolved in corn oil (50 mg B[a]P/kg BW) was given by oral gavage on day 16. Mice were sacrificed, and blood samples were collected on day 17.

Determination of cytokine production The levels of IL-6, IL-1β, and TNFα present in the culture media (collected from A549 cultures and co-cultures of A549 and THP-1 cells) or in the plasma (collected from the sacrificed mice) were measured by enzyme-linked immunosorbent assay (ELISA) using a commercial diagnostic kit (Mouse IL-6, IL-1β, or TNFα ELISA Sets, BD Biosciences, San Diego, CA, USA).

Statistical analysis The obtained data were analyzed by one-way analysis of variance using the SPSS statistics 22 software (SPSS Inc., Chicago, IL, USA). Statistical significance among treatment groups was determined by Duncan's multiple range test at $p < 0.05$. Statistically significant differences were displayed in different alphabetical letters.

Results and Discussion

Ten different kinds of fruit and vegetable that are potential ingredients for DJ were extracted and tested for their capability to induce NQO1 activity in Hepa1c1c7 cells (data not shown). Blueberry, raspberry,

Fig. 1. Quinone reductase activity-inducing capability of fruit-based detox juice. (A) The ability to induce NQO1 enzyme activity of each DJ formulation was tested in Hepa1c1c7 cells. (B) The levels of a proinflammatory cytokine IL-6 in the conditioned media from A549 cultures treated with DJ and/or LPS were determined by ELISA. (C) Culture sketch for THP-1 and A549 co-cultures. (D) The levels of cytokines, IL-6, IL-1β, and TNFα, in the conditioned media from THP-1 and A549 co-cultures treated with DJ and/or LPS were analyzed. n (independent experimental sessions)=3. Error bars, mean±SD. Different letters indicate significant differences ($p < 0.05$).

Fig. 2. Anti-inflammatory effect of detox juice treatment in C57BL/6J mice. (A) Experimental scheme for treatment of animals with DJ (eight times for 15 days) and B[a]P (once 24 h before sacrifice). Sixty mice were randomly grouped for six conditions (10 mice per group): (1) no treatment (negative control), (2) DJ administration (1.6 g DJ/kg BW), (3) B[a]P treatment with no DJ, (4) B[a]P treatment after low dose of DJ (0.4 g DJ/kg BW), (5) B[a]P treatment after medium dose of DJ (0.8 g DJ/kg BW), and (6) B[a]P administration after high dose of DJ (1.6 g DJ/kg BW). (B) Proinflammatory cytokine levels in the mouse plasma. Error bars, mean $\pm$ SD. Different letters indicate significant differences.

mulberry, and garlic extracts caused a significant increase of NQO1 activity in Hepa1c1c7 cells compared to control. This finding indicates that those extracts are able to promote intracellular detoxification.

Four different formulations for detox juice, DJ-1, 2, 3, and 4, were established by combining various fruit and vegetable extracts showing relatively high NQO1-inducing activity (Table 1). Their abilities to induce NQO1 enzyme activity were examined in Hepa1c1c7 cells (Fig. 1A). DJ-4 showed the highest NQO1-inducing activity. More specifically, intracellular NQO1 activity increased by 2.4-fold in Hepa1c1c7 cells by treatment with DJ-4, whereas NQO1-inducing activities of DJ-1, 2, and 3 were not significantly different from the negative control. The relatively higher ability of DJ-4 to induce NQO1 is most likely attributable to blueberry and/or grape seed extracts that are not included in DJ-1 formulation. In particular, blueberry is well known to have strong antioxidant and detoxifying enzyme-inducing activities (17).

Multiple studies have reported that the fruits used in DJ preparations contain various bioactive substances that have a strong antioxidant activity (18-23). Thus, it is irrefutable that the ability to highly induce NQO1 activity is attributed to the antioxidant constituents in the fruit materials. However, it requires further physicochemical study to quantify and identify the functional components in the formulated juice.

Since the DJ-4 formulation (labeled as DJ in Fig. 1B-D and Fig. 2) showed significantly higher NQO1-inducing activity than the other formulations, we then examined whether DJ modulates inflammatory cytokine production as well. A549 cells were cultured with various

concentrations of DJ (0, 100, 200, and 400 μ g/mL medium) that did not exhibit any cytotoxic effect (data not shown). After 24 h-pretreatment, cellular inflammatory responses were provoked by LPS treatment (2 μ g/mL) for 24 h. The conditioned media from A549 cell cultures were then collected and IL-6 levels were measured in the media by ELISA (Fig. 1B). Interestingly, DJ treatment at concentrations of 200 μ g/mL or higher suppressed LPS-induced IL-6 secretion in A549 cultures.

In addition, A549 cells were co-cultured with human monocytic THP-1 cells to mimic the cellular environment of the lung in an *in vitro* system (Fig. 1C) (24). THP-1 cells were differentiated into macrophage-like cells by TPA treatment for 48 h and combined for co-culture with A549 cultures that were pretreated with DJ for 24 h. To induce inflammatory responses, the co-cultures were treated with LPS. After 4 h incubation, the levels of inflammatory cytokines (IL-6, IL-1 β , and TNF α) secreted to the conditioned media were determined (Fig. 1D). The levels of three kinds of cytokine, particularly IL-6, dramatically increased with LPS treatment. However, cytokine levels induced by LPS treatment were significantly diminished by pretreatment with DJ in a concentration-dependent manner.

To examine the anti-inflammatory effect of DJ *in vivo*, C57BL/6J mice were orally administered DJ (at 0, 0.4, 0.8, and 1.6 mg/kg BW/day). At 24 h before sacrifice, mice were treated with B[a]P (Fig. 2A). The cytokine levels in the plasma were determined by ELISA (Fig. 2B). After treatment with B[a]P, proinflammatory cytokine levels in the mouse plasma dramatically increased, whereas they were significantly lowered by the pretreatment with DJ in a dose-dependent manner.

B[a]P is a well-known carcinogen and an agonist for aryl hydrocarbon receptor (AhR) that activates phase I detoxifying enzymes including CYP1A1 (5,25). CYP enzymes dynamically function in B[a]P metabolism, and the activated AhR induces T cell differentiation, influencing systematic inflammation (5,26,27). In contrast, dietary curcumin, sulforaphane, and resveratrol can inhibit B[a]P-induced AhR activation and CYP1A1 transactivation and enhance Nrf2-mediated phase II detoxifying enzyme levels and activities (13,28,29). In addition, numerous studies are supportive of antioxidant and anti-inflammatory effects of phytochemicals, including resveratrol and quercetin (4,30,31).

Taken together, our *in vitro* and *in vivo* studies demonstrate that the dietary intake of DJ composed of NQO1 activity-inducing fruit extracts can suppress the LPS-induced inflammatory responses in lung cells and the systematic inflammation provoked by exposure to environmental air pollutants such as B[a]P. However, further biochemical study will be necessary to reveal the molecular mechanisms by which DJ or its constituents function against inflammatory responses.

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Disclosure The authors declare no conflict of interest.

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