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Antioxidant and anti-ageing activities of citrus-based juice mixture

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ABSTRACT

The production of excessive reactive oxygen species by exposure to oxidative stress and solar radiation are primary factors in skin damage. We examined the effects of a citrus-based juice mixture and its bioactive compounds on antioxidant and anti-ageing activities in human dermal fibroblasts and hairless mice via the regulation of antioxidant enzymes and the mitogen-activated protein kinase pathway. The citrus-based juice mixture reduced H_2O_2-induced cell damage and intracellular reactive oxygen species production in human dermal fibroblasts. Citrus-based juice mixture pretreatment suppressed the activation of the H_2O_2-mediated mitogen-activated protein kinase pathway by activating the expression of activator protein 1 and matrix metalloproteinases. Moreover, it increased the expression levels of antioxidant enzymes such as glutathione reductase, catalase and manganese superoxide dismutase. In addition, oral administration of the citrus-based juice mixture decreased skin thickness and wrinkle formation and increased collagen content on an ultraviolet light B-exposed hairless mouse. These results indicate that the citrus-based juice mixture is a potentially healthy beverage for the prevention of oxidative stress-induced premature skin ageing.

1. Introduction

Ageing is a complex process, and various theories have therefore emerged to help explain it (Harman, 2002). Among these many studies, the cellular manifestations theory of ageing proposes that reactive oxygen species (ROS) generated inside the cell will lead to ageing (Marchi et al., 2012). ROS are byproducts of aerobic respiration that are involved in the modification of multiple cellular reactions. They are also produced as a cellular response to inflammation, nutrient deprivation, ionizing radiation, ultraviolet light (UV), hypoxia, and exposure to heavy metals and oxidants. Cells are equipped to address ROS with a substantial number of antioxidants (e.g., vitamin C, vitamin E, reduced glutathione and carotenoids) as well as an antioxidant defence system that includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (MatÉs, Pérez-Gómez, & De Castro, 1999). The excessive generation of ROS could cause significant apoptotic or necrotic cell death. These processes lead to some of the cellular changes observed during differentiation, ageing and malignant transformation. During these cellular changes, ROS and free radicals influence the expression of various genes and signal transduction pathways. The most significant effects are observed in the mitogen-activated protein kinase (MAPK)/activator protein-1 (AP-1) (Pacurari et al., 2008) and matrix metalloproteinase (MMP) activation signalling pathways (Rittié & Fisher, 2002).

Recently, many studies have reported that plant bioresources (e.g., fruits and vegetables) can effectively reduce the risk of skin disease. Interestingly, numerous reports have focused on the beneficial dermal effects of dietary plant resources due to their antioxidant and anti-inflammatory activities in the skin (Bae et al., 2009).

Citrus fruit is one of the most popular fruits and is widely used for fruit bars, juices, pies, ice creams, and jams all over the world. It can be an excellent dietary source of beneficial substances. It also contains many nutrients and has a very low fat content. Specifically, the organic acids of citrus fruits such as citric acid and malic acid are easily metabolized and are key components of many metabolic pathways in humans.

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Several studies demonstrated that citrus fruits, citrus fruit extracts and citrus flavonoids have a preventative effect on ROS generation, inflammation (Choi, Hwang, Ko, Park, & Kim, 2007), cancer incidence (Frydoonfar, McGrath, & Spigelman, 2003) and various chronic diseases.

This study was undertaken to investigate the antioxidant and anti-ageing effects of a citrus-based juice mixture (CBJM) and its bioactive compounds (narirutin; NAR, hesperidin; HES, and ascorbic acid; ASA) on the H_2O_2-induced oxidative stress in human dermal fibroblasts (HDFs) and UVB-exposed hairless mice. The findings presented herein demonstrate the H_2O_2-induced collagen degradation and induction of MMP activity via the regulation of the antioxidant defence system and the MAPK/AP-1 signalling pathways.

2. Materials and methods

2.1. Chemicals and antibodies

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (P/S), phosphate buffered saline (PBS), and trypsin–EDTA were obtained from Gibco (Gaithersburg, MD, USA). H_2O_2, gelatin, acetic acid, sodium carbonate, Folin–Ciocalteu's phenol reagent, gallic acid, potassium ferrocyanide, potassium ferricyanide, sodium phosphate dibasic, 6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid (Trolox), and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma–Aldrich Co. (Saint Louis, MO, USA). Coomassie Brilliant Blue G-250 and Triton X-100 were acquired from Bio-Rad Laboratories (Hercules, CA, USA). Potassium phosphate monobasic and fluorescein (sodium salt) were obtained from Junsei Chemical (Tokyo, Japan). Dichlorodihydro-fluorescein diacetate (H_2DCFDA) was purchased from Molecular Probes (Carlsbad, CA, USA). Antibodies to GR and Mn-SOD were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-CAT was purchased from Abcam (Cambridge, UK). Antibodies against p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (p-ERK), c-Jun NH(2)-terminal kinase (JNK), phospho-JNK (p-JNK), p38 MAPK (p38), phospho-p38 MAPK (p-p38), phospho-c-Jun (p-c-JUN), phospho-c-Fos (p-c-Fos), β-actin and all secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Sample preparation

The *Citrus sunki* Hort. ex Tanaka (Jingyul), *Citrus unshiu* Marcov × *Citrus sinensis* Osbeck × *Citrus reticulata* Blanco (Hallabong) and *Vitis vinifera* L. (white grape) were supplied by a farm located on Jeju Island, Korea. Jingyul and Hallabong were used for CBJM. The fruits were cleaned and thoroughly washed, and the peels were removed before blending the fruits into a mixture of pulp, juice and water. CBJM are complex mixtures consisting of 34% Jingyul juice, 20% Hallabong puree, 12% white grape concentrate and pure water. The optimized CBJM recipe was selected as a representative of 20 different juice mixtures with high antioxidant activity from a pilot study. CBJM was freeze-dried (Ilshinbiobase Co., Ltd, Gyeonggi-do, Korea) and preserved at −20 °C. A combined bioactive compound mixture (MIX) was prepared by the ratios of the amounts of major compounds in the CBJM (NAR:HES:ASA = 2:5:47 w/w%, respectively).

2.3. Physicochemical properties of the CBJM

Water content was analysed by an MB45 moisture analyser (Ohaus Corporation, Greifensee, Switzerland). The °Brix was measured by a hand reflectometer (PAL-α Brix 0–85%, Atago, Tokyo, Japan). The pH value was investigated at 25 °C by a pH meter (pH510, EUTECH, Co., Anyang, Korea), and the total titratable acidity was determined by titrating 10 mL of bath against 0.1 N sodium hydroxide (NaOH) using phenolphthalein with the end-point indicated by the emergence of a pink colour. The total titratable acidity was calculated by the following equation, where V is titer volume of 0.1 N NaOH and M is the weight of CBJM (mL).

$$\text{Total titratable acidity } (\%) = \frac{V \times (0.1\,\text{N NaOH}) \times (0.067) \times (10)}{M}$$

2.4. Determination of the phenol compounds in the CBJM

ASA analyses were performed using a high-performance liquid chromatography (HPLC)/PDA system (NANOSPACE SI-2 series, Shiseido Co. Ltd., Tokyo, Japan). Separation was performed on a Shiseido CAPCELL PAK C_{18} column (250 × 4.6 mm, 5 μm particle size) (Shiseido Co. Ltd., Tokyo, Japan). A sample volume of 20 μL was injected into the column and eluted with a constant flow rate of 1.0 mL/min. The mobile phase was composed of 60% acetonitrile with 50 mM KH_2PO_4. Continuous scanning was performed by UV detector, and chromatograms were acquired at 254 nm for 15 min. NAR and HES analyses were performed using an HPLC system (Waters 2695 Separation Module, Waters Co., Milford, MA, USA). Separation was performed on a SunFire™ C_{18} column (250 × 4.6 mm, 5 μm particle size) (Waters Co., Milford, MA, USA). The sample volume of 10 μL was injected into the column and eluted at a constant flow rate of 1.0 mL/min. The mobile phase was composed of acetonitrile (0.1% phosphoric acid) and water. Continuous scanning was performed by a Waters 996 Photodiode Array Detector (Waters Co., Milford, MA, USA) in the range of 200–400 nm. The chromatograms were acquired at 280 nm for 20 min.

The total phenolic content was determined using Folin–Ciocalteu's colorimetric method. The sample was appropriately diluted with distilled water. One millilitre of the sample or standard (gallic acid) was mixed with 1 mL of 10% sodium carbonate and 1 mL of 2% Folin–Ciocalteu's phenol reagent. After 90 min, the absorbance was measured at 750 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The results are expressed in gallic acid equivalents (GAE) using a gallic acid (0–0.2 mg/mL) standard curve ($y = 17.531x + 0.0016$).

2.5. Oxygen radical absorbance capacity (ORAC)

The ORAC assay was analysed according to Prior et al. (2003) with some modifications. Samples were diluted with a potassium sodium phosphate buffer (75 mM, pH 7.4). We used 25 μL of diluted samples, Trolox (0–10 μM) or potassium sodium phosphate buffer (blank) and 150 μL fluorescein (40 nM) in black-walled 96-well plates. Finally, we pre-incubated 25 μL AAPH (18 mM) at 37 °C for 15 min, which was then transferred to each well. The plate was immediately carried to a fluorescence microplate reader (SpectraMax GEMINI EM, Molecular Devices, Sunnyvale, CA, USA), and fluorescence was measured. The analyser was designated to register at an excitation wavelength of 485 nm and at an emission wavelength of 530 nm every 3 min for 90 min at 37 °C. The results of the ORAC values were calculated by using a calibration curve of Trolox and area under the fluorescence decay curve. The ORAC value is expressed as Trolox equivalents as micromole per millilitre.

2.6. Cell culture and cell viability

HDFs were provided from Professor E. K. Hong (Kangwon National University, Chuncheon, Korea). HDFs were maintained