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Protective effect of bioaccessible fractions of citrus fruit pulps against H_2O_2-induced oxidative stress in Caco-2 cells

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ABSTRACT

Fruit pulps from Navel (N) and Cara Cara (CC) oranges, and Clementine mandarin freshly harvested (M) and refrigerated stored (M12) were used to evaluate the cytoprotective effect of their bioaccessible fractions (BF) against H_2O_2-induced oxidative stress in Caco-2 cells. BF of samples preserved viability *vs.* H_2O_2 treated cells, reaching values similar to controls. Lipid peroxidation was reduced to levels of control cells, but M did not reach control values. ROS and mitochondrial membrane potential changes ($\Delta\psi_m$) values were reduced compared with H_2O_2 treated cells, but without achieving control levels. A significant reduction in cell proportions in G1 phase and a significant increase in sub-G1 phase (apoptosis) of cell cycle was shown in H_2O_2 treated cells, and BF allowed a recovery close to control levels. Thus, BF of samples protect the cells from oxidative stress by preserving cell viability, mitochondrial membrane potential and correct cell cycle progression, and diminishing lipid peroxidation and ROS.

1. Introduction

The gastrointestinal tract is the first site where interaction between endogenously generated reactive oxygen species (ROS) and dietary antioxidants takes place *in vivo* (Halliwell, Zhao, & Whiteman, 2000). At elevated levels, ROS can adversely affect cell function leading to cell damage and cell death; thus, body's antioxidant system, which is incomplete without exogenous antioxidants such as vitamin C, carotenoids and polyphenols, has to be efficient avoiding the onset of oxidative stress (Bouayed & Bohn, 2010).

Citrus fruits and juices of orange and mandarin are widely consumed worldwide and have attracted attention since a high intake may reduce the risk of degenerative diseases including cardiovascular, neoplastic, and nervous system related diseases (Silalahi, 2002), and also colon carcinogenesis (Miyagi, Om, Chee, & Bennink, 2000; Tanaka et al., 2000). These products are rich sources of a complex mixture of antioxidants such as vitamin C, carotenoids (namely β-cryptoxanthin, lutein, zeaxanthin and β-carotene) and polyphenols (mainly flavanones such as hesperidin and naringenin) (Guarnieri, Riso, & Porrini, 2007) which may act in concert (additively or synergistically) to exert their antioxidant and anticancer activities (Liu, 2004).

A spontaneous mutant variety from sweet orange, termed Cara Cara, has been characterized and shows distinctive red pulp coloration due to the accumulation of lycopene in the juice vesicles (Alquezar, Rodrigo, & Zacarias, 2008; Brasili et al., 2017; Lee, 2001). In addition, it has been reported that certain postharvest conditions promote the accumulation of carotenoids in the skin and pulp of citrus fruits, specifically β-cryptoxanthin in mandarin fruits (Carmona, Zacarias, & Rodrigo, 2012; Rodrigo, Cilla, Barberá, & Zacarías, 2015). Based on these data, the profile and bioaccessibility of bioactive compounds (vitamin C, flavonoids and carotenoids) in fruit juices and fruit pulps from Navel and Cara Cara oranges, and Clementine mandarin freshly harvested and postharvest stored 5 weeks at 12 °C has been determined. In general, vitamin C contents and bioaccessibility were similar in the 4 varieties studied, but higher bioaccessibility on average for pulps *versus* juices was observed. Likewise, much more content of flavonoids and carotenoids were noticed

Abbreviations: N, Navel orange; CC, Cara Cara orange; M, mandarin freshly harvested; M12, mandarin refrigerated stored; BF, bioaccesible fractions; ROS, reactive oxygen species; t-BuOOH, tert-Butyl hydroperoxide; $ABTS^{\cdot+}$, 2,2′-azino-bis [3 ethylbenzothiazoline-6-sulfonic acid] anion radical; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; TEER, transepithelial electrical resistance. MTT: 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide; MDA, malondialdehyde; DHR, dihydrorhodamine; PI, propidium iodide; $\Delta\psi_m$, mitochondrial membrane potential changes; DMEM, Dulbecco's Modified Eagle Medium

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in pulps than in juices, and higher bioaccessibility was achieved in oranges or mandarins when flavonoids or carotenoids were considered, respectively (De Ancos, Cilla, Barberá, Sánchez-Moreno, & Cano, 2017; Rodrigo et al., 2015). Therefore, next step would be the evaluation of the bioactivity of citrus fruit pulps (due to their major bioaccessibility of vitamin C, flavonoids and carotenoids *versus* fruit juices) in a biological relevant model (Caco-2 cells), to further characterize their potential functional health benefits.

To this end, fully differentiated Caco-2 cells may serve as a good and valuable model for assessing the physiological response of enterocytes to various oxidant stresses (Bedoya-Ramírez, Cilla, Contreras-Calderón, & Alegría-Torán, 2017). The protective effects of citrus fruits or related antioxidants against oxidative stress using Caco-2 cells have been documented in previous studies. In these sense, pre-incubation (24 h) of Caco-2 cells with β-carotene (0.1–50 μM) failed to inhibit 10 mM H_2O_2-derived oxidative stress (Bestwick & Milne, 1999). On the contrary, Tarozzi et al., 2006 reported that pre-incubation for 24 h with blood orange extracts (6.25–50 mg/mL) protected Caco-2 cells from 3 mM tert-Butyl hydroperoxide (t-BuOOH) oxidative stress decreasing cytotoxicity and ROS levels. Similarly, pre-incubation (1 h) with β-cryptoxanthin (1–25 μM) protected Caco-2 cells from DNA damage generated by 15 μM H_2O_2 (Lorenzo et al., 2009). Other cytoprotective studies against oxidative stress with citrus fruits or related bioactive components such as sweet orange peel extracts (Chen, Chu, Chyau, Chu, & Duh, 2012) or sweet orange and citrus fruit unshiu mikan juice powders and their major flavonoids (Jannat, Ali, Kim, Jung, & Choi, 2016) in HepG2, flavonoid fraction of bergamot and orange juices in A549 cells (Ferlazzo et al., 2015), citrus flavanones in PC12 cells (Hwang & Yen, 2008) and lutein and zeaxanthin in HLEC cells (Gao et al., 2011) have been reported. However, these studies have not considered the fact that food after consumption undergoes a gastrointestinal digestion process that may affect the native antioxidant potential of the complex mixture of bioactive compounds present in the food matrix before reaching the proximal intestine, thus, probably overestimating their biological activity (Cilla, Perales et al., 2011). In this respect, to our knowledge, only two studies involving digested fruit beverages made of grape-orange-apricot have evaluated the cytoprotection against H_2O_2-induced oxidative stress in Caco-2 cells, based on cell viability maintenance, GSH-related enzymes activation, more preserved mitochondrial membrane potential and cell cycle distribution, and inhibition of caspase-3 activation (Cilla, Laparra, Alegría, & Barberá, 2011; Cilla, Laparra, Alegría, Barberá, & Farré, 2008).

Thus, the aim of the present study was to evaluate the cytoprotective effect of bioaccessible fractions obtained after simulated gastrointestinal digestion of citrus fruit pulps from Navel and Cara Cara oranges, and Clementine mandarins freshly harvested and postharvest stored, against H_2O_2-induced oxidative stress in Caco-2 cells.

2. Materials and methods

2.1. Reagents

2.1.1. Simulated gastrointestinal digestion

Pepsin (porcine, 975 units per mg protein), pancreatin (porcine, activity equivalent to 4 × USP specifications) and bile extract (porcine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1.2. Carotenoids

HPLC-grade methanol, chloroform and acetone were supplied by Scharlau (Barcelona, Spain) and methyl tert-butyl ether (MTBE) by Merck (Darmstadt, Germany). Petroleum ether and diethyl ether were of analytical grade and were supplied by Scharlau (Barcelona, Spain). Commercial standards of β-carotene (≥ 97%), lutein (≥ 95%) and lycopene (≥ 90%) were purchased from Sigma-Aldrich, and β-cryptoxanthin (≥ 97%) and zeaxanthin (≥ 98%) from Extrasynthese (Lyon, France). Standards of phytoene and phytofluene were obtained from peel extracts of Pinalate orange fruits, and of all-*E*-violaxanthin and 9-*Z*-violaxanthin from peel extracts of Navel orange fruits and HPLC purified.

2.1.3. Ascorbic acid analysis

Glacial acetic acid, metaphosphoric acid and formic acid and L(+) ascorbic acid (≥ 99% purity) were purchased from Panreac Química (Barcelona, Spain).

2.1.4. Flavonoid analysis

Methanol and acetonitrile (HPLC-grade) were provided by Lab-Scan (Dublin, Ireland). Formic acid and hydrocloric acid were purchased from Panreac Química (Barcelona, Spain). Narirutin (naringenin-7-*O*-rutinoside) was obtained from Extrasynthese (France); hesperidin (hesperitin-7-*O*-rutinoside) and apigenin were purchased from Sigma (St. Louis, MO, USA).

2.1.5. Hydrophilic antioxidant capacity analysis

2,2′-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] ($ABTS^{\bullet+}$) anion radical, potassium persulfate ($K_2S_2O_8$), iron (III) chloride hexahydrate, phosphate buffered saline, hexadecyltrimethyl-ammonium bromide, and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\blacksquare}$), were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(1-naphtyl)ethylenediaminedihydrochloride and 2,4,6-tris (2–pyridyl)-s-triazine (TPTZ) were obtained from Fluka Chemie AG (Buchs, Switzerland). Ascorbic acid (≥ 99% purity) and ethanol were obtained from Panreac Química (Barcelona, Spain).

2.1.6. Caco-2 cell culture

DMEM + GlutaMAX™-I, phosphate buffered saline (PBS), and trypsin-EDTA solution (2.5 g/L trypsin and 0.2 g/L EDTA) were purchased from Gibco Invitrogen (Carlsbad, USA). Absolute methanol was obtained from Merck (Darmstadt, Germany). The reagents used for preparing buffers were purchased from Panreac Química (Barcelona, Spain), and the rest of reagents used throughout the assays were from Sigma Chemical Co. (St. Louis, MO, USA), including the hydrogen peroxide solution 30% (w/w) used as inducer of oxidative stress.

Water of cellular grade was used for the preparation of reagents used in Caco-2 cells and throughout the *in vitro* digestion assay (Aqua B Braun, Braun Medical, Barcelona, Spain). The rest of the reagents were prepared with Milli-Q water (18.2 MΩ cm resistivity).

2.2. Samples

Fruit pulps from freshly harvested Clementine mandarin (*Citrus clementina* L) (M) and postharvest stored at 12 °C (90–95% relative humidity) for 5 weeks (M12), rich in β-cryptoxanthin (Carmona et al., 2012) and from Navel orange (*Citrus sinensis* L) (N) and its spontaneous mutant Cara Cara (CC) with high lycopene and other linear carotenes (phytoene and phytofluene) content (Alquezar et al., 2008), were used to evaluate the cytoprotective effect of their bioaccessible fractions (BF) (the maximum soluble fraction of food-derived compounds in the simulated gastrointestinal media that would be available for absorption) obtained by *in vitro* digestion against H_2O_2-induced oxidative stress in Caco-2 cells. Bioaccesible bioactive compounds contents have been analyzed in previous reports (De Ancos et al., 2017; Rodrigo et al., 2015).

2.3. In vitro digestion

An *in vitro* gastrointestinal digestion procedure mimicking the physiological situation in the upper digestive tract (stomach and small intestine) was used to obtain bioaccessible fraction from 80 g of citrus fruit pulps in order to evaluate BF of carotenoids, polyphenols, ascorbic acid and hydrophilic antioxidant capacity according to the procedure