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Original article

Orange juice and its major polyphenol hesperidin consumption do not induce immunomodulation in healthy well-nourished humans

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SUMMARY

Background & aims: Polyphenols exert a variety of biological properties, including antioxidant, immunomodulatory and antigenotoxic effects. In a randomized crossover study in healthy men, we investigated the effects of orange juice and its major polyphenolic compound hesperidin on a panel of immune cell functions, including cytokine secretion by leukocytes, lytic activity of NK cells, and the Reactive Oxygen Species (ROS) burst by polymorphonuclear neutrophil cells (PMN).
Methods: The protocol design was divided into three 4-week treatment periods separated by 3-week wash-out intervals, for total study duration of 18 weeks. During treatment periods, volunteers ($n = 24$) consumed daily 500 mL of orange juice, or an isocaloric control beverage with hesperidin (292 mg in a capsule), or of the same control beverage with a placebo.
Results: Whatever the intake was, no variations were recorded on leukocyte subset distributions (PMN, B and T lymphocytes, NK cells and monocytes), ROS production by stimulated PMNs, lytic activity of NK cells or cytokine production capacity of leukocytes in well-nourished healthy volunteers.
Conclusions: We show that consumption within the usual daily intake range of orange juice and its major polyphenol hesperidin do not induce immunomodulation of cell immune function in healthy well-nourished humans.

1. Introduction

Polyphenols are low-molecular-weight compounds resulting from the secondary metabolism of plants that both constitute the major phytochemicals in fruits and vegetables, and the main antioxidants in our food. A growing number of epidemiological and clinical studies suggest that these bioactive compounds could be involved in the cardiovascular health effects associated with a high consumption of plant foods rich in polyphenols.[1] By their ability to modulate oxidative process, polyphenols may protect cellular protein, lipid or nucleic acid and thus contribute in lowering mutagenesis, carcinogenesis and coronary heart disease.[2] Indeed, several *in vitro* and *in vivo* studies have shown that polyphenols, such as flavonoids, display antioxidant as well as immunomodulator activities. For example, cacao liquor polyphenols inhibited both hydrogen peroxide and superoxide anion production by phorbol myristate acetate (PMA)-activated granulocytes from human healthy subjects.[3] Enhanced activation of Natural Killer (NK) cell cytotoxicity in response to IL-2 was observed in healthy volunteers consuming polyphenol-rich food (fruits, nuts, and vegetables).[4]

It has been proposed that fruits (especially citrus and apple) favorable effects on the cardiovascular system, on inflammation and against cancer, are due to vitamins, soluble sugars, organic acids and polyphenols.[5, 6] For example, orange juice consumption has been associated with a reduction of a lipid peroxidation blood marker.[7] Citrus constitute the unique significant source of dietary

Abbreviations: ECD, PE-Texas red; EGFP, Enhanced Green Fluorescent Protein; FITC, Fluorescein IsothioCyanate; NK, Natural Killer; PBMC, Peripheral Blood Mononuclear Cells; PC5, PE-Cyanine 5; PE, PhycoErythrine; PHA, PhytoHemAgglutinin; PMN, PolyMorphoNuclear neutrophil cells; PMA, Phorbol Myristate Acetate; ROS, Reactive Oxygen Species.

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flavanones, a subclass of flavonoids. Due to the high content of flavanones in citrus fruits and juices, these compounds may highly contribute to the total flavonoid intakes, particularly in citrus consumers.[8] Hesperidin (hesperetin-7-*O*-rutinoside) represents more than 90% of the flavanones found in orange and orange juice.[9]

Besides the effects on the cardiovascular system and oxidative stress, it has been proposed that polyphenols play a role in the immune system *via* oxidative mechanisms involved in immune defenses such as phagocytosis and inflammation. In young subjective-stressed adults, consumption of fruit and vegetable juices (encapsulated juice concentrated) has been associated with an increase in circulating γδ-T cells, classically involved in innate and acquired immunity, protection of epithelial linings, and wound healing.[10] Similarly, Bub et al. (2003) demonstrated that some parameters of lipid metabolism and immune status in healthy volunteers were modulated by the consumption of two different mixed fruit juices delivering about 230 mg of polyphenols per day (a mixture of apple, mango and orange juices with added anthocyanins from aronia, blueberries and boysenberries or flavonols from green tea, apricot and lime).[11] They reported that polyphenol-rich fruit juices led to fewer oxidized DNA bases in peripheral blood mononuclear cells (PBMCs) and higher lytic NK cell activity.[11] Contrariwise, *in vitro* experiments in mouse and rat models have shown that quercetin and catechin decrease NK cell activity. In a murine inflammatory model (endotoxin-induced acute lung injury), hesperidin (200 mg/kg) downregulated the LPS-induced expression of proinflammatory cytokines, decreased total leukocyte counts and nitric oxide production, and induced the expression of cell adhesion molecules.[12] The oxidative and inflammatory stresses induced by high-fat and high-carbohydrate meals were prevented in healthy subjects by the consumption of orange juice.[13] However, the mechanisms underpinning the molecular action of polyphenols in immune cells remain unidentified.

Prompted by the lack of data on the specific impact of hesperidin on human immune cells, the purpose of this study on healthy volunteers was to characterize the effect of a daily consumption of hesperidin as a purified molecule and of orange juice on a panel of immune cell functions including pro- and anti-inflammatory cytokines secretion (IL-2 and IL-4, respectively) by leukocytes, lytic activity of NK cells, and polymorphonuclear neutrophil cells (PMN) cell-induced reactive oxygen species (ROS) burst.

2. Materials and methods

2.1. Subjects

Twenty-four men (Table 1) were recruited for the study, as previously published.[14] All volunteers were healthy and well nourished, as determined by screening medical history and a medical check-up. None of the volunteers were taking vitamin supplements or medication before or during the study. The study was approved by the local Medical Ethics Committee (CPP Sud-Est 6, France) and all the participants gave their written consent (Trial Registration: ClinicalTrials.gov NCT 00983086). Similar study design (4 weeks treatment and the 3 weeks washout) was already use in the nutrition intake study.[11, 14]

Table 1
Characteristics of the healthy volunteers.

n	24 (male)
Age (years)	56 ± 3
Height (cm)	175 ± 6
Body mass (kg)	84 ± 6
Body mass index (kg/m^2)	27 ± 1

Results are expressed as mean ± SEM.

2.2. Study design and diet intakes

This study consisted in 3 treatment periods (P1, P2 and P3) each lasting 4 weeks and separated by 3-week wash-out periods, resulting of a total study duration of 18 weeks (Fig. 1). During periods P1, P2 or P3, volunteers consumed per day in a randomized order (A) orange juice (500 mL, naturally providing 292 mg hesperidin), (B) an isocaloric control drink (500 mL) together with 292 mg of pure hesperidin, or (C) the same control drink (500 mL) with a placebo (292 mg starch). The compositions of the orange juice (provided by the Florida Department of Citrus, Lake Alfred, FL, USA) and of the control drink are given in Table 2. The control beverage was a sweet drink which had a sugar composition similar to that of orange juice. Thus the energy contents brought by 500 mL of orange juice or of control drink were quite close to each other, 180 and 194 kcal respectively. Pure hesperidin was packaged in capsules, each containing 146 mg of Orange Bioflavonid Complex (99.2% hesperidin, Nutrafur, Murcia, Spain) and volunteers had to consume daily two of these capsules. Placebo capsules were filled with 146 mg of starch and were visually identical to those of hesperidin. Like for hesperidin, volunteers had to consume daily 2 capsules of placebo. In practice, they were instructed to divide the total daily dose of each dietary treatment into 2 equal intakes (250 mL beverage plus one capsule, according to the experimental group), one with the breakfast and the other one at lunchtime.

During periods P1, P2 and P3, volunteers were limited in their consumption of polyphenol-rich drinks like coffee, tea, wine and cocoa (less than 200 mL/day). During orange juice diet periods, subjects consumed daily 180 mg of ascorbate, an intake representing more than 1.5 fold the Recommended Daily Intake.[15] Compliance to diet recommendation was determined by examining diaries in which volunteers recorded daily the consumption of study products (beverages and capsules) and by counting the returned unconsumed products at the end of the dietary periods.

2.3. Collection and preparation of blood samples

Fasting blood samples were taken at the beginning (V2, V4 and V6) and at the end (V3, V5 and V7) of each experimental period (P1, P2 and P3) in the morning between 7 and 9 a.m. (Fig. 1). Blood was drawn from an antecubital vein into pre-chilled tubes containing ethylenediamine tetra-acetic acid (EDTA) (phenotyping and determination of NK cell lytic activity) and/or lithium-heparin (whole blood culture and cytokines secretion) and immediately placed on ice in the dark until analysis.

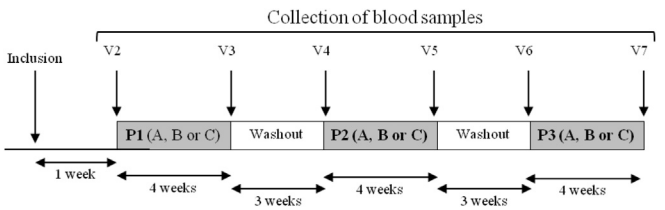

Fig. 1. Study design and diets. This randomized crossover study was divided into three treatment periods (P1, P2 and P3) each lasting 4 weeks, split by with three 3-week washout periods, *i.e.* a total study period of 18 weeks. In each treatment period (P1, P2 and P3), volunteers consumed (A) orange juice (500 mL/day containing 292 mg hesperidin), (B) a similar quantity of hesperidin with an isocaloric drink as orange juice (500 mL/day; 292 mg hesperidin) or (C) a placebo with a similar quantity of starch as hesperidin and isocaloric drink (500 mL/day).The functional immune status of each volunteer was evaluated at the beginning (V2, V4, V6) and at the end (V3, V5, V7) of each treatment period.