Orange juice decreases low-density lipoprotein cholesterol in hypercholesterolemic subjects and improves lipid transfer to high-density lipoprotein in normal and hypercholesterolemic subjects

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Abstract

Orange juice (OJ) is regularly consumed worldwide, but its effects on plasma lipids have rarely been explored. This study hypothesized that consumption of OJ concentrate would improve lipid levels and lipid metabolism, which are important in high-density lipoprotein (HDL) function in normolipidemic (NC) and hypercholesterolemic (HCH) subjects. Fourteen HCH and 31 NC adults consumed 750 mL/day OJ concentrate (1:6 OJ/water) for 60 days. Eight control subjects did not consume OJ for 60 days. Plasma was collected before and on the last day for biochemical analysis and an in vitro assay of transfers of radioactively labeled free-cholesterol, cholesteryl esters, phospholipids, and triglycerides from lipoprotein-like nanoemulsions to HDL. Orange juice consumption decreased low-density lipoprotein cholesterol (160 ± 17 to 141 ± 26 mg/dL, P < .01) in the HCH group but not in the NC group. HDL-cholesterol and triglycerides remained unchanged in both groups. Free-cholesterol transfer to HDL increased (HCH: 4.4 ± 2 to 5.6 ± 1%, NC: 3.2 ± 2 to 6.2 ± 1%, P < .05) whereas triglyceride (HCH 4.9 ± 1 to 3.1 ± 1%, NC 4.4 ± 1 to 3.4 ± 1%, P < .05) and phospholipid (HCH 21.6 ± 2 to 18.6 ± 3%, NC 20.2 ± 2 to 18.4 ± 2%, P < .05) transfers decreased in both groups. Cholesteryl-ester transfer decreased only in HCH (3.6 ± 1 to 3.1 ± 1%, P < .05), but not in NC. In control subjects, plasma lipids and transfers were unaltered for 60 days. Thus, by decreasing atherogenic low-density lipoprotein cholesterol in HCH and increasing HDL ability to take up free cholesterol in HCH and NC, OJ may be beneficial to both groups as free-cholesterol transfer to HDL is crucial for cholesterol esterification and reverse cholesterol transport.

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Abbreviations: apo A1, Apolipoprotein A-I; apo B, Apolipoprotein B; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; NC, Normocholesterolemics subjects; OJ, Orange juice; PMF, Polymethoxylated flavones; PON1, Paraoxonase 1.

1. Introduction

Orange juice (OJ) consumption has become a worldwide dietary habit and, as a result, the consumption of frozen concentrated juice has increased steadily over the years. Not surprisingly, the market share of this product is now much greater than that of fresh fruit, especially in developed countries [1]. The concentrated product has a greater
flavonoid content; such as polymethoxylated flavones (PMF), hesperitin and naringin, when compared to fresh juice. This is due to the manufacturing grinding process which uses the entire fruit to produce the juice [2]. Pectin and essential oils contained in the peel are also found in greater amounts in the concentrated juice [2].

In animal studies, supplementation with PMF, hesperitin, and naringin reduced low-density lipoprotein (LDL) cholesterol, apolipoprotein B (apo B), and triglycerides [3,4]. The few studies involving OJ consumption or orange extract of flavonoids have reported beneficial effects on plasma lipids [5,6]. In hypercholesterolemic subjects, consuming 750 mL orange juice daily increased high-density lipoprotein (HDL) cholesterol concentrations by 21% [5].

High-density lipoprotein, as other lipoprotein classes, is synthesized in the liver or intestinal cells. However, it can also be synthesized in the intravascular compartment as well [7]. Apolipoprotein A1 (apo A1), which is synthesized by the liver or by the small intestine, receives phospholipids and cholesterol from cell membranes and from apo B containing lipoproteins in the plasma [8]. This results in discoidal HDL; which is converted to a larger, spherical HDL through cholesterol esterification by lecithin:cholesterol acyltransferase, using apo A1 as a cofactor [8,9]. Cholesterol esterification occurs mostly in the HDL fraction and is a key process for cholesterol plasma pool stabilization [9].

Lipid transfer is facilitated by the so-called transfer proteins, cholesteryl ester transfer protein and phospholipid transfer protein, and involves all lipoprotein classes [10]. The transfer of lipids to HDL is an essential metabolic step for reverse cholesterol transport in which cholesterol from peripheral tissues is shuttled back to the liver and excreted in the bile. Lipid transfer also determines HDL composition and, thus, the adhesion to the lipoprotein surface of proteins, cholesteryl ester transfer protein and phospholipid transfer protein, and involves all lipoprotein classes [10]. The transfer of lipids to HDL is an essential metabolic step for reverse cholesterol transport in which cholesterol from peripheral tissues is shuttled back to the liver and excreted in the bile. Lipid transfer also determines HDL composition and, thus, the adhesion to the lipoprotein surface of proteins related with HDL atheroprotection, such as the antioxidative, anti-inflammatory, antiapoptotic, vasodilatory, and anti-thrombotic, and anti-infection functions of the lipoprotein [11,12].

Despite the great importance of OJ as a widely consumed dietary item, its effects on serum lipids have rarely been investigated. The importance of alterations in the plasma lipid profile and lipid metabolism in atherogenesis is well established. This study investigated whether the ingestion of considerably large amounts of concentrated OJ could affect the lipid profile of hypercholesterolemic and normolipidemic subjects. Furthermore, it was hypothesized that OJ consumption would improve the transfer of lipids (free cholesterol, cholesteryl esters, phospholipids and triglycerides) to HDL.

2. Methods and materials
2.1. Participant subjects and study design
Thirty-one normocholesterolemic (NC; 12 males and 19 females) and 14 hypercholesterolemic (HCH; 6 males and 8 females, with LDL cholesterol >130 mg/dL) subjects were evaluated on the first and last day of the 60-day period of orange juice consumption (750 mL/d). To monitor seasonal variations, blood samples were collected at a 60-day interval for biochemical analysis from a non OJ consuming control group of 8 subjects: 3 normocholesterolemic (2 men and 1 women) and 5 hypercholesterolemic (1 man and 4 women) individuals. An estimate by dietary inquiry of daily diet composition of all participants is in Table 1.

Data on their physical characteristics are presented in Table 2. The following inclusion criteria were observed: little or no consumption of OJ, no vitamin or mineral supplementation, no lipid-lowering medication or hormone replacement therapy, fasting glucose below 100 mg/dL and no kidney diseases, no alcohol drinking, no thyroid problems, and no diabetes or any other metabolic diseases.

Both NC and HCH subjects were submitted to a 60-day period of dietary supplementation with 750 mL/d frozen concentrated OJ/water at 1:6 dilution, consumed at breakfast, lunch, and dinner. Food intake of each participant was assessed by means of a 3-day dietary inquiry before and after the experimental period as previously described [13]. Food intake was described in terms of portion sizes, and the individual sugar and oil intake was calculated from the questionnaire. Estimates of dietary energy and nutrient intake obtained with the 24-hour recalls and food frequency questionnaires were calculated by the NUTRI software, version 2.5 (CIS, School of Medicine, Federal University of São Paulo, SP, Brazil). Concentrated OJ was furnished to the participants on a weekly basis. A dietitian maintained contact with the participants to ensure comprehension of and compliance with the dietary regimens. Participants were advised not to change their normal diet or physical activities during the study period.

The study protocol was approved by the Ethics Committee of the Pharmaceutical Science Department of the Sao Paulo State University. An informed written consent was obtained from each participant.

2.2. Supplementation with orange juice
A single batch of frozen concentrated OJ (65° Brix) was provided by Citrusuco-Fischer Group S/A company (Matão, Brazil). The OJ was stored in 900 mL bottles at 20°C. Participants received one bottle of concentrated juice per week and instructed on how to dilute it to 12° Brix for consumption (1:6 OJ/water). No sugar was added. The flavonoid content in 750 mL of OJ (chilled from concentrate) was 42 mg of hesperitin and 12 mg of naringin [2]. Each daily serving of OJ contained approximately 258 mg of vitamin C, 135 μg of folate, 64 g of total sugar (2:1:1 of sucrose:fructose:glucose), and 1.36 MJ (1318 KJ).

2.3. Plasma lipid determinations
Blood samples were collected in tubes without anticoagulant, on day zero and on day 60 of the OJ supplementation period. Serum was separated by centrifugation at 2500 rpm
for 15 minutes and then stored at −80°C for 10 days for biochemical analysis. Total plasma cholesterol and triglycerides were determined after a 12 h fast with commercial enzymatic kits (CHOD-PAP, Merck, Darmstadt, Germany and Abbott Laboratories, Abbott Park, Ill, respectively). High-density lipoprotein cholesterol was determined by the same method after precipitating LDL and very low-density lipoprotein with MgCl₂ and phosphotungstic acid. Low-density lipoprotein was estimated by the Friedewald equation [14].

2.4. Assay for the lipid transfer from the donor nanoemulsion to HDL

Blood samples were collected in tubes containing 0.1 % EDTA as anticoagulant. Plasma was separated by centrifugation at 2500 rpm for 15 minutes and then stored at −80°C until analysis. The nanoemulsion was prepared from lipid mixtures composed of 40 mg cholesteryl oleate, 20 mg egg phosphaditylcholine, 1 mg triolein and 0.5 mg cholesterol purchased from Sigma (St Louis, Mo). Radioactive lipids were purchased from Amersham International (Little Chalfont, Buckinghamshire, UK) and were added to the lipid mixtures. Two sets of nanoemulsions were prepared: one labeled with ³H-cholesteryl oleate and ¹⁴C-cholesterol and the other with ¹⁴C-phosphatidylcholine and ³H-triolein. Lipid emulsification by prolonged ultrasonic irradiation in an aqueous media and a two-step ultracentrifugation procedure of the crude emulsion and addition of KBr to adjust density in order to obtain the nanoemulsion was carried out as described by Maranhão et al [15].

The in vitro assay of the transfer of the radioactive lipid nanoemulsion to the HDL fraction has been described elsewhere [16]. Briefly, the assay consisted of a 1-hour incubation period for the 2 sets of nanoemulsions with plasma followed by chemical precipitation of the apo B containing lipoproteins and the nanoemulsion. The supernatant containing the HDL fraction was transferred to vials with scintillation solution and radioactivity was counted in a Packard 1660 TR model Liquid Scintillation Analyzer (Palo Alto, CA). The transfer to HDL of the nanoemulsion lipids was calculated as a percentage of the radioactivity of a given labeled lipid in the nanoemulsion found in the HDL plasma fraction.

2.5. Paraoxonase 1 activity

Paraoxonase 1 (PON1) activity was measured by adding serum to 1 mL Tris-HCl buffer (100 mmol/L, pH 8.0)
containing 2 mmol/L CaCl₂ and 5.5 mmol/L paraoxon (Sigma). The generation of p-nitrophenol was measured at 405 nm, at 37°C, for 6 minutes in 1-minute intervals, in a Bio-Rad Benchmark Microplate Reader (Nippon Bio-Rad, Tokyo, Japan) [17].

2.6. Statistical analyses

All values are expressed as means ± SD. The paired Student t test was used to compare the means obtained before and after the experiment period for the 3 groups. Statistical analysis of the data was obtained with the Sigma Stat software version 3.11 (Sigma Stat for Windows, Systat Software Inc, San Jose, Calif). Statistical significance was set at P < .05.

3. Results

An energy intake increase of about 38% occurred after the introduction of the OJ in the NC subjects (Table 1). After the OJ intake period, the NC and HCH subjects presented an increase in the ingestion of carbohydrates of 77% and 55%, respectively (P < .001).

Body mass index and waist circumference of the subjects of the three study groups were not altered during the study period, as can be seen in Table 2.

OJ supplementation had no effect on LDL or HDL-cholesterol or triglycerides in the NC subjects. However, in the HCH subjects, there was a 12% decrease in LDL-cholesterol after the OJ intake period; whereas HDL-cholesterol and triglycerides remained unchanged (Table 2).

In the NC subjects, OJ intake elicited a 48% increase in free cholesterol transfer and 9% decrease in the transfer of phospholipids (P < .001). As for the transfer of core lipids in this group, the triglyceride transfer diminished by 23%, but the cholesteryl ester transfer remained unaffected by OJ (Table 3).

In the HCH subjects, free cholesterol transfer increased by 22%; whereas the phospholipid transfer decreased 10% (P < .05), triglyceride transfer diminished 23% and cholesteryl ester transfer decreased 14% (P < .01) after OJ consumption (Table 3).

The activity of the antioxidative enzyme PON1 decreased only in the NC subjects and was unchanged in the HCH subjects (Table 3).

In the control group, the lipid profile and transfer of lipids to HDL and PON1 activity were similar at the first evaluation and second evaluation after 60 days, without OJ consumption.

4. Discussion

In this study, the ingestion of 750 mL/day of OJ concentrate for two months resulted in a reduction of LDL-cholesterol in HCH subjects; whereas in NC subjects, this reduction was not statistically significant. In both groups, serum triglycerides and HDL-cholesterol were unchanged.

It was recently shown that 500 mL/day of OJ associated with aerobic training decreased LDL-C levels and increased HDL-C in normolipidemic, overweight, middle-aged women. However, these effects were not observed in women under the same aerobic training program who did not consume orange juice [18]. Kurowska et al [5] reported that consuming 250 or 500 mL/d of orange juice did not alter lipid levels in hypercholesterolemic subjects. However, the consumption of 750 mL/day, in contrast with our data, did not decrease LDL-cholesterol levels but increased HDL-cholesterol. On the other hand, plasma triglycerides increased, an undesirable effect which was not observed in our study. Roza et al [6] supplemented hypercholesterolemic subjects with PMFs extracted from orange peels. They reported that supplementation lead to a reduction of both LDL-cholesterol and triglycerides but did not significantly alter HDL-cholesterol. In studies performed in rats, the hypocholesteremic effect of PMFs were obtained by inhibiting 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase and increasing the expression of LDL receptors in the liver [3,4]. This is essentially the same mechanism whereby cholesterol reduction is achieved through treatment with PMFs.

### Table 3

<table>
<thead>
<tr>
<th>Lipid Transfer to the HDL Fraction %</th>
<th>NC First day</th>
<th>Last day</th>
<th>HCH First day</th>
<th>Last day</th>
<th>Control First day</th>
<th>Last day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipids</td>
<td>20.2 ± 2</td>
<td>18.4 ± 2 ††</td>
<td>21.6 ± 2</td>
<td>18.6 ± 3 †</td>
<td>18.9 ± 1</td>
<td>18.6 ± 3</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>3.2 ± 2</td>
<td>6.2 ± 1 ††</td>
<td>4.0 ± 2</td>
<td>5.6 ± 1 †</td>
<td>6.7 ± 1</td>
<td>6.0 ± 2</td>
</tr>
<tr>
<td>Cholesteryl esters</td>
<td>3.1 ± 1</td>
<td>3.0 ± 1</td>
<td>3.6 ± 1</td>
<td>3.1 ± 1 †</td>
<td>3.9 ± 1</td>
<td>3.6 ± 1</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>4.4 ± 1</td>
<td>3.4 ± 1 ††</td>
<td>4.9 ± 1</td>
<td>3.1 ± 1 †</td>
<td>4.9 ± 1</td>
<td>4.4 ± 1</td>
</tr>
<tr>
<td>PON1 (nmol/mL/min)</td>
<td>64 ± 44</td>
<td>54 ± 42 †</td>
<td>38 ± 25</td>
<td>40 ± 17</td>
<td>97 ± 43</td>
<td>96 ± 46</td>
</tr>
</tbody>
</table>

* Values expressed as means ± SD. Comparison between first and last day in the same group.
* † P < .05.
†† † P < .01.
††† P < .001.
statins. The reduction in LDL-cholesterol elicited by OJ consumption in the HCH group cannot be ascribed to changes in dietary cholesterol: those changes were not detected in the dietary questionnaire and slight changes in cholesterol intake are unlikely to influence LDL-cholesterol levels.

As hypothesized, in respect to the lipid transfers to HDL, OJ consumption elicited marked changes in this process despite the HDL cholesterol remaining unchanged. The most relevant change occurred in the transfer of free cholesterol, which increased in both the HCH and NC groups. As is commonly known, the free form of cholesterol is unstable and is stored in cells in its esterified form. In the plasma, cholesterol is esterified by lecithin:cholesterol acyltransferase [8]. This reaction occurs mainly in HDL and apolipoprotein A1, the main HDL apolipoprotein, being the cofactor. Substrate free cholesterol is transferred to HDL by transfer from other lipoprotein classes or from cells, by the adenosine triphosphate-binding cassette transporter A1 complex mediating by the cholesterol efflux after hydrolysis of the esterified form, the initial step of reverse cholesterol transport [19,20]. Therefore, despite our findings of increased free cholesterol transfer to HDL, which may be interpreted as a favorable effect of OJ intake, it appears to assist in the esterification process.

A further potential benefit of OJ consumption on lipid transfers (which occurred in both the NC and HCH subjects in our study) was the decreased triglyceride transfer to HDL. Increase in triglyceride transfer to HDL, as occurs in hypertriglyceridemia, may determine a greater instability of the lipoprotein particles, which, in turn, may affect HDL antiatherogenic properties [21]. Hepatic lipase hydrolyzes triglyceride-enriched HDL to form small dense HDL particles. Such small dense HDL particles of abnormal composition (high triglyceride/cholesterol ester ratio) have a shorter half-life in the plasma than larger HDL particles. As a result of this, HDL levels fall [21]. Therefore, a decrease in triglyceride transfer to HDL may be a favorable effect of OJ consumption. The reduction of phospholipids by OJ intake is complex to interpret, as is the reduction of cholesteryl ester transfer in the HCH group. A recent study reported that HDL enrichment with phospholipids may facilitate reverse cholesterol transport [22].

Alterations in lipid transfer can be ascribed to changes in the action of cholesteryl ester transfer protein and phospholipid transfer protein; the status of lipoprotein composition and concentration [23]. This last factor can be discarded since study subjects of both groups did not have their HDL-cholesterol affected by the orange juice intake. As PMFs contained in OJ concentrate may affect lipid regulatory proteins such as 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase in the liver, the possibility may exist that PMFs may also influence the hepatic synthesis of transfer proteins. It should be noted that those transfer changes did not alter PON1 activity. Paraoxonase 1 is associated with the HDL fraction and is related to the antioxidant functions of HDL [24]. The fact that no alterations of the enzyme activity were found suggests that whatever changes occurred in HDL composition as a consequence of the changes in lipid transfer to the lipoprotein were not sufficient to influence enzyme activity.

Recruitment difficulties and drop-outs were limitations to this study, thus the HCH group was pronouncedly smaller than the NC group. As there are clear OJ dose-response effects, lower OJ intakes should also be tested. Differences in fructose and glucose intake, eventually resulting from the introduction of OJ supplementation, did not cause changes in triglycerides.

In conclusion, OJ consumption leads to a reduction of LDL-cholesterol in HCH, an effect that is undoubtedly anti-atherogenic. On the other hand, OJ consumption did not alter HDL-cholesterol. Nonetheless, the changes in HDL metabolism by OJ, i.e., the increase in the transfer of free-cholesterol and the decrease in transfer of triglycerides, may favor HDL function in cholesterol reverse transport and cholesterol esterification. These changes may also protect the lipoprotein from rapid degradation in the plasma and prevent a fall in HDL-cholesterol levels. These effects were observed regardless of the presence of hypercholesterolemia or not. Considering the results of this study, the effects of OJ consumption can be considered beneficial to both subjects with normal lipids and those with hypercholesterolemia.

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