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Research Paper

Hydroxytyrosol supplementation increases vitamin C levels *in vivo*. A human volunteer trial

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ABSTRACT

Hydroxytyrosol (HT) is a main phenolic component of olive oil. In this study, we investigated the safety and effects produced by HT purified (99.5%) from olive mill waste. HT was administered at a daily dosage of 45 mg for 8 weeks to volunteers with mild hyperlipidemia (n=14). We measured markers of cardiovascular disease risk, enzyme markers of several clinical conditions, hematology, antioxidant parameters, vitamins and minerals at baseline (T0), 4 weeks (T4) and 8 weeks (T8). The values obtained at T4 and T8 were compared with baseline. We found that the HT dose administered was safe and mostly did not influence markers of cardiovascular disease, blood lipids, inflammatory markers, liver or kidney functions and the electrolyte balance. Serum iron levels remained constant but a significant ($P < 0.05$) decrease in ferritin at T4 and T8 was found. Serum folate and red blood cell folate levels were also reduced at T4 and T8. Finally, vitamin C increased by two-fold at T4 and T8 compared with levels at baseline. These results indicate a physiologically relevant antioxidant function for HT through increasing endogenous vitamin C levels.

1. Introduction

Hydroxytyrosol (3,4-dihydroxyphenyl-ethanol, HT) is one of the main phenolic components of virgin olive oil and olive mill waste [1] that has demonstrated the strongest reactive oxygen species (ROS) scavenging properties *in vivo and vitro* among all the olive oil polyphenols [1,2]. HT is the only phenolic compound that has received a European Food Safety Authority (EFSA) health claim approval. According with EFSA, the consumption of olive oil polyphenols contributes to the protection of blood lipids from oxidative damage [3, and references therein]. This claim refers mainly to the effects of HT and its derivatives (e.g. oleuropein complex and tyrosol) on the protection of low-density lipoproteins (LDL) from oxidation. As oxidative modification of LDL plays a central role in the development of cardiovascular disease (CVD), natural antioxidants are a main target for the nutraceutical industry and several HT preparations are commercially available.

Apart from LDL protection, several human studies have indicated other possible beneficial effects of olive oil phenolic compounds, including antioxidant effects in plasma [2,4,5], antithrombotic effects [4,6], increased HDL-cholesterol [7], inhibition of platelet aggregation [8,9], reduction of inflammatory markers and reduction of blood pressure [10]. These studies have all been carried out with polyphenols administered in olive oil, or with olive polyphenol extracts of complex origin, where other components (e.g. fatty acids, terpenoids) may also have a role.

In a previous study carried out in our laboratory, we showed that supplementation with 4 mg/Kg of pure HT improved blood lipids, the antioxidant status and reduced the size of atherosclerotic lesions in a rabbit model of diet-induced atherosclerosis [11]. We also purified large amounts of HT from olive mill waste aimed to produce an almost pure (99.5%) water-soluble pharmacological formulation and characterised its absorption profile in humans [12]. So far, no chronic studies reporting effects of pure HT in human volunteers have been published. In this study, we investigated the safety and health effects of a daily dose of 45 mg of HT administered for 8 weeks in markers of CVD risk, enzyme markers of several clinical conditions, hematology, antioxidant markers, vitamins and minerals.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CPK, creatine phosphokinase; CRP, ultrasensitive C-reactive protein; GGT, Gamma glutamyl transferase; HDL-C, high-density lipoprotein cholesterol; HCT, hematocrit; Hcy, homocysteine; ICAM, intercellular cell adhesion molecule 1; LD, lactate dehydrogenase; LDL-C, low-density lipoprotein cholesterol; Lpa, lipoprotein a; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MDA, malondialdehyde; ox-LDL, oxidized LDL; TAC, total antioxidant capacity; TC, total cholesterol; TG, triacylglycerols; VCAM, vascular cell adhesion molecule 1

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2. Materials and methods

2.1. Subjects and study design

Fourteen healthy volunteers (11 men and 3 women) who were resident in Granada (Spain) participated in the study. The subjects were recruited from volunteers who responded to an advertisement about dietary intervention studies. We advertised for subjects preferably with borderline high levels of total cholesterol, as defined by the National Cholesterol Education Program-Adult Treatment Panel III (total blood cholesterol values between 200 and 239 mg/dL), not suffering from other cardiovascular risk factor. The subjects were given a physical examination and their medical history was consulted before they were included in the study. The subjects were not suffering from any chronic or metabolic disease, had no medical history of gastrointestinal, liver or kidney disease and were not taking any medication or antioxidant supplement. The Ethical Committee of Virgen de las Nieves Hospital Foundation approved the study. Informed written consent was obtained from the volunteers. The study was conducted in accordance with The Code of Ethics of the World Medical Association (Helsinki Declaration, Fortaleza revision 2013), following the EEC Good Clinical Practice guidelines (document 111/3976/88 of July 1990) and current Spanish law, which regulates clinical research in humans (Royal Decree 561/1993 regarding clinical trials). Sample size for before-after study (Paired *T*-test) was calculated using α (two-tailed) =0.05. The study was designed to have 80% power to detect an effect size of a 15 mg/dL difference in mean blood cholesterol using variance estimates from our previous cholesterol lowering studies.

The volunteers were instructed not to consume olive oil, olives or olive-derived products and to refrain from alcohol consumption for at least 14 days prior to the beginning of the study. The subjects and their partners attended a dietary counselling session on general aspects of food composition, food processing, adequate portions, the effects of alcohol consumption, and the beneficial effects of the Mediterranean diet. The subjects were also advised not to eat fast food or precooked meals and to avoid smoky places or to reduce or stop smoking. The subjects were asked not to change their physical activity pattern during the entire duration of the study. Dietary intake was assessed at baseline and at week 7 of the study with a 7-d self-administered food-frequency questionnaire. Subjects were requested to fill in a food diary according to instructions from the principal investigator, where they recorded all food consumption during the week. Once the subjects completed the first week of the study, they were asked to use the food diaries as a reference to repeat the intake of the same foods registered in the diaries, during the remaining weeks of the study. Spanish food composition tables [13] were used to estimate dietary intake.

The HT supplement was administered in the morning between 10 and 12 a.m., as a single oral dose of 45 mg in 40 mL of sterile saline in a Falcon tube. The supplement was prepared daily and transported on ice to the subject's location where it was administered. On Fridays, the subjects received the oral dose and two other doses in an ice box for the weekend days. Compliance with consumption of the HT supplement at weekend days was ensured by collecting the empty containers. The subjects consumed the HT supplement every day for 8 weeks.

Peripheral blood (120 mL) was drawn from each volunteer at baseline (T0) and at times 4 weeks (T4) and 8 weeks (T8) of the study. Anthropometric data (height, weight and body mass index), systolic and diastolic blood pressure were monitored at each visit. The blood extractions were made by qualified personnel under medical supervision. Plasma was obtained by centrifugation at 1600×*g* for 10 min at 4 °C, and immediately frozen at −80 °C. For analysis of hematology parameters, blood was withdrawn in EDTA-containing vacutainers (S-Monovette, Sarstedt, Germany) and was sent to the laboratory. 24-h urine was collected in acid-washed containers the day before and on the test day itself. Urine aliquots were obtained and frozen at −80 °C until used.

2.2. Preparation of the HT supplement

The HT administered to the volunteers was obtained by the Process Engineering Department of Puleva Biotech SA, Granada (Spain). HT was purified from an olive mill waste water extract using food grade solvents, preparative chromatography and further desiccation. The HT obtained was a white powder soluble in water with a purity of 99.5% (see reference 12 for details). The HT used for analytical determinations was synthesised by reducing 3,4-dihydroxyphenylacetic acid with $LiAlH_4$ in tetrahydrofuran under refluxing for 4 h. The reaction product was purified by chromatography on silica gel with $CHCl_3$:MeOH (7:1) as the eluting solvent. The identity of the HT obtained was verified by ^1H nuclear magnetic resonance and gas chromatography-mass spectrometry (GC–MS). The purity of the HT obtained using this method was 99%. The HT purified was dissolved in sterile saline and stored at −80 °C until use. The concentrations of HT in the aqueous solutions used in the study were measured by high performance liquid chromatography (HPLC) as described in [14].

2.3. Analytical determinations

The biochemistry parameters glucose, total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), triacylglycerols (TG), total protein, albumin, liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), direct and indirect bilirubin, alkaline phosphatase (ALP), lactate dehydrogenase (LD), creatine phosphokinase (CPK), amylase, urea, creatinine, uric acid, lipoprotein (a) (Lp(a)), vitamins A, C, D, E, B_6, B_{12}, ferritin, transferrin, iron, calcium, phosphorous, chloride, sodium and potassium, were measured in serum by a routine laboratory at Reference Laboratory (Barcelona, Spain), with certified quality which follows the UNE-EN ISO 15189:2007 directives. Vitamin C was measured by HPLC [15]. Urine analysis was also carried out at Reference Laboratory. Hematology analysis was carried out in Lara Laboratories (Granada, Spain) using an automatic hematology analyser (Beckman Coulter, USA).

Total fasting plasma homocysteine concentration was measured by high-performance liquid chromatography with fluorescence detection [16]. The serum total antioxidant capacity (TAC) was determined as described in [17], using TROLOX as standard. Plasma malondialdehyde (MDA) concentrations were measured using a HPLC separation described in [18] that is based on the thiobarbituric acid reaction and reverse-phase separation with fluorescence detection. Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were measured with commercial enzyme-linked immunosorbent assay kits from Biosource International (Camarillo, California, USA) according to the manufacturer's instructions. Oxidized LDL (ox-LDL) in plasma was quantified using an ELISA kit using the monoclonal antibody mAb-4E6 (Mercodia AB, Sweden).

2.4. Statistical analyses

All the data are expressed as means ± standard error of the mean (SEM) and differences of $P < 0.05$ were considered significant. Normality was assessed by the Kolmogorov-Smirnov test. The longitudinal effect of the HT supplement at the various time points of the study was analysed by one-way repeated-measures ANOVA followed by Tamhane's T2 posthoc test for not assumed equal variances. For the non-Gaussian variables, Wilcoxon test was performed to assess differences. The data were analysed using SPSS statistical software version 23.0; SPSS Chicago, USA).

3. Results

The HT supplement used in the study was well tolerated, compliance was good and the fourteen subjects completed the 8-week study.