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Hydroxytyrosol ameliorates metabolic, cardiovascular and liver changes in a rat model of diet-induced metabolic syndrome: Pharmacological and metabolism-based investigation

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ABSTRACT

Metabolic syndrome is a clustering of interrelated risk factors for cardiovascular disease and diabetes. The Mediterranean diet has been proposed as an important dietary pattern to confer cardioprotection by attenuating risk factors of metabolic syndrome. Hydroxytyrosol (HT) is present in olive fruit and oil, which are basic constituents of the Mediterranean diet. In this study, we have shown that treatment with HT (20 mg/kg/d for 8 weeks) decreased adiposity, improved impaired glucose and insulin tolerance, improved endothelial function with lower systolic blood pressure, decreased left ventricular fibrosis and resultant diastolic stiffness and reduced markers of liver damage in a diet-induced rat model of metabolic syndrome. These results were accompanied by reduced infiltration of monocytes/macrophages into the heart with reduced biomarkers of oxidative stress. Furthermore, in an HRMS-based metabolism study of HT, we have identified 24 HT phase I and II metabolites, six of them being over-produced in high-starch, low-fat diet fed rats treated with HT compared to obese rats on high-carbohydrate, high-fat diet. These results provide direct evidence for cardioprotective effects of hydroxytyrosol by attenuation of metabolic risk factors. The implications of altered metabolism of HT in high-carbohydrate, high-fat diet fed obese rats warrant further investigation.

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1. Introduction

The Mediterranean diet has been proposed as an important dietary pattern to confer cardioprotection by attenuating risk factors of metabolic syndrome such as adiposity, insulin resistance and type II diabetes, atherogenic dyslipidaemia, hypertension and chronic low-grade inflammation [1]. Olive oil is an important component of the Mediterranean diet being the primary source of monounsaturated fatty acids (MUFA; predominantly oleic acid) [2]. The decreased incidence of cardiovascular diseases in cohorts consuming large amounts of olive oil was first reported in the landmark Seven Countries study [3]. Studies in cells in culture, animals and humans have since confirmed the cardioprotective actions of the Mediterranean diet and olive oil components, mediated by attenuating the risk factors of metabolic syndrome [1,4,5]. Although these effects have been attributed, at least in part, to the high MUFA content of olive oil in the Mediterranean diet, this cannot fully explain

Abbreviations: ALP, plasma alkaline phosphatase; ALT, plasma alanine transaminase; AST, aspartate transaminase; C, corn starch; CHT, corn starch + HT; ESI, electrospray ionisation; FCPC, fast centrifugal partition chromatography; H, high carbohydrate, high-fat; HHT, high-carbohydrate, high-fat + HT; 4-HNE, 4-hydroxynonenal; HPLC, high performance liquid chromatography; HRMS, high-resolution mass spectrometry; HT, hydroxytyrosol; IVSd, interventricular septal diameter in diastole; IVSs, interventricular septal diameter in systole; LDH, lactate dehydrogenase; LDL, low-density lipoproteins; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; LVPWd, left ventricular posterior wall diameter in diastole; LVPWs, left ventricular posterior wall diameter in systole; MDA, malondialdehyde; NEFA, non-esterified fatty acids; NMR, nuclear magnetic resonance.

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the cardioprotective effects of olive oil. Total MUFA intake is similar in Italy and France to countries such as United States, United Kingdom, Australia and Canada at 12–13% E but markedly lower than in Spain (around 16% E) and Greece (around 22% E) [6]. More recent evidence emphasises the role of minor though highly bioactive phenolic components of olive oil and olive fruits [7–9]. In 2011, the European Food Safety Authority published its scientific opinion recognising that the prevention of oxidative damage to low-density lipoproteins (LDL) by hydroxytyrosol (HT) from olive oil and its precursors or derivatives such as oleuropein complex and tyrosol may have beneficial physiological effects [10].

Thus, the cardioprotective actions of olive oil may be mediated by phenolic compounds such as HT and its precursors or derivatives [11]. The main precursors of HT are oleuropein, ligstroside, as well as their aglycones oleacein and oleocanthal or other opened and closed forms belonging to the secoiridoids. Other polar constituents of olive oil include lignans, flavonoids and triterpenoids. These compounds are usually referred to as olive oil phenols or biophenols. After ingestion, the secoiridoids are hydrolysed in the gastrointestinal tract to several derivatives, such as HT, elenolic acid, oleuropein, ligstroside aglycone and glucose, depending on their structures [12,13]. Both *in vitro* and *in vivo* studies have shown that oleuropein and its metabolites, particularly HT, protect against ischaemia/reperfusion-induced myocardial damage and may also have anti-atherosclerotic and anti-thrombotic effects primarily mediated by reduced myocardial oxidative stress and injury [14]. Additionally, oleuropein prevents diet-induced and carbon tetrachloride-induced hepatic damage and steatosis in mice [15]. Synthetic HT was effective in preventing high-fat diet-induced obesity, insulin resistance and hepatic steatosis in mice by reducing oxidative stress and mitochondrial dysfunction [16,17].

Biological responses to HT have been attributed to potent anti-inflammatory effects in addition to free radical scavenging properties [11]. HT lowered the expression of vascular and intracellular cell adhesion molecules by blocking the activation of the transcription factor NF-κB, reducing LDL oxidation and inhibiting the expression of pro-inflammatory genes including genes for nitric oxide synthase and cyclooxygenase-2 [11]. In cultured endothelial cells, HT up-regulated components of antioxidant pathways such as Nrf-2 and heme-oxygenase-1, most likely through the upstream activation of PI3 K/Akt and ERK1/2 pathways [11]. However, the physiological and pharmacological changes due to these mechanisms in response to HT, particularly in the cardiovascular system, are not clear due to the paucity of studies that have tested the physiological and pharmacological effects of pure HT in studies on animal or human. One recent clinical trial suggested that HT at 5 and 25 mg/d doses does not affect body weight and adiposity, blood pressure, plasma lipids, circulatory cytokines or Nrf2-driven Phase II enzyme expression in peripheral blood mononuclear cells [18]. However, the participants in this study were treated with HT for one week so the effects of chronic dosing are unknown. Further, the pharmacological effects of HT in metabolic syndrome have largely been implied from the reduced risk factors for cardiovascular diseases by extra virgin olive oil rich in phenolic compounds and the isolated phenolic fraction of olive oil or leaf including HT as part of a complex mixture [5,19], rather than from studies on the pure compound.

Chronic feeding of a high-carbohydrate, high fat diet to rats produces the signs of metabolic syndrome including impaired glucose and insulin tolerance, elevated plasma lipid concentrations, hypertension, abdominal adiposity and remodelling of the heart, liver, kidneys and pancreas therefore mimicking the human condition [5,20]. We have previously shown that, in this model of diet-induced metabolic syndrome, olive leaf extract containing phenolic compounds such as oleuropein and HT improved glucose tolerance, normalised abdominal adiposity, reduced plasma triglyceride and total cholesterol concentrations, reduced plasma uric acid and malondialdehyde (MDA) concentrations, normalised inflammation and fibrosis in the heart and the liver, and improved left ventricular and hepatic function without changing blood pressure [5].

In this study, we have evaluated in rats the effects of pure HT isolated from olives on cardiovascular, hepatic and metabolic parameters in a high-carbohydrate (mainly fructose and condensed milk), high-fat (mainly beef tallow) diet with a corn starch-rich, low fat diet as the control diet. We have assessed the responses to 8 weeks' treatment with HT on body composition, abdominal adiposity, glucose and insulin tolerance, systolic blood pressure, vascular reactivity, plasma lipid concentrations, and structure and function of the heart and liver. Further, we have quantified HT and its metabolites in the plasma from these rats.

2. Materials and methods

2.1. Extraction and purification of hydroxytyrosol

Olive fruits of Amfissis variety were debittered for a period of 4 months, using an aqueous solution of 8% NaCl (w/v), yielding the Amfissis-Table Olive Processing Wastewater [21]. HT was isolated from olive fruits, as described in the Supplementary Methods section, based on in-house methods, with a purity of more than 98% (HPLC-UV). The structure was identified by high-resolution mass spectrometry (HRMS) and 1 & 2D Nuclear Magnetic Resonance (NMR). NMR spectra were recorded with the aid of a Bruker Avance III spectrometer operating at 600.11 MHz (Bruker Biospin GmbH, Reinsteten, Germany). HRMS spectra were recorded using a hybrid LTQ-Orbitrap Discovery XL mass spectrometer (ThermoScientific, Bremen, Germany).

2.2. Rats and diets

The experimental groups consisted of 48 male Wistar rats (9–10 weeks old) supplied by The University of Queensland Biological Resources unit and individually housed in a temperature-controlled ($20 \pm 2\,^{\circ}C$), 12-h light/dark cycle environment with *ad libitum* access to water and the group-specific rat diet at the University of Southern Queensland Animal House. All experimentation was approved by the Animal Experimentation Ethics Committees of The University of Queensland and the University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. The rats were randomly divided into four separate groups (n = 12 each) and fed with corn starch (C; 330 ± 3 g), corn starch + HT (CHT; 324 ± 3 g), high-carbohydrate, high-fat (H; 336 ± 2 g), high-carbohydrate, high-fat + HT (HHT; 336 ± 2 g).

The preparation and macronutrient composition of basal diets, including the dietary fatty acid profiles, have been described in detail [5,20,22]. HT (20 mg/kg) dissolved in distilled water was administered daily by oral gavage for 8 weeks starting 8 weeks after the initiation of the corn starch or high-carbohydrate, high-fat diet in CHT and HHT groups. The drinking water in both high-carbohydrate, high-fat diet-fed groups (H and HHT) was augmented with 25% fructose for the duration of the study.

2.3. Measurements of cardio-metabolic variables

Body weight and food and water intakes were measured daily and feed efficiency (%) was calculated [22]. Body composition was assessed using dual energy X-ray absorptiometry at 16 weeks. Oral glucose and intraperitoneal insulin tolerance were measured using standard techniques. Systolic blood pressure was measured using a non-invasive computerised tail-cuff system. Thoracic aortic

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